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Laboratory Diagnosis of Brucellosis in Man*

M. RUIZ CASTAÑEDA, M.D.[1]

CONTENTS

INTRODUCTION

The more cases of human brucellosis one sees, the more apparent is the fact that the diagnosis can be made only with the help of the laboratory. Of the known laboratory procedures, only the isolation of the responsible micro-organism from the blood or other material from infected organs or tissues of the patients is of definite value. However, the isolation of *Brucella* is not always successfully accomplished, and the clinican often requires immediate information which may be given by means of serological and other immunological studies, but no single test is free from pitfalls.

The most common procedure for isolating *Brucella* is blood cultivation. The serological tests are the agglutination, the complement-fixation, and the recently introduced antiglobulin tests. Some workers still favour the opsonocytophagic test, and certain clinicans, and particularly research laboratory epidemiologists, derive important information from allergic tests. Of the above-mentioned serological tests, the sero-agglutination test is the most widely used. Complement-fixation is a satisfactory method, although too complicated for routine work, and the antiglobulin test cannot be recommended for small laboratories. The opsonocytophagic test was not considered to be suitable by the Joint FAO/WHO Expert Panel on Brucellosis (1951) or the Joint FAO/WHO Expert Committee on Brucellosis (1953), and the intradermal test should be used with great discrimination.

The study of the blood picture is frequently of considerable assistance and may suggest the possible presence of *Brucella* infection.

In the body of the present paper are described methods which might be considered applicable in non-specialized laboratories. For laboratories with more complete equipment, additional information is given in the annexes.

* This is one of a series of studies on the laboratory diagnosis of various diseases which appear from time to time in the *Bulletin of the World Health Organization*. An effort is made to ensure that the diagnostic methods recommended in these studies are as internationally representative and acceptable as possible by securing the co-operation of a number of experts from different countries. A list of the reviewers of the study presented here is given in Annex 7 (page 83). To all of these, and to the author, the World Health Organization is greatly indebted.—ED.

[1] Departamento de Investigaciones Médicas, Hospital General, Mexico, D.F., Mexico.

COLLECTION OF SPECIMENS

In human brucellosis the usual material to be cultivated is blood, but in certain instances investigation for the presence of *Brucella* in spinal fluid, urine, pus from abscesses, articular fluid, faeces and even milk from nursing mothers may be required.

Blood is obtained from a vein in the arm or in some patients, particularly in children, from the jugular vein. The syringes and needles must be sterilized either by dry heat at 180°C for one hour or by autoclaving. In the latter case, care must be taken to allow the material to become completely dry. It is advisable to protect the syringes with thick cloth or strong paper.

The skin must be cleaned with a suitable antiseptic. It is advisable to allow a piece of cotton, soaked in the antiseptic, to remain on the selected place for a short time prior to the puncturing of the vein.

For a whole battery of tests, about 20 ml of blood should be obtained, of which some 4 ml are deposited in a flask containing anticoagulant [1] to be used for the study of the blood picture, sedimentation rate, some rapid tests (see Annex 3), etc. A further 4 ml are poured into a test-tube and allowed to clot for serological tests, and the remainder may be used for blood cultivation.

The collection of certain specimens, such as peritoneal, pleural, articular and spinal fluids, requires the same precautions as those recommended for obtaining the blood.

ISOLATION OF *BRUCELLA* : CULTURE MEDIA AND METHODS

The following media are considered satisfactory for the isolation of *Brucella*: trypticase soy (Difco) Albimi and selected batches of Bacto-tryptose (Difco). Although these media have acquired wide acceptance, particularly in North and South America, there are areas where these products are not readily available and therefore the following home-made media can be employed.

[1] Anticoagulants ;

Potassium oxalate	0.8 g
Ammonium oxalate	1.2 g
Distilled water	100 ml

Use 0.5 ml in a vial and evaporate until dry. This is enough for 5 ml of blood.

An excellent anticoagulant is ethylenedinitrotetracetic acid disodium salt (Eastman Kodak) in a 10% solution, of which 0.2 ml is sufficient for 5 ml of blood (Schmidt, Hane & Gomez, 1953).

Liver medium of Stafseth

This is a medium which has been commonly used. Grind 500 g of beef liver, mix it with 500 ml of tap water, heating in the autoclave under atmospheric pressure (or in an Arnold sterilizer) for one hour and shaking from time to time. Filter the hot mixture through a layer of cotton, kept between two layers of gauze, and add to the filtrate 10 g of sodium chloride, 10 g of peptone and distilled water to complete 1 litre. If a solid medium is to be prepared, 25 g of agar should be added. Heat the mixture to ensure solution of all ingredients and adjust the pH to 7.0. Heat in the autoclave at 120°C for 15 minutes, and allow the medium to stand while still hot in order to facilitate sedimentation of the insoluble materials. After sedimentation, decant the supernatant and distribute it according to requirements. Sterilization of the final product should be made at 115°C for 15 minutes. It should be emphasized that best results have been obtained with beef liver.

