Determination of the most accurate diagnostic approach for the diagnosis of human brucellosis in Lankien, South-Sudan

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Study protocol

MSF International

Epicentre

Institute for Tropical Health,

University of Navarra, Pamplona, Spain

Ministry of Health, South-Sudan

October 2016

Study	**Determination of the most accurate diagnostic approach for the diagnosis of human brucellosis in Lankien, South-Sudan**		
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Contents

Acronyms and abbreviations

ANC	Ante natal care
GMP	Good Manufacturing Practice
ITH	Institute for Tropical Health at the University of Navarra, Pamplona, Spain
IPD	In-patient department
KIT	Koninklijk Instituut voor de Tropen, Amsterdam, Netherlands
LPS	Lipopolysaccharide antigen
MSF	Médecins sans Frontières
MTA	Material transfer agreement
OCA	Operational Centre Amsterdam
OPD	Out-patient department
PNC	Post natal acre
RDT	Rapid diagnostic test
SAT	Serum agglutination tests
WHO	World Health Organization

1.

1. Background

Brucellosis is caused by facultative intracellular, gram-negative partially acid-fast coccobacilli. Three species are the most common agents of human disease (*B. abortus, B. melitensis, B. suis*). Professional contact (e.g. shepherds, farmers, veterinarians and butchers) and food borne transmission (general public) is the main source of human brucellosis.

Brucellosis can be found worldwide. However, it is more common in countries that do not have good standardized and effective public health and domestic animal vaccination programs. There are only a few countries in the world that are officially free of the disease although cases still occur in people returning from endemic countries.

The reported incidence in endemic areas varies between 0.3 to >1,600 per 1,000,000. The true incidence, however, is suspected to be much higher due to misdiagnosis. Brucellosis affects people of all age groups and of both sexes.

Incubation period usually is 2-3 weeks in acute cases (approx. 50 %). In the other half, the onset is insidious, with signs and symptoms developing over a period of weeks to months from the infection.

The clinical manifestations vary, are non-specific and include symptoms such as: inconstant fevers, headache, anaemia, fatigue, weakness, sweats, myalgia, arthralgia, abdominal pain, weight loss, constipation, spontaneous abortions etc. Usually, the patients' symptoms worsen towards the evening. The desire to rest can be profound, and depression is pervasive. If untreated, the fever can increase and wane over several days ('undulant fever') but other forms such as a constant fever are observed as well when the disease evolves. Complications may affect any organ (Food and Agriculture Organization of the United Nations, 2006). Long evolution (undiagnosed and untreated brucellosis) cases more often develop into chronic forms and then an afebrile pattern may appear with a history of myalgia, fatigue, depression, and arthralgia (chronic fatigue syndrome is the most important disease in the differential diagnosis). The chronic form is primarily caused by *B melitensis* and usually affects adults older than 30 years. Mortality varies from 1 to 5% in untreated cases and is almost always the result of Brucella endocarditis. The chronic form is rare in children (Vircell, 2008).

1.1 Diagnosis of brucellosis

The diagnosis of human brucellosis cannot be made solely on clinical grounds due to the wide variety of clinical manifestations of this disease, and it is essential to perform serological tests and wherever possible bacteriological tests.

Isolation of *Brucella spp*., usually from blood and occasionally from other organ tissues, is definite but not available in resource-constrained settings. In addition, blood culture is often

negative in long evolution cases, has to be repeated sequentially and requires biosafety practices (Diaz et al., 2011). Serology is generally considered as the most useful diagnostic procedure approach as some of these tests are easy to perform and implement (Diaz et al., 2011; Diaz et al., 1989). Different tests are available (e.g. Rose Bengal test [RBT], serum agglutination test [SAT], Coombs test, Brucellacapt and several ELISA based tests) that detect either agglutinating or non-agglutinating antibodies or both. Agglutinating antibodies (IgM antibodies but also agglutinating fractions of IgG and IgA) are normally present in acute (short evolution) brucellosis, but they disappear as the disease progresses. Thus, in the chronic forms of brucellosis the antibodies are predominantly non-agglutinating IgG and IgA (Vircell, 2008). All of these tests have in common that they detect antibodies to the major surface antigen (i.e. smooth [S] lipopolysaccharide [LPS]).

Serological assays against S-LPS

Commonly, a rapid sensitive screening test, as the Rose Bengal test (RBT) (produced by several manufacturers, e.g. Spinreact, Sant Esteve de Bas, Spain) is performed. The RBT detects anti-*Brucella* IgM, IgG and IgA antibodies (Spinreact, 2013). This test was originally developed for the serological diagnosis of cattle brucellosis and is performed in lactate buffer at pH 3.7 in order to remove the unspecific agglutinations observed with some cattle sera. Studies carried out with human sera show that at this pH non-agglutinating human antibodies become agglutinating. Nevertheless, the pH and other conditions have not been optimized for human sera. The sensitivity of RBT is close to 100% but it can give false positive reactions with *Y. enterocolitica* O:9 infected patients and perhaps other cross-reactive organisms, but not with sera from patients with other common infectious conditions such as typhus, malaria and others (Mert et al., 2003). Also, weak positive results can be observed upon repeated contact not followed by clinical disease in endemic areas (Ruiz-Mesa et al., 2005). However, these two specificity problems are not fully solved by any standard brucellosis test, as all detect antibodies to the S-LPS, the immunodominant *Brucella* antigen and the cross-reacting molecule. The results of RBT are usually supplemented in resourced settings with one or several of the following tests that use serum dilutions to obtain a quantitative picture that complements the clinical history and presentation. These tests are the following:

a. The modified RBT is adapted from RBT to test serum dilutions made directly on a glossy plate. A positive test at dilutions (titres) higher than 1:4 allows discriminating contacts from infected individuals with high specificity and sensitivity (Diaz et al., 2011; Dabdoob et al., 2000). In hot climates the plate can be covered in a plastic chamber to avoid sera and reagents drying out. The test requires almost no infrastructure but recent experience in Nigeria stresses the need for proper training and adherence to all details of the test procedure.