Other infusions, such as veal or beef infusion, may be prepared in a similar way, but to these media, whether liquid or solid, 10% horse serum should be added.

Potato medium

A medium prepared with potato infusion has been recommended for routine work by several authors. It is particularly useful for the preparation of antigens. Care must be taken in the selection of potatoes since in some countries they are not properly " cured " and this is a prerequisite for a good medium. The potatoes should be cleaned, the skin removed and 250 g sliced and submerged in 1 litre of water. The mixture is infused for 24 hours in a covered container heated at 60°C, filtered through gauze, and the volume is brought to the original amount. The ingredients to be added are: 10 g of peptone, 5 g of beef extract, 5 g of sodium chloride, 20 ml of glycerine and 25 g of agar. After heating to achieve solution of these substances, the pH is adjusted to 6.8-7.0 and then autoclaved at 120°C for 30 minutes. The medium is allowed to stand until sedimentation of the precipitate is complete, and the supernatant is then distributed according to requirements. Considerable improvement of this medium can be obtained by addition of 10% horse serum. For this purpose the supernatant. after sedimentation, should be mixed with the serum and distributed in sterile glassware under aseptic precautions.

Any one of the commercial or home-made media described or mentioned here is suitable for the growth

of *Brucella*, but for primary isolation from blood or other fluids (not usually contaminated with other bacteria) the first step is cultivation in a fluid medium and, at chosen intervals of incubation, transference to a solid medium in order to detect the slow-growing colonies of *Brucella*.

Double-phase medium

In order to facilitate handling of blood cultures and to minimize the danger of infecting personnel during the transfers from the liquid to solid media, a special outfit has been developed (Castañeda, 1947) and recently improved (Castañeda, 1956) in which both liquid and solid media are placed together. The solid medium is allowed to harden on the side of the container, while a certain amount of liquid remains at the bottom.

Preparation of the double medium is as follows.

Solid medium. Prepare a 2% tryptose, trypticase or Albimi broth in distilled water and add 0.5%-0.7% of sodium citrate. Add 2.5% of agar, heat to dissolve the ingredients and adjust the pH to 7.4. While it is still liquid, distribute the medium in bottles in convenient amounts to obtain suitable slants (our standard outfit requires some 15 ml of medium). Sterilize the bottles at 120°C for 20 minutes and allow to cool in a horizontal position.

Liquid medium. This is a 2% solution of the same product as used for the slant, containing 2% sodium citrate, adjusted to pH 7.4 and sterilized at 120°C for 20 minutes.

Procedure. By means of an adequate distributor and under aseptic precautions, the broth is added to each bottle in amounts of 10-15 ml. The mouth of the bottle is protected against contamination either by means of cotton stoppers or with rubber caps. Our standard equipment is protected with rubber stoppers kept in place by a perforated metal support, which is protected by another metal cap (Castañeda, 1956). If the bottles are stoppered with rubber and metal protectors, CO_2 may be introduced either at the time of distribution of the broth, which requires special equipment, or after the blood has been inoculated. If the bottles are stoppered with cotton, the incubation can be made in a jar containing a mixture of air with 10% CO_2.

The addition of CO_2 after the inoculation of sealed bottles can be made by injecting the calculated proportion of gas by means of a syringe. A practical procedure in our service after several bottles have been arranged has been the use of a 20-ml syringe connected to a double valve manufactured by Becton Dickenson. This is connected through the " inlet valve " to a rubber bulb filled with CO_2. The " outlet valve " is connected with a Swinny filter adapter, also manufactured by Becton Dickenson. The filter is sterilized, as are as many needles as required. For each bottle a sterile needle is fitted to the filter and inserted through the rubber stopper. By withdrawing and pressing the plunger of the syringe, a calculated amount of gas may be introduced into each bottle.

If the special valve is not available, a filter can be arranged using a tuberculin syringe filled with absorbent cotton and stoppered with a rubber stopper through which a needle has been inserted. The syringe, with another needle fitted at the tip, is sterilized in the autoclave, and the free needle inserted through the stopper of the bottle. With a 20-ml syringe, CO_2 may be taken from its source (a CO_2 tank or gas produced in a flask by chemical reaction) and injected into the bottle through the filter. If necessary, the inside pressure may be re-established by working the syringe in reverse a few seconds after the injection.

The preparation of a jar containing the proper concentration of CO_2 is not as easy as one might expect. The common practice of burning a candle inside the jar, burning cotton or even mixing carbonate and sulfuric acid has been considered by experts as rather inconvenient. If the jar is not too big, one may proceed as follows. The jar can be stoppered by means of a metal cap through which two tubes or faucets are passed and soldered. One of the faucets or tubes is connected with a rubber tube which reaches the bottom of the jar, and the other is free. By means of a 50-ml syringe, CO_2 is taken from a tank and injected into the jar through the large tube, leaving the short tube open to allow air from the upper part to be expelled. When the calculated amount of gas has been introduced, both tubes or faucets are properly sealed and the lid of the jar is secured by means of a strip of adhesive tape.