b. The serum /standard/slow (tube) agglutination test (SAT) (e.g. BioSystems, Barcelona, Spain) similar to the other tests described here, detects agglutinating antibodies against the S-LPS of the outer cell membrane. The SAT is generally used in combination with tests detecting non-agglutinating antibodies as non-agglutinating antibodies increase during the evolution of the disease. Therefore, the combination of tests allows assessing the stage of disease evolution and therefore possibility of focal forms and complications. SAT titres can be expressed in International Units (IU). This test requires a laboratory technician and overnight incubation.

c. The Coombs test is one of the tests that can be used in combination with SAT because it detects non-agglutinating (also known as incomplete) antibodies in the supernatant of a SAT test by adding serum containing anti-human immunoglobulin, which then causes the agglutinations of brucellae carrying specific but non-agglutinating antibodies. Accordingly, the Coombs test is necessarily carried out after SAT but not directly because it requires a washing step of the bacteria in SAT supernatants to remove unspecifically absorbed immunoglobulins (Vircell, 2008). Non-agglutinating antibodies increase progressively as the infection evolves so that the SAT/Coombs combination varies from high SAT / negative or low Coombs tires in recent (acute) cases to low or negative SAT / high Coombs in the long evolution cases. In addition, an increase of titres in the Coombs test may indicate a reactivation of the disease. As for the SAT, the Coombs test requires some expertise and a laboratory equipped with incubators and a centrifuge. There are no commercial kits available and the test must be standardized in each laboratory.

d. The Brucellacapt assay (Vircell, Granada, Spain) is a single-step immunocapture assay for the detection of total anti-Brucella antibodies. The test consists of U-bottom well-strips coated with anti-human immunoglobulins. After addition of serum dilutions, the antigen (prepared in a special buffer at pH 5.0) is added and strips are incubated for 24 hours to allow for immunoglobulin-coated bacteria to settle. The bacteria carrying specifically bound immunoglobulins are then captured by the anti-human immunoglobulins on the walls of the wells. This allows the reaction to be visualized. The assay allows the detection of both agglutinating and non-agglutinating (incomplete) antibodies and reflects closely the results of the SAT and Coombs test combined (Orduña-Domingo et al., 2008). Also this test requires some expertise and a laboratory equipped with incubators.

e. A lateral flow immunoassay rapid diagnostic test has been developed by the Koninklijk Instituut voor de Tropen (KIT), Amsterdam, Netherlands. This test allows the semi-quantitative detection of IgM and IgG on separate strips and is therefore useful to assess the state of evolution of the infection. It has been manufactured under the name Test-it™ Brucella IgM / IgG Lateral Flow Assay by Life Assay, South

Africa (Life-Assay) and evaluation results were very encouraging (Smits et al., 2003; Irmak et al., 2004). However recent quality problems during the manufacturing process resulted in interruption of the test production.

f. ELISA tests detect antibodies against *Brucella* spp and are commercially available (e.g. Brucella-Ab cELISA, Svanova Biotech, Uppsala, Sweden). However, to our knowledge none of the commercial tests have been rigorously evaluated using reference standard sera. In theory ELISA tests could measure IgM and IgG separately, which could aid the disease staging: 'acute' versus 'chronic'.
ELISA tests can only be performed in a well-equipped laboratory with well-trained staff.

Serological assays against cytosoluble proteins

Contrary to S-LPS, *Brucella* cytosoluble proteins are not a source of unspecific reactions created by *Y. enterocolitica* O:9 or other bacteria that show cross-reactivity in the standard tests. These cytosolic antigens have been shown of diagnostic value in a counter-immunoelectrophoresis set up (Diaz et al., 1976), which requires appropriate equipment in a well-equipped laboratory. Antibodies to *Brucella* cytosolic proteins can be tested in other formats, the simplest ones being a gel immunoprecipitation and passive agglutination (such as latex) tests. However, there is a paucity of studies on these potential applications.
No commercial serological assays against cytosoluble proteins are available.

1.2 Treatment of brucellosis

The essential element in the treatment of all forms of human brucellosis is the administration of effective antibiotics for an adequate length of time. Treatment of uncomplicated cases in adults and children of ≥8 years of age is doxycycline 100 mg twice a day for six weeks plus streptomycin 1 g daily for two to three weeks. Alternatively, doxycycline 100 mg twice a day for six weeks plus rifampicin 600–900 mg daily for six weeks can be administered. Special regimes are necessary for pregnant women, children of <8 years of age and in some complications of brucellosis disease (e.g. focal forms, endocarditis) (Zinsstag et al., 2011; Ariza et al., 2007) – see Annex 9.

2. Study rationale

Approximately 5-8 years ago, an Irish company produced a Brucella test based on the prototype (i.e. lateral flow immunochromatography [LFI] assay) developed by the KIT (Koninklijk Instituut voor de Tropen), Amsterdam, the Netherlands, which was used with great satisfaction within MSF.
However, approx. 4 years ago this company (Organon, Ireland) was sold and the new owner decided to no longer produce the Brucella test. Another manufacturer in South-Africa (Life-Assay) was found to produce the test but technical problems due to poor manufacturing forced MSF to abandon the test from its programmes. Left without a LFI for brucellosis testing, MSF decided to introduce the more complex RBT (Vircell, Granada, Spain) (Vircell, 2005). Unfortunately, some of our programmes were facing an unrealistically high positivity rate of up to 80% when using the RBT suggesting a low specificity of the test when used undiluted in this context (e.g. South-Sudan). In part, this could be because sera from chronic cases produce rim-like agglutination reactions and to distinguish them from negative results is often not straightforward, in particular when the temperature is high and evaporation creates artifacts. Other tests such as the Brucellacapt (Vircell, 2008), ELISA, SAT or cultures are too complex to be used in resource-constrained settings (Food and Agriculture Organization of the United Nations, 2006).
Thus, MSF is facing once again a situation where no easy, resource-constrained setting appropriate diagnostic test for brucellosis is available.