Blood culture

If the operator decides to use liquid medium for preliminary isolation, any one of the commercial or home-made media may be used, provided that some sodium citrate is added to prevent coagulation of the blood. Amounts of from 30 to 75 ml of broth are distributed in suitable containers, which are sterilized as usual. Into this broth, 5-10 ml of blood are

inoculated and the flask is incubated in a CO_2 jar at 37°C. Subcultures to agar slants are made on the 5th, 15th and the 30th days. Not less than two tubes must be inoculated, so that one or more may be incubated in the CO_2 jar and others (at least one) under ordinary atmospheric conditions.

If the double medium is preferred for isolation, the blood is introduced through the rubber stopper in amounts which may be from 5 to 10 ml. CO_2 is added as indicated and transfers are performed at 48-hour intervals by the simple procedure of tilting the bottle to a horizontal position to allow the mixture of blood and broth to cover the agar slant for a few seconds. The bottle is returned to the incubator and observed at the end of 48 hours. If there is no apparent growth in the agar slant the transfer is repeated. Since the cultures are incubated for a minimum of 30 days, at least 14 transfers may be performed before apparently negative bottles are discarded.

Positive cultures must be handled with extreme care to avoid accidental infection to the operator. It would be preferable to refer such material to specialized laboratories, but if one is in a position to attempt the identification of the isolated microorganism, the following procedure is recommended.

The transfers to agar slants can be made by means of a syringe inserted into the bottle through the rubber stopper and into which enough fluid is drawn to inoculate about 0.5 ml into each tube. This method, recommended by Spink (1956), is quite satisfactory, but a further period of 48 hours of incubation of the transfers will delay identification of the cultures. In order to save time one may open the bottles and use the colonies from the slant for microscopic examination, slide agglutination or transfers to differential media. Care must be taken to operate the bottles under the protection of a hood.

Selective media

In ordinary routine work one is seldom requested to isolate *Brucella* from contaminated material such as urine, faeces, pus or milk. This is a delicate procedure which is rather the work of specialized laboratories. However, for those laboratories which may be interested in such work, Annex 1 describes one of the most recently reported media for selective cultivation of *Brucella*.

Special methods, such as cultivation of material from liver biopsy, bone marrow and lymph glands, which may be required in certain cases, are hardly within the scope of this study.

Fertile eggs

Some brucellosis workers favour the use of fertile eggs for the isolation of *Brucella* from the blood. However, this method has certain inconveniences, namely, the tendency to produce dissociated cultures and the limited amount of blood which can be inoculated into the eggs (Joint FAO/WHO Expert Panel on Brucellosis, 1951 ; Joint FAO/WHO Expert Committee on Brucellosis, 1953). Nevertheless, the method has some advantages when large numbers of specimens are to be cultivated, particularly in research laboratories.

Animal inoculation

The inoculation of guinea-pigs is a valuable procedure for the isolation of *Brucella* from highly contaminated material. A penicillin solution (500 IU/ml) mixed with the inoculum is recommended to prevent the growth of certain contaminants. Local laboratories may try to detect agglutinin response two weeks or more after inoculation, but for further work the animals should be referred to specialized laboratories.

IDENTIFICATION OF THE ISOLATED ORGANISMS

There are some clues which may help to indicate whether a positive culture belongs to the *Brucella* group. The operator, therefore, should proceed with due care. The following data are given for this purpose.

(*a*) *Brucella* are slow-growing organisms which seldom appear in the slant of the double medium before the sixth day of incubation. (The bottles may have been brought to transfer position at least twice.)

(*b*) The colonies are small, with a tendency to be discrete rather than confluent ; they are transparent and require at least 48 hours to be developed fully.

(*c*) If significant agglutinin titres are found in the serum of the patient and the culture shows the characteristics (*a*) and (*b*) above, suspicion of the presence of *Brucella* is strengthened.

If the operator decides to proceed with identification, transfers can be performed and the atmospheric requirements of the isolated organism determined.

From the bottle or from the subcultures, smears are prepared which are stained by the Gram method.

For those familiar with the morphology of *Brucella* the organism is not difficult to recognize. It is the smallest Gram-negative bacillus of all pathogenic bacteria which can be isolated from the blood, it is coccobacillary in shape, and often appears in pairs. On other slides, using drops of saline containing 10% formalin, the culture, carried with a wire loop, is emulsified and a droplet of anti-*Brucella* serum is added to the emulsion. In a similar way the culture is tested with normal serum. The control should remain in a homogeneous suspension, while brucellae are readily agglutinated by specific serum.

This is as far as a non-specialized laboratory can go with regard to the diagnosis of a suspected *Brucella*. Better-equiped laboratories may proceed further by cultivation in media containing bacteriostatic dyes and with other complementary tests, such as detection of H_2S (see details in Annex 5).