While waiting that the KIT finds a good manufacturing facility for its test, we aim to to store specimens for future testing of a possible commercial product after technology transfer to a permanent manufacturer and/or of other tests for brucellosis.

In the meantime, the most promising alternative option to the LFI in this type of settings is the modified RBT using dilutions to increase specificity. As indicated above in an evaluation on sera from culture-confirmed brucellosis patients and healthy contacts or healthy controls, the specificity of RBT was improved to 100 % when a serum dilution of ≥1:4 was used, and the sensitivity of the RBT test at this dilution was 87.4 % (Diaz et al., 2011). In this evaluation, the modified RBT showed the best performances compared with other methods used on the same panel of specimens, including SAT, Coombs, competitive ELISA, Brucellacapt and lateral flow assay (Diaz et al., 2011).
In addition, we would like to optimize the buffer used in the RBT to improve specificity and optimize reading of the RBT as the RBT tends to produce rim-like reactions (see above). To date the buffer conditions are those used for diagnosis in cattle and they have not been optimized for diagnosis in humans. In fact, the conditions used in the Brucellacapt (i.e. a

special buffer at pH 5.0) also render all antibodies agglutinating. Therefore, some simple modifications of the RBT conditions (i.e. pH and ionic strength) may improve the performance of RBT and produce a similarly simple but better test.

Finally, we would also like to test an in-house latex-agglutination test against Brucella-specific proteins, which would also be a good option for field use if performances are correct.

3. Objectives

3.1 Primary objective

To estimate the diagnostic accuracy (sensitivity, specificity, positive and negative predicative values and likelihood ratios) of the modified RBT method and the rapid diagnostic test developed by the KIT tests performed (if commercially available) at Institute for Tropical Health (ITH), the University of Navarra, Pamplona, Spain for the diagnosis of brucellosis.

Specimens collected in an endemic region (South Sudan) will be used and characterized at the ITH at the University of Navarra, Pamplona, Spain with undiluted RBT, SAT, Coombs test, Brucellacapt and when necessary an indirect ELISA used as the reference tests.

3.2 Secondary objectives

- To assess the diagnostic accuracy (sensitivity, specificity, positive and negative predicative values and likelihood ratios) of the Rose Bengal test (Spinreact, Spain) at the study site.
- To assess inter-user agreement of the RB test performed on site and at ITH.
- To optimize the buffer used in the RBT using characterized sera available at ITH and evaluate the diagnostic performance of the modified method with serum dilution using specimens collected in this study. To date the buffer conditions are those used for diagnosis in cattle and they have not been optimized for diagnosis in humans. In fact, the conditions used in the Brucellacapt (i.e. a special buffer at pH 5.0) also render all antibodies agglutinating. Therefore, some simple modifications of the RBT conditions (i.e. pH and ionic strength) may improve the performance of RBT and produce a similarly simple but better test.
- To estimate the diagnostic performance of an ‘in-house’ latex-agglutination test against Brucella-specific cytosoluble proteins.
- To describe the clinical characteristics of brucellosis suspects and confirmed cases
- To assess/identify risk factors for brucellosis in the study population

4. Methods

4.1 Study design

This is a prospective evaluation of brucellosis diagnostic assays.
Samples of patients presenting with symptoms suggestive of acute brucellosis at Lankien, South Sudan will be collected and shipped to the Institute for Tropical Health at the University of Navarra, Pamplona, Spain for analysis with both index and reference tests.

4.2 Sample size

To estimate an expected test sensitivity of 85% with a precision of 6% and a specificity of 90% with a precision of 5%, a minimum of 136 positive specimens and 138 negative specimens will be needed. In the absence of good test in the field, the prevalence of brucellosis is not known. Considering that the prevalence of brucellosis is probably comprised between 5% and 15%, the total sample size can vary from 906 to 2720 patients. We propose to do an interim analysis after 50-100 patients and complete the inclusions based on the prevalence found in this interim analysis, if necessary.

At the interim analysis it will also be assessed if the RBT undiluted or the modified RBT has the best diagnostic accuracy. If it is shown that the modified RBT has a better diagnostic accuracy the protocol will be amended and treatment will be provided based on the modified RBT result.

4.3 Study site

The study will take place in Lankien located in Jonglei state, or a similar project site where MSF will be operational South Sudan, depending on population movement and security situation.
Médecins Sans Frontières Operational Center Amsterdam (MSF-OCA) has been working in Lankien since 1995 to reduce the mortality and morbidly in the general population. The overall population size is estimated at 100,000-250,000.
Lankien hospital is a comprehensive health care facility covering both primary and secondary health care needs. There is an out-patient department (OPD), an in-patient department (IPD), a TB/HIV unit, a Kala-Azar unit, a maternity unit with pre- and post- natal care (PNC/ANC), an emergency room, a therapeutic feeding unit, a mental health program and a surgical ward. The hospital is also involved in surveillance and response to outbreaks which have included Kala Azar, cholera and malaria in the past.

In 2013, 71,087 consultations were carried out in the OPD and 1,839 patients were admitted in the IPD. Basic laboratory services are available to support diagnostic activities.
Brucellosis has been a significant problem in the catchment area which is made up mainly of pastoralists with the following diagnosis over two years.

Brucellosis has been a significant problem in South-Sudan. In Lankien, the brucellosis testing with the RDT developed by KIT and manufactured previously in South Africa by Life Assay and Organon in Ireland, has been carried out and the following outcomes were recorded:

Year* **	**# tests performed**	**IgM -/IgG -**	**IgM +/ IgG-**	**IgM-/ IgG+**	**IgM+ /IgG+**	**Total # (%) positive***	**Total # (%) positive****
2008	383	253	27	19	84	130 (34 %)	111 (29 %)
2009	37	23	3	3	8	14 (38 %)	11 (30 %)
2011	366	273	25	12	56	93 (25 %)	81 (22 %)
2012	796	583	78	41	94	213 (27 %)	172 (22 %)
2013	1090	844	67	64	115	246 (23 %)	182 (17 %)
Total	**2627**	**1976**	**200**	**139**	**357**	**696 (27 %)**	**557 (21 %)**

*Positive was defined as IgM+/IgG-, IgM-/IgG+ or IgM+/IgG+

** Positive was defined as IgM+/IgG- or IgM+/IgG+

*** No testing was carried out with RDTs in 2010 and only briefly in 2009.

4.4 Study population

The study population will be patients with clinical symptoms suggestive of brucellosis accessing the healthcare facilities in the MSF-OCA Hospital in Lankien, South-Sudan (or a similar site depending on population movement and security).

4.4.1 Inclusion criteria

- Age ≥ 5 years (to ensure intravenous blood sampling)
- Self-reported fever within the last 7 days plus at least one of the following criteria
    - Lack of energy/fatigue
    - Body aches
    - Sweats
    - Joint or back pain
    - Worsening condition when day progresses
- No other confirmed diagnosis (e.g. malaria, tuberculosis)
- Written informed consent obtained

4.4.2 Exclusion criteria

- Insufficient specimen collected
- Acute danger signs such as convulsions, non-ability to drink, repeated vomiting, inability to walk/sit/speak, severe dehydration, severe diarrhea or signs for kidney failure (e.g. production of only small quantities of urine).

4.5 Study procedures

4.5.1 Inclusion and initial evaluation

Patients presenting with a history of fever within the last 7 days will be seen by a clinician. If the patient presents with inclusion criteria, he/she will be sent to the study coordinator, who will inform the patient on the study and study procedures according to the Information Note (Annex 4). After answering any question from the patient, the study coordinator will ask him/ her to sign the informed consent if (s)he agrees to participate.

The study coordinator will collect the sociodemographic and clinical information (Annex 5 or see below).

The study coordinator, i.e. laboratory technician, will collect 8 mL of blood in two plain tubes for serum preparation.

4.5.2 Laboratory methods on site

The Rose Bengal test (SpinReact, Spain) will be performed on site according to manufacturer's instructions.

On all initial

Serum aliquots will be prepared from blood by centrifuging the sample (see Annex 3)

8 mL will be separated in 2 aliquots and stored at -20°C until shipment to ITH, Spain.

4.5.3 Laboratory methods at ITH

Both reference methods and index tests will be performed at ITH.

<u>Reference methods</u>

All sera will be characterized using following tests (see annex 8 for package inserts and SOPs), performed according to the manufacturer's recommendations:

1. Rose Bengal test (Spinreact, Sant Esteve de Bas, Spain, # 1200901, 50 tests)
2. Serum agglutination test (SAT) (BioSystems, Barcelona, Spain, # 33309, 1 x 5 mL; 50 µL per sample)
3. Coombs test (in house assay from Institute for Tropical Health at the University of Navarra, Pamplona, Spain)
4. Brucellacapt (Vircell S.L, Santa Fe, Granada, Spain; # Brucapt, 24 tests per kit)
5. Brucella-Ab cELISA Svanovir (Svanova Biotech, Uppsala, Sweden; # 10-2701-10 10 plates for max. 880 samples excl. controls, 960 tests)

Note: Although generally preferred for the reference standard, culture and molecular testing are not an option to perform in humans as sample must be taken from fresh blood, or less often from organs that are directly affected by brucellosis e.g. cerebrospinal fluid, abscesses, etc.

Index tests

6. Modified RBT assay with dilutions (dRBT) from Spinreact and possibly other manufacturers.
7. The lateral flow immunoassay developed by the KIT, Amsterdam, Netherlands for detecting IgM and IgG antibodies against brucellosis will be performed according to the manufacturer's recommendations once commercially available under good quality manufacturing conditions.

All specimens found positive by RBT (undiluted serum; 1:2 titer upon mixing with the RBT antigen) will be used to perform the RBT on two-fold serial dilutions from 1:2 to 1:16 on the serum made with sterile saline. The plain serum and these dilutions will be used to perform the RBT test as recommended by the manufacturer. The last dilution that gives a positive result will be considered as the serum titer (plain serum 1:2; titer; dilutions, titers from 1:4 to 1:32 (See protocol in Annex 8) (Díaz et al 2011; Mantur et al. 2014)

8. RBT assay with optimized buffer (obRBT)

The use of pH 3.6 in RBT was originally optimized for the diagnosis of brucellosis in cattle as a method to remove non-specific agglutinations while preserving most of the agglutinating activity of the anti-Brucella specific immunoglobulins and abrogating the prozone effect (i.e. absence of agglutination when testing plain sera) (Rose and Roepke, 1957; Davies, 1971) . However, a similar study has not been performed with human immunoglobulins and two observations indicate that a pH better than 3.6 may be found. First, in human brucellosis a positive RBT correlates with SAT titers better than with Coombs titers (Díaz et al., 1976) suggesting that RBT may be missing a fraction of immunoglobulins. Second, Brucellacapt employs a buffer at pH 5 and it is not affected by prozones. Although this shows that this pH is enough to promote the agglutinating activity of human IgG (Díaz et al., 2011), some SAT positive sera are not detected by Brucellacapt (Orduña-Domingo et al., 2000). Therefore, an in-house (IHT) modification of the RBT based on the use of a range of buffers (pH 4-6) will be tested with reference sera available at IHT, to obtain the optimal pH conditions for the RBT, defined as increased titers and a more refined specificity/sensitivity ratio or as sera positive in at a given pH but not at other. Then, the test with optimized buffer will be used on all sera undiluted and, when positive, with dilutions as described above.