It must be kept in mind that even with experience and technical skill these tests are particularly liable to give rise to error. For this reason it has been recommended that the study of each new strain should be controlled by cultivating it simultaneously with reference strains. The reference strains recommended by the Joint FAO/WHO Expert Committee on Brucellosis (1958) are :

Br. abortus 544, Central Veterinary Laboratory, Weybridge, Surrey, England;

Br. melitensis 16M, United States Department of Agriculture, Beltsville, Md., USA;

Br. suis 1330, Medical School, University of Minnesota, Minneapolis, Minn., USA.

As a final step, serological typing may be carried out with monospecific sera, prepared and used as indicated in Annex 6.

Briefly, the minimum requirements which permit identification and typing of the isolated strains are as follows:

Brucella melitensis. Grows well either in ordinary atmosphere or in the presence of CO_2; is not inhibited in the presence of fuchsin or thionin; fails to produce H_2S or produces a trace only; and is agglutinated to a higher titre in the presence of monospecific anti-*melitensis* serum.

Brucella abortus. Recently isolated typical strains are CO_2-dependent; they are inhibited in the presence of thionin; produce H_2S in a moderate amount for two days or longer; and are agglutinated to a higher titre by monospecific anti-*abortus* serum.

Brucella suis. Grows well in both atmospheric conditions; is inhibited in the presence of fuchsin; and, except for certain strains (Danish), produces rather strong blackening of the acetate paper for four or five days; behaves like *Br. abortus* in the presence of monospecific sera.

SERODIAGNOSTIC TESTS

Agglutination test

It is most unfortunate that many laboratories at the present time use antigens for rapid tests which were originally devised for the detection of bovine brucellosis. Such antigens, when properly standardized, are useful in veterinary work, but the low range of titres has no value in human brucellosis, although it is quite satisfactory for the detection of animal reactors, for which, according to recommendations of the Joint FAO/WHO Expert Committee on Brucellosis, the titre must be over 100 IU to give any serological support to a diagnosis of brucellosis.

These reasons have been taken into consideration in recommending the tube dilution method as the only reliable procedure for agglutinin titration.

If possible, the antigens should be obtained from official laboratories or FAO/WHO brucellosis centres. If such antigens are not obtainable, homemade antigens may be prepared and standardized according to the procedures outlined in Annex 2.

Since locally prepared antigens differ from laboratory to laboratory, precise information must be obtained concerning their relationship to the International Standard Serum in order to determine their sensitivity (Joint FAO/WHO Expert Committee on Brucellosis, 1958). International Standard Serum, containing 1000 Units per ml, is obtainable from the Central Veterinary Laboratory, Weybridge, Surrey, England, or the Statens Seruminstitut, Copenhagen, Denmark.

The ideal antigen should give complete agglutination when mixed with an equal amount of a 1 : 500 dilution of the International Serum. While the antigens used in North America conform to this degree of sensitivity, those used in other countries may become agglutinated in higher or lower dilutions. Titres of unknown sera of 640–320→40, will correspond to 640–320→40 International Units. The following formula converts to IU the titres obtained with such locally produced antigens of different sensitivity:

$$\frac{\text{Titre of unknown serum with local antigen} \times 1000}{\text{Titre of standard serum with local antigen}} = \text{International Units}$$

Some authors object to this manner of recording agglutinin titres, but since there are no uniform criteria concerning this matter and the above formula has been recommended by the Joint FAO/WHO Expert Committee on Brucellosis (1958), the inclusion of this information is considered justifiable here.

The agglutination test may be performed as follows. To a series of 6-10 tubes of the size usual in serological reactions (13 mm × 100 mm) add 0.9 ml of isotonic saline in the first tube and 0.5 ml in each of the remaining tubes. To the first tube add 0.1 ml of the serum to be tested and, after proper mixture, transfer 0.5 ml to the second tube; mix, and from the second tube transfer 0.5 ml to the third and so on, discarding the 0.5 ml removed from the last tube. To each tube add 0.5 ml of the antigen, diluted to the specified concentration. Mix by shaking and incubate in the water-bath at 37°C.

The results of the test may be read after 24 hours or, better, 48 hours of incubation. The end-point is the last tube showing 50% agglutination, although some authors prefer to take the last tube with complete clearing as the end-point. It is advisable to set up a series of control tubes containing antigen with and without immune serum.

Rapid tests

There are certain discrepancies between agglutination and blood culture results which might be taken as an argument in favour of the use of the rapid tests. Although the latter seem to be less influenced by factors interfering with the clumping of bacteria in the test-tube, slide titrations are subject to many inaccuracies. This led us to investigate the possibilities of certain rapid tests which, when used with properly standardized material, might be reliable at least as screen tests. One of these tests is a bedside test which has been found to be satisfactory in our service (Castañeda, 1953a) and elsewhere (Spink & Anderson, 1952). Another, which we call the surface-fixation test (or strip test), allows the detection of antigen-antibody reactions without much risk of error from factors interfering with agglutination.