9. Latex agglutination assay against Brucella-specific proteins

The *Brucella* cytosolic protein fraction, obtained as described previously (Blasco et al., 1994)] will be used in a reverse radial immunoprecipitation test and a used to sensitize latex beads, as described previously for S-LPS rich extracts (Iannelli et al., 1976; Abdoel et al., 2007). These tests will be first evaluated with reference sera available at IHT in order to define optimal conditions (i.e., plain or modified latex, latex bead size, protein concentration, sensitization buffer, temperature and time). Then, the modified test will be used on all sera.

4.5.4 Treatment

Patients suspected of brucellosis will be treated by MSF following the protocol described in Annex 9. Results of the Rose Bengal test will be used together with the clinical picture for treatment management.

If the LFI used at site is negative for acute brucellosis but the reference standard results are positive (i.e. false negative result at site), the patient will be traced, if s/he agreed so, to provide appropriate treatment upon reception of the laboratory results.

4.6 Reference standard definition

A patient (specimen) will be considered positive for brucellosis if

- RBT positive (undiluted) plus
- At least one of the tests is positive:
  SAT titer ≥ 160, Coombs IgG positive or Brucellacapt titer ≥ 320.

A patient (specimen) will be considered negative for brucellosis if

- All tests are negative in undiluted form (Note: the lateral flow-immunoassay from KIT is not considered here) or
- Only RBT is positive.

In all other cases, the patient will be considered indeterminate for brucellosis.

4.7 Data collection

Socio-demographic and clinical data will be collected on site using a standardized case report form (CRF – Annex 5). Laboratory data (Annex 7) will be collected at ITH using a laboratory form.

Data will be entered using EpiData 3.1 software (EpiData, Odense, Denmark). Variables from Annex 5 will be collected in South-Sudan and double entered on site for verification.

The variables from Annex 7 will be generated at the reference in the laboratory in Spain. The data sets will be merged using the identifier variable: study inclusion number.

4.8 Analysis

The socio-demographic characteristics will be described for the overall population, as well as confirmed cases only (positive reference standard). All dichotomous variables will be described by their percentage and 95% confidence intervals.
The results from the different tests under evaluation (i.e. modified RBT with dilutions, LFI) will be compared to the results of the reference standard, as defined above (4.6), to calculate sensitivity, specificity, positive and negative predictive values, likelihood ratios and their 95% confidence intervals. Patients with indeterminate brucellosis diagnosis by the reference standard, as defined in 4.6, will be excluded from the analysis.

Risk factors will be first assessed by univariate analysis. Factors associated with brucellosis in the univariate analysis will be included in a multivariate analysis using logistic regression. The crude and adjusted relative risks will be presented with their 95% confidence intervals.

Analysis will be carried out using STATA version 13 (StataCorp, College Station, Texas, USA).

5. Formal and ethical approval

The study will be approved by the MSF Ethical Review Board. It will be carried out after approval at the project site in Sudan and by the Ethical Committee of the Medical School and the Institute for Tropical Health at the University of Navarra, Pamplona, Spain as well as the ethical review board in South-Sudan.

The study will be carried out in accordance with the Declaration of Helsinki concerning medical research in humans. All enrolled clients will sign or fingerprint the informed consent. Minors below the legal age of consent in the study country will only be included if their parent or culturally acceptable guardian consents to the study. Mature minors (age 14-18) will be asked in addition to give oral consent.

The study consent from will be read to illiterate patients by the local study coordinator in the presence of a witness. Each study participant will be given the possibility to ask questions. The study coordinator will address those.

Participation will be voluntary. All results will be made available to the clinicians associated with the health facility.

Risks

A risk compared to normal procedures is associated with the additional venous blood puncture where most current test protocols require only a finger prick. Venous blood collection is a very common procedure, which may cause minor pain.

Benefits

- Personal benefits: Study participants will have the opportunity to know their results of the rapid and the reference standard testing which otherwise would not be available to them. Treatment decision will be made based on the rapid diagnostic test result and clinical grounds at the health facility.
- Communal benefits: The local community will be informed of the study and its rationale through the local health care facilities. Written materials in the local language will be made available. The results will be shared with the community leaders upon completion, again through meetings with the community, and local health professionals.

  The study is expected to directly benefit the study community by contributing to the knowledge of brucellosis prevalence. As stated above, the information will be used to change MSF practice to improve the quality of brucellosis screening as well as inform MoH and other local testing centres on the study outcome and implications on MSF testing strategy for brucellosis.

Local collaboration

The Ministry of Health at local and national level will be consulted prior to implementation of the study, and the research findings will be discussed with relevant Ministry of Health bodies on completion of the study.

In South-Sudan, MSF staff work alongside Ministry of Health staff and the protocol will be implemented in conjunction with them.

The investigators will also aim to publish results in a peer-reviewed journal.

6. Collaboration and financing the study

The Institute for Tropical Health at the University of Navarra, Pamplona, Spain is MSF's and Epicentre's counterpart. MSF will be in charge of patient screening and inclusion, specimen collection and shipment of all required samples to the Institute for Tropical Health at the University of Navarra, Pamplona, Spain.

Employees of the the Institute for Tropical Health at the University of Navarra, Pamplona, Spain will carry out laboratory testing as described in the protocol.

All costs related to testing at programme site, treatment and the shipment will be covered by MSF.

The cost for testing at the Institute for Tropical Health at the University of Navarra, Pamplona, Spain will be covered by the ITH.

ITH will report results to MSF/Epicentre, who will analyse the data and prepare the final report with support of the ITH counterpart.

More details are outlined in the material transfer agreement (MTA), see annex 1.

7. References

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Annexes

Annex 1: Material Transfer Agreement (MTA)

MTA - Brucellosis study MSF Epicentre ar

Annex 2: Order list of items for study site and Laboratory in Spain

The study site has to order the following items:

	Item	Manufacturer	Cat number MSF	Amount
Sample collection				
1	Gloves, size M	N/A	SMSUGLOE1M-	1200
2	Coat, medical, white, medium	N/A	ELINCOAW1M-	2
3	Tray, dressing, 30 x 20 x 3 cm ,stainless steel	N/A	EMEQTRAD3--	3
4	Tube holder with needle injector, reusable	Becton Dickinson	ELAEBSVV1H-	20
5	Cotton wool, hydrophilic, roll, 500g	N/A	SDRECOTW5R-	2
6	Tourniquet, elastic, 100 x 1.8 cm	N/A	EMEQTOUR1--	1
7	Tube vacuum, serum	Becton Dickinson	ELAEBSVT5P-	1500
8	Needle for blood sampling system 21G	Becton Dickinson	ELAEBSVV21N	500
9	Polyvidone iodine, 10%, solution, 200 ml	N/A	DEXTIODP1S2	2
10	Marker, permanent, black, fine point	N/A	ELAEMARK1B-	3
11	Rack, tube, 5 ml,	N/A	ELAETUHA1R-	2
12	Reusable sharps container, 1.2 litre	N/A	SINSCONT1R-	1
13	Detergent, desinfectant for surfaces 5 l tin + pumping dose	N/A	DDISSURF5B-	1
Sample preparation				
14	Centrifuge, Hettich EBA	Hettich	ELAECENT4E-	1
15	Pipette blue, 100-1,000	Eppendorf	ELAEPIAA100	1
16	Pipette yellow, 10-100	Eppendorf	ELAEPIAA1--	1
17	Tips blue	Eppendorf	ELAEPIAATB-	1500
18	Tips yellow	Eppendorf	ELAEPIAATY-	1500
19	Tip, yellow, rack	Eppendorf	ELAEPIAATYR	1
20	Tip, blue, rack	Eppendorf	ELAEPIAATBR	1
21	Microtube,flat bottom, non-sterile, screw cap, 2 ml	Not specified	ELAETUBE2--	1500
22	Box with stand for 81 cryotubes	Not specified	ELAETUMI1B	20
23	Rotator orbital		ELAEROTE2--	1
Sample testing				
24	Rose Bengal test, 50 tests (cat # 1200901)	Spinreact	No MSF cat number yet	100 (5000 tests)
Sample storage				
25	Freezer, 111 litres (Vestfrost MF 114)	Vestfrost	PCOLFREE1E-	1
26	Ice pack, empty for water, 0.4 l, gio-style	N/A	PCOLPACKW04	30
Sample shipment				

	Item	Manufacturer	Cat number MSF	Amount
27	Box isotherm, triple pack, biological substance [UN3373]; keeping samples between 2 - 8 °C for 72 hours	Not specified	PPACUN62DSI	5
28	Freezing indicator, (freeze-tag)	N/A	PCOLCONT3FT	5
29	Temperature monitor, Log Tag, TRID30-7R	N/A	PCOLCONTLT3	5
Data entry				
30	Computer, laptop, entry-level	N/A	ADAPCOLA-	1
Local purchase				
31	Sticker for cryotubes labelling	N/A	N/A	5000
32	Notebook A4			2
33	Pens			5
34	Ruler			1
35	Stapler			1
36	Whole puncher			1
37	Folder A4			3

The laboratory in Spain has to order the following items:

	Item	Manufacturer	Cat number manufacturer	Package size	Amount
1	Rose Bengal test	Spinreact, Sant Esteve de Bas, Spain	1200901	50	50*
2	SAT**	BioSystems, Barcelona, Spain	# 33309	1 x 5 mL; 50 uL per sample	1-2
3	Coombs test	In-house assay	-	-	For 100 tests
4	Brucellacapt**	Vircell S.L, Santa Fe, Granada, Spain	Brucapt	24 tests per kit	4-5
5	Brucella-Ab cELISA Svanovir	Svanova Biotech, Uppsala, Sweden	10-2701-10	96 per kit	10
6	Pipette tips, yellow, 10-100 uL	Eppendorf	003000870	500	3
7	Other laboratory equipment and consumables	-	-	-	-

*Based on eth assumption that 4 dilutions per sample are required average.

** On positives only. Estimated a max. of 100 positive samples (min. of 73 per sample size calculation).

MSF will order the following items for the laboratory in Spain:

	Item	Manufacturer	Cat number manufacturer	Package size	Amount
1	Brucella IgM/IgG assay 25 tests per pack	unkown	Brucella IgM/IgG assay	25	112 (i.e. 2800 tests total)

Annex 3: Workflow at study site

1. Ask consecutively all patients that get tested for Brucellosis infection to participate in the study.
2. Explain the study and take inform consent of all patients agreeing to participate in the study until 1000 patients are included for the interim analysis.
3. If patient agrees collect required information on the study form.
4. Collect an additional tube of serum next to the specimen required for the LFI.
5. Prepare 2 serum aliquots and label specimen with the study number.
6. Freeze the specimen at -20 °C.
7. Once all specimens are collected ship all specimens to ITH (see SOP transportation to laboratory in Spain).