Since rapid tests are in fairly wide use and since many small laboratories may not be able to adopt or perform the tube agglutination test, it may be of value to give some information on the two rapid tests which in our experience have been found to be the least liable to error.

Spot test. A spot test with the blue antigen described in Annex 3 and whole blood may be performed by placing a drop of antigen carried with a 4-mm wire loop on to a clean slide to which a drop of blood (taken from the finger or from an oxalated sample) is added with a 3-mm loop. The mixture is made to rotate by moving the slide. If agglutination occurs, the clumped particles gather at the periphery of the mixture, forming a blue ring which surrounds a reddish centre owing to liberation of the red cells from the mixture. With a negative reaction the red cells may accumulate at the periphery, forming a reddish ring surrounding the greenish mixture. Weak or doubtful reactions should be confirmed by repetition of the test.

In view of the care taken in the standardization of the antigen, some workers use this test as a screen test with serum instead of whole blood.

Surface-fixation test. The surface-fixation test or strip test is a method of detecting antigen-antibody reactions on a piece of filter-paper (Castañeda, 1953b). The antigen (see Annex 4) is printed in spots near the end of a strip of filter-paper; for a single test, three spots are used. With a wire loop or capillary pipette a drop of anti-*Brucella* serum is placed over the spot at the left side and a drop of normal serum on the spot at the right. The serum under test is placed in the middle. The paper is set up in a tray containing isotonic saline, as in paper chromatography. Care must be taken that the spots remain slightly above the fluid. By capillary absorption the fluid will pass beyond the spots in 20-30 minutes. The negative control spot will be displaced, leaving a comet-like trail of antigen; in the positive control, the antigen will remain unaffected. The serum under test may be judged by comparison with the two controls, graduations of the intensity of the reaction being expressed as from 1+ to 4+ or by approximate percentages of fixation.

INTRADERMAL TEST

Allergens

Among the substances recommended for skin testing, the most commonly used is the brucellergen of Huddleson (1943). The purified protein of Morales, Otero & Gonzalez (1938) has been found to be acceptable as have our own polyvalent aqueous *Brucella* extracts (Castañeda & Carrillo-Cardenas, 1941; Castañeda, 1953a). Information concerning brucellergen may be obtained from Dr Huddleson[1]

[1] Dr I. F. Huddleson, Department of Microbiology and Public Health, Michigan State University, East Lansing, Mich., USA.

and about the *Brucella* extracts from the present author.

The allergens, inoculated intradermally into the forearm, produce a delayed tuberculin-like reaction in previously infected persons. Readings are usually made 24-48 hours after the injection, but in some instances the reaction may reach a peak several days later.

HAEMATOLOGY

The blood picture has become such a necessary adjunct to the clinical history, particularly in infectious diseases, that there is no justifiable argument against its inclusion in the battery of tests for the study of brucellosis patients.

EVALUATION OF LABORATORY METHODS

Blood culture

A positive blood culture is definite evidence of active brucellosis regardless of the clinical picture or the stage of the disease. It may be obtained in all cases of acute brucellosis, particularly those caused by *Br. melitensis*, if frequent cultures are performed. The chances of successful isolation decrease in the later stages of the infection and considerable interference with the culture should be expected if the patients have been submitted to treatment with antibrucellar drugs.

In our experience, a single culture performed on admission of patients suffering from *Br. melitensis* infection (provided that no antibiotic drugs had been administered shortly before bleeding) yielded the following percentages of isolations: from 15 days to 3 months since the onset of the disease, 89%; from 3 to 6 months, 70%; in chronic cases 20%. Less success has been reported in *Br. abortus* infection, although authors such as Spink (1956) record successful isolation in 50% of the cases. Spink finds it necessary to perform multiple cultures on successive days, since it has been demonstrated that, if one distributes 30-40 ml of blood in three or four bottles, only one or two of them will probably show a positive culture, owing to the small numbers of circulating bacteria.

Agglutination test

Infection with *Brucella* stimulates an antibody response which is one of the most constant features of the disease in man. Cross-reactions may occur with sera from patients infected with *Pasteurella tularensis* or *Vibrio cholerae* or from individuals vaccinated against cholera owing to some similarity in the antigenic structure of *Brucella* and these organisms. We have no conclusive evidence of any other infections capable of producing cross-reactions. However, agglutinins have been found in persons who have recovered from brucellosis, and in a relatively large percentage of individuals from endemic areas, even when no signs of *Brucella* infection have been detected.

Surveys in large groups of healthy persons have shown that in individuals with a positive agglutination test the titres are seldom above 100 IU, while in patients suffering from brucellosis the titres are usually higher. In the interpretation of these results titres of 100 IU should be regarded as suggestive and only titres of 320 or higher are to be considered significant. Some authors, however, believe that agglutination reactions of 1:80 should be further investigated.