Annex 4: Informed Consent

Informed Consent (English)

INFORMED CONSENT FOR PATIENTS: Diagnostic approach brucellosis Lankien, South-Sudan

To be translated by local staff and read to the patient in the local language. Patients will then be asked for consent by signature or fingerprint. Mature minors will be asked to give oral consent in addition to the parent or culturally acceptable guardian. The consent form will be signed twice: one copy will be given to each participant and one copy will be kept at the study site.

Brucellosis is a disease that causes fever, fatigue, loss of appetite and weight and has been common in this region in the past. Testing for brucellosis is currently not available in this region.

Brucellosis requires complex tests to be diagnosed. We aim to introduce the first-line test (Rose Bengal) for brucellosis in Lankien and treat you based on the results of this test.

Furthermore, we aim to aim to look for alternative tests and adjustment of current tests for brucellosis, that could maybe be used here or in similar settings in the future. We hope that these tests are easier to be carried out and better (i.e. more accurate) than the currently recommended first-line test (Rose Bengal).

Therefore, we aim to collect some of your blood and send it to a specialized laboratory in Spain in order to:

- Examine your blood with a number of tests to determine if you are infected with brucellosis.
- Use your blood on other more simple tests to check how well they are working in comparison to test used now at this clinic/hospital.
- Use your blood to adapt the first-line test for brucellosis now being used at this clinic/hospital to make it better (i.e. increase its accuracy).

In order to carry out all these examinations, especially examine newly developed simple tests; it may be necessary that your blood will be stored for up to 5 years, perhaps even longer.

We would ship your blood to a specialized laboratory in Spain, where testing for brucellosis will be done.

Procedures and risks
Therefore, if you accept to participate in the study, we would like your permission to take some extra blood (2 tubes, each 4 mL). The drawing of the blood for the test is slightly painful for a short moment but has no serious side effects.

As explained above, we aim to introduce the first-line test screening test for brucellosis in Lankien and treat you based on the results of this test. This test is fairly accurate according to previous studies but has the disadvantage that it can show false-positive results. Thus, you may be treated although you are not infected with brucellosis. However, if this is the case and your symptoms remain after the treatment has been completed, we ask you to return to the clinic/hospital, for further assessment.

Privacy and confidentiality
The blood used for this study will not be labeled with your name so that your privacy will be guaranteed. The blood will be exclusively used for this study.

Also, study records will contain only ID codes but will NOT show individual names. This will be ensured for the counselling and testing process as well as for all analysis linked to the study.

Voluntary participation
Your participation to this study is voluntary. If you do not wish to participate, you do not have to, and you do not have to give a reason. You can also withdraw your consent at any time during the testing procedure. Your decision to participate or not has no influence to your further treatment. There will not be any financial expenses for you to participate in the study. You will also not be paid to participate.

Benefits
Treatment decision will be made based on the result of a test used in the laboratory here in Lankien and on clinical grounds at the health facility. However, if the test result at the reference laboratory in Spain shows a positive result but do not start treatment today after the physician's examination, you can be traced to receive this information (e.g. by phone, at your home or at another address), and you will be treated by MSF for brucellosis. The treatment is free of charge.

Alternatively to being traced, you can make a new appointment to receive the test results obtained at the reference laboratory or it can be revealed to you at your next follow-up appointment.

If we find a testing method that can determine well if you are infected with brucellosis and if this testing method is fully validated by the Medical Directors in MSF, we commit to make this test(s) immediately available in Lankien and other locations in South Sudan, once the study is completed.

If you agree to participate in the study, we will ask you to fill in and sign/fingerprint the form below in 2 copies. One copy will be kept by us and one copy is for you.
For additional information, you can ask your doctor.

The investigator team

CONSENT PAGE
BRUCELLOIS STUDY IN LANKIEN, SOUTH-SUDAN

Principal Investigator: Cara Kosack, MSF International, Amsterdam, Netherlands
Email: cara.kosack@amsterdam.msf.org

Local contact: Medical Coordinator Assistant, MSF, South Sudan
Phone number: +254 700 861 742

I have read or listened and understand the above information. I have been advised of the goals and procedures of the study, and the benefits and risks of participating in the study.
I hereby give my consent to participating in the study.

_____

_

Name of patient Date Signature/fingerprint

Witness

I certify that the person named above has been given an opportunity to understand the above given information and ask questions, that he or she understands the issues discussed, that his or her decision to participate in the study is an informed and voluntary one, and that I have witnessed his or her signature.

_____

_

Name of witness Date Signature/fingerprint

_____

_

Name of study coordinator/doctor Date Signature

CONSENT PAGE

TRACING CONSENT - BRUCELLOSIS STUDY IN LANKIEN, SOUTH- SUDAN

Principal Investigator: Cara Kosack, MSF International, Amsterdam, Netherlands
Email: cara.kosack@amsterdam.msf.org

Local contact: Medical Coordinator Assistant, MSF, South Sudan
Phone number: +254 700 861 742

I have read or listened and understand the above information. I have been advised of the goals and procedures of the study, and the benefits and risks of participating in the study.
I hereby give my consent to be traced:

O by phone (phone number): _____

O at my house (provide address): _____

_____

_____

O or another location (please provide details):_____

_____

_____

if I have not been diagnosed with brucellosis today but the results of the laboratory tests in Spain suggest a diagnosis of brucellosis.