In regard to the correlation of the agglutination test with the blood culture it should be pointed out that some patients, in spite of a positive blood culture, may not produce agglutinins detectable by standard methods. Although this discrepancy is not frequently found, it should be borne in mind; the causes of interference with a complete antigen-antibody reaction require investigation.

The prozone phenomenon may be detected by using a large series of dilutions in the agglutination test, and other procedures such as complement-fixation and the antiglobulin tests may be useful to cover other deficiencies in the standard agglutination test. The latter two procedures are not sufficiently convenient to be recommended as routine for small laboratories; on the other hand, the rapid tests described above, which are in use at the FAO/WHO Brucellosis Centre in Mexico, have been found to be less liable to interference and, even if only used as screen tests, they correlate well with the standard methods and with clinical findings.

The spot test performed with whole blood is particularly useful as a bedside test in febrile patients. It has been found to be positive in almost every case of acute brucellosis, and a negative reaction tends to exclude a possible diagnosis of brucellosis. The fact that the antigen is not sensitive to agglutinin in low titre is an advantage in eliminating false positives.

The strip test, if positive to at least 40% of the maximum intensity, is an indication of present or recent infection. (The percentage of fixation is the inverse of the length of the trace left by the antigen, as compared with the negative control). Percentages under 40 have been found in active brucellosis, but, on the other hand, surveys in a healthy population

group attending one of the blood banks in Mexico City have shown percentages of fixation from 10 to 30 in about 0.7% of the individuals tested. Those low-titred strip tests have the same meaning as the low agglutinin titres.

Both rapid tests may prove to be useful as preliminary tests even in well-equipped laboratories.

Allergic test

This is not a procedure to be recommended for diagnostic purposes and should be used only under special circumstances. This test must not be performed with impure substances, such as filtrates from broth cultures, since antibody production may be stimulated or irritation may be produced which could be confused with a positive allergic response. On the other hand, the allergens mentioned previously are not likely to produce such an effect unless the patient has had a previous exposure to *Brucella* infection.

With regard to the interpretation of a *Brucella* skin test, it must be remembered that the mechanism involved in a positive or negative response resembles that of the tuberculin reaction and should therefore be evaluated accordingly.

A positive reaction of moderate intensity is a common finding in late stages of the disease as well as in cases which have recovered and even among healthy residents of endemic areas, but an extremely severe skin reaction to a properly standardized antigen is not so frequent and may therefore be related to some abnormal condition in the subject tested. Particular difficulty occurs when such hyperergic reactions appear in individuals suffering from obscure clinical manifestations, such as low grade fever, irritability, fatigue, etc., which are described by some authors as indicative of chronic forms of brucellosis. The possibility of a relationship between such reactions and chronic brucellosis may be strengthened when the patient has a past history of active brucellosis and no other cause can be blamed. Many hyperergic patients of the type just described lack other laboratory data supporting a diagnosis of active *Brucella* infection.

Haematology

Although there is no special feature of the blood picture which could be said to be indicative of brucellosis, it is useful to remember that in acute brucellosis and even in the later stages of the disease, the number of white cells, if not reduced, remains normal and is seldom increased. In most cases there is a tendency to neutropenia with relative or absolute lymphocytosis. The sedimentation rate has no significance other than corresponding to the general condition of the patient.

SUMMARY AND CONCLUSIONS

No single test is, by itself, sufficient to detect all cases of human brucellosis, and it is therefore advisable to use as many tests as possible, in the hope that one at least may lead to a diagnosis of *Brucella* infection.

With the exception of a positive culture there is no test which, by itself, could be taken as evidence of actual infection. A blood culture should be made whenever possible, and the more cultures can be performed, the better the chances of success. If it is not possible to perform blood cultures, the detection of *Brucella* antibodies may be of considerable help, provided that the titres are significant.

A convenient battery of tests is as follows. When the blood is obtained, cultures may be performed by one of the recommended methods. Then a screen test may be of assistance, while more classic tests are going on. Because of the good correlation with the standard tests, the rapid methods used at the Brucellosis Centre of Mexico may be useful at least as preliminary tests. A clear-cut spot test with whole blood may help to decide on early treatment in acute cases, while a negative reaction justifies a delay until better information becomes available.

There is no objection to the use of more sensitive tests, such as complement-fixation or the antiglobulin test, but their use will depend on the equipment and experience of each laboratory.

Whatever the method employed to detect circulating antibody, it must be borne in mind that, even where brucellae have been isolated, it is not rare to find that antibodies are not readily detected. It is therefore advisable to withhold final reports until one or more attempts to isolate the organism have given information one way or another.

Skin testing is a delicate procedure to be used only by experienced workers; it is particularly of value for epidemiological purposes.

Annex 1

SELECTIVE MEDIA

The isolation of *Brucella* from contaminated material is always a difficult procedure. Inoculation into guinea-pigs may help but is slow and expensive. Several media have been used, among which the most popular in the past has been a suitable agar medium to which crystal violet was added in a proportion of 1 : 700 000. A medium which at present seems quite satisfactory is one developed by Kuzdas & Morse (1953) and modified by Renoux (1954) which has the following formula:

Albimi agar	1000 ml
Ethyl violet	1 : 800 000
Actidione	100 mg
Polymyxin B	6000 units
Bacitracin	25 000 units

Actidione, polymyxin B and bacitracin are prepared in stock solutions which are sterilized by filtration through a Seitz or Chamberland filter and kept under refrigeration no longer than one week. The proper concentration of ethyl violet is added to the melted agar before this is autoclaved. Later, when the temperature of the agar has dropped to about 50°C the calculated amounts of the other ingredients are added to the medium and distributed in Petri dishes.

There are certain difficulties in the preparation of this medium, particularly in regard to the dye, for which it is advisable to follow the detailed instructions in Annex 10 of the report of the Joint FAO/WHO Expert Committee on Brucellosis (1958).

Annex 2

ANTIGEN FOR THE TUBE AGGLUTINATION TEST

The antigen may be prepared according to procedures adopted by international agreement (Joint FAO/WHO Expert Committee on Brucellosis, 1958).

Several strains of *Br. abortus* have been used but those better known are: Strain 1119 of the United States Department of Agriculture; Strain 99 of the Central Veterinary Laboratory, Weybridge, England; and Strain S 6 of Montpellier, France. The selected strain may be cultivated in plates containing a suitable medium; after three to four days of incubation, these are observed with the aid of incident light as described by Huddleson (1943) and in most monographs dealing with brucellosis. This method permits the selection of smooth or intermediate colonies, which are transferred to slants prepared with liver or potato medium. The tubes are incubated for 48 hours and kept in the refrigerator for further use.

Liver or potato medium is distributed in Roux bottles or other containers suitable for mass cultivation. From the tubes, kept under refrigeration, transfers are made to as many further tubes as there are Roux bottles to be inoculated. These tubes are incubated for 48 hours and transfers then made to the Roux bottles, which are incubated at 37°C during 72 hours; the condensate is removed before addition of some 50 ml of isotonic NaCl solution to each bottle. The growth is carefully removed and filtered through sterile absorbent cotton, then heated at 60°C for 60 minutes. To this suspension enough phenol is added to obtain a concentration of 0.5%. The material is centrifuged and the sediment resuspended in enough phenolized saline to obtain a bacterial concentration which, diluted to 1 : 100 gives a turbidity comparable to No. 2 or 3 on the scale of McFarland. When proper equipment is available the diluted antigen may show an optical density between 0.10 and 0.18 in a Coleman Junior spectrophotometer model 6 B at 610 mμ.

In order to standardize the antigen it is advisable to follow the instructions in Annex 9 of the report of the Joint FAO/WHO Expert Committee on Brucellosis (1958). Although a local antigen may be adjusted to any desired concentration, the least complicated procedure seems to be to report the results of titrations with such an antigen in International Units according to the instructions given in that annex.

Annex 3

ANTIGEN FOR THE SPOT TEST

The simplicity of the spot test makes it necessary to use carefully standardized antigens, as described below.

Mass cultures may be prepared as indicated in Annex 2 but, instead of phenol, 5%-10% formalin is added to the bacterial suspension. After 24 hours the suspension is centrifuged and the sediment resuspended in a small volume of isotonic saline. The bacterial content should be adjusted to a concentration close to that of the finished antigen. For this purpose, 0.1 ml of the suspension is diluted with water until a turbidity measuring 2 cm with the Gate's wire loop is obtained. The material is adjusted to produce the indicated turbidity when the water is added in a volume of 20 ml. Further steps will be easier if a sample of a previously standardized antigen is available. The suspension is stained with methylene blue to a rather deep colour, the aim being to obtain mixtures of antigen and blood which will produce good contrast in the test. After 24 hours the material is centrifuged and the sediment resuspended in 1.1% sodium citrate containing thiomersal in a proportion of 1 : 10 000. The volume of the bacterial suspension must be a little less than before centrifugation.

If a standard antigen is not available for comparison, the local product may be adjusted by addition of diluent or by concentration through centrifugation, the aim being to obtain a reagent which produces a positive reaction in a test with whole blood only when the antibody content has a titre of 1 : 100 or above.

If a sample of known antigen is available, the standardization of the local product can be performed by comparative tube dilution tests with sera of titres from 1 : 160 to 1 : 640 against a 1 : 50 dilution of the standard antigen and several dilutions above or below that figure of the local antigen. The dilution which compares best with the standard will serve as the basis for correcting the concentration of the local antigen.