_____

Name of patient Date Signature

_____

Name of study coordinator/doctor Date Signature

Annex 5: Patient form

MSF Program, country **Lankien, South-Sudan**

Date **|__|__| / |__|__| / |__|__| [dd/mm/yy]**

Patient number **|__|__|__|__|**

Client initials **|__|__| [last name/first name]**

Gender **❒ Male** **❒ Female**

Age in years **|__|__|**

Informed consent taken **❒ Yes** **❒ No**

Tracing offered **❒ Yes** **❒ No**

Study number **|__|__|__|__| [given by local study coordinator]**

Occupation ❒ Butcher ❒ Housewife/houseman

❒ None ❒ Other, please name_____

Cattle at compound where participant lives

❒ Cows, number: _____

❒ Sheep, number: _____

❒ Goats, number: _____

❒ Pigs, number: _____

❒ Camels, number: _____

Contact to (Outside of living compound)

Cows: Yes ❒ No ❒ Unknown ❒

Sheep: Yes ❒ No ❒ Unknown ❒

Goats: Yes ❒ No ❒ Unknown ❒

Pigs: Yes ❒ No ❒ Unknown ❒

Camels: Yes ❒ No ❒ Unknown ❒

Handling animals at parturition (birthing process in cattle), <u>or</u> handle abortion materials <u>or</u> new born animals in the past year Yes ❒ No ❒ Unknown ❒

Milking animals

❒ Yes, more than once (1x) per week
❒ Yes, more than once (1x) time per month
❒ Yes, less than once (1x) time per month
❒ No

Drinking blood of animals
e.g. cow, sheep, goats, pigs

❒ Yes, more than once (1x) per week
❒ Yes, more than once (1x) time per month
❒ Yes, less than once (1x) time per month
❒ No

Drinking milk of animals
e.g. cows, goats?

❒ Yes, more than once (1x) per week
❒ Yes, more than once (1x) time per month
❒ Yes, less than once (1x) time per month
❒ No

Signs and symptoms

History of fever in 7 days
(reported by patient) Yes ❒ No ❒ Unknown ❒

Number of days since onset of fever # of days

_____

Sweating **Yes ❒** **No ❒** **Unknown ❒**
Presence of chills **Yes ❒** **No ❒** **Unknown ❒**
Weakness **Yes ❒** **No ❒** **Unknown ❒**
Rigor* **Yes ❒** **No ❒** **Unknown ❒**
Malaise** **Yes ❒** **No ❒** **Unknown ❒**
Headache **Yes ❒** **No ❒** **Unknown ❒**

Lack of appetite **Yes ❒** **No ❒** **Unknown ❒**

Weight loss **Yes ❒** **No ❒** **Unknown ❒**

Constipation **Yes ❒** **No ❒** **Unknown ❒**

Abdominal pain **Yes ❒** **No ❒** **Unknown ❒**

Vomiting **Yes ❒** **No ❒** **Unknown ❒**

Diarrhea **Yes ❒** **No ❒** **Unknown ❒**

Abdominal tenderness **Yes ❒** **No ❒** **Unknown ❒**

Arthralgia/joint pain **Yes ❒** **No ❒** **Unknown ❒**

Aches (muscle) **Yes ❒** **No ❒** **Unknown ❒**

Back pain **Yes ❒** **No ❒** **Unknown ❒**

Enlarged spleen **Yes ❒** **No ❒** **Unknown ❒**

Enlarged liver **Yes ❒** **No ❒** **Unknown ❒**

Cough **Yes ❒** **No ❒** **Unknown ❒**

Sore throat **Yes ❒** **No ❒** **Unknown ❒**

Bronchitis **Yes ❒** **No ❒** **Unknown ❒**

Epistaxis/Nose bleeds **Yes ❒** **No ❒** **Unknown ❒**

Hemoptysis **Yes ❒** **No ❒** **Unknown ❒**

Orchitis **Yes ❒** **No ❒** **Unknown ❒**

Adenitis/Lymphadenitis **Yes ❒** **No ❒** **Unknown ❒**

Rash **Yes ❒** **No ❒** **Unknown ❒**

Visual disturbances **Yes ❒** **No ❒** **Unknown ❒**

Photophobia ❒	**Yes ❒**	**No ❒**	**Unknown ❒**
Depression ❒	**Yes ❒**	**No ❒**	**Unknown ❒**
Insomnia ❒	**Yes ❒**	**No ❒**	**Unknown ❒**
Irritability ❒	**Yes ❒**	**No ❒**	**Unknown ❒**

Result RB **Positive ❒** **Negative ❒**
Invalid ❒ **Unknown ❒**

Diagnosis at site **Brucellosis ❒** **Kala Azar ❒** **Malaria ❒** **Tb ❒**
Urinary tract infection ❒ **Pneumonia ❒** **Meningitis ❒**
Fever of unknown origin ❒
Other ❒ **If other, please specify**_____

Data and specimen collected: **Name**
_____

Date: |__|__| / |__|__| / |__|__| [dd/mm/yy] **Signature** _____

*Rigor: sudden feeling of cold with shivering accompanied by a rise in temperature, often with copious sweating, especially at the onset or height of a fever.

** Malaise: a general feeling of discomfort, illness, or uneasiness whose exact cause is difficult to identify.

*** Adenitis: inflammation of a gland. Often it is used to refer to lymphadenitis which is the inflammation of a lymph node.

Annex 6: Shipment information

1. All collected and aliquoted specimens must have been stored by -20 °C until day of shipment.
2. All aliqouted specimens must be labeled with the study number and the date of collection only.
3. Place them in the inner