Annex 4

ANTIGEN FOR SURFACE-FIXATION (STRIP) TEST

The antigen is a thick suspension of *Br. abortus* in sugar syrup stained with haematoxylin and ferrous sulfate to give a black colour (Castañeda, 1953b).

Mass cultures may be obtained as described in Annexes 2 and 3 but only 1% formalin is added to kill the organisms. The suspension is kept for 24 hours at room temperature and then centrifuged and the sediment resuspended in isotonic saline. A 2% solution of ferrous sulfate is added in amounts of 2 ml for each 100 ml of suspension, and then about 4 ml of a 1% solution of haematoxylin (Coleman & Bell). The process of staining may require several days until a colour as near as possible to black is obtained. The material is filtered through cotton and centrifuged at low speed to remove impurities. The supernate is centrifuged at high speed and the sediment resuspended in saline. It may be necessary to repeat centrifugation and resuspension several times in order to remove all traces of colour in the washings. The packed sediment is transferred to a mortar and emulsified with about four volumes of saturated sugar syrup. To obtain a proper concentration of the antigen it is necessary to try several combinations of syrup and antigen, which are printed on filter paper, and to test the various samples with sera of known titres.

The filter paper which has been found satisfactory for the detection of *Brucella* antibody is Eaton Dikeman No. 609, which is cut in leaves of 5 × 12 inches (about 13 cm × 30 cm). Close to one of the edges, and by means of a glass rod ground to produce a seal of about 3 mm in diameter, spots of antigen are printed about 2 cm apart. The antigen retains its sensitivity for several months provided the printed paper is kept in a dry place. If a negative control fails to displace the antigen when submitted to saline treatment, the paper should be discarded.

Annex 5

DYE-INHIBITION TEST AND HYDROGEN SULFIDE TEST

Dye-inhibition test

Albimi or trypticase agar may be used to which at least three concentrations of fuchsin and thionin are added—for instance, 1 : 50 000, 1 : 100 000 and 1 : 200 000. Each concentration is tested simultaneously with the reference strains. It is recommended that aniline dyes previously tested by a reliable laboratory should be used. Stock solutions may be used at either 1% or 0.1%; from there, calculated amounts are added to the melted medium, which is distributed in Petri dishes. Several strains, including the controls, are inoculated on each plate.

Cruikshank (1948) has developed an ingenious simplification of the dye test. Strips of filter paper previously sterilized are impregnated with a solution of fuchsin and thionin at a concentration of, say, 1 : 200 and 1 : 600 respectively, or whatever may be convenient. The stained strips may be kept in stock. For use, one of each is placed at the bottom of a Petri dish which is covered with melted Albimi agar. The strips should be laid parallel in order to facilitate comparison of the growth of organisms which are spread in a uniform line across the plate.

Hydrogen sulfide test

The reagent which is employed to detect the production of H_2S is prepared with a 10% solution of lead acetate with which strips (1 cm × 5 cm) of filter paper are impregnated; these are allowed to dry before use. A strip is placed at the upper part of a test-tube and held in place by a cotton stopper. The strip is replaced by a new one after the first 24 hours of incubation of the inoculated tube and so on at 24-hour intervals until the fourth or fifth day. If hydrogen sulfide is produced, the paper becomes more or less intensely blackened and the duration and intensity of these changes are recorded and interpreted as indicated in the text (page 77).

Annex 6

MONOSPECIFIC SERA

Monospecific sera are difficult to prepare. Animals are inoculated with carefully selected strains of *Br. abortus* and *Br. melitensis* and the sera absorbed with the heterologous antigen. Because of the irregularity in results, reported by several laboratories, it is advisable to request samples of sera from Dr A. W. Stableforth, Central Veterinary Laboratory, Weybridge, Surrey, England, or from Dr G. Renoux, Laboratoire de Microbiologie, Faculté de Médecine, Université de Montpellier, France. Both preparations have been found to be quite satisfactory for the differentiation of strains isolated in Mexico.

A recommended method for the preparation of monospecific sera has recently been published (Jones, 1958), giving details which enable better and more regular results to be obtained.

Unknown *Brucella* strains are tested against serial dilutions of both *abortus* and *melitensis* sera. The serum which, after 24-48 hours of incubation, produces the higher titres of agglutination will indicate the type of the strain under test.

In our experience with tehse sera we have been impressed with the results of a preliminary slide test, in which a drop of serum is mixed with another drop of an emulsion of the culture prepared on the same slide. The differentiation is usually sharp and, so far, has always been corroborated by the tube dilution method.

Annex 7

REVIEWERS

Professor A. Grumbach, Hygiene Institute of the University, Zurich, Switzerland

Professor G. Löfström, Department of Bacteriology and Hygiene of the University, Uppsala, Sweden

Professor J. F. Murray, Dr L. Schrire and Dr V. Bokkenheuser, The South African Institute for Medical Research, Johannesburg, Union of South Africa

Dr G. Pacheco, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

Dr G. S. Wilson, Public Health Laboratory Service, London, England

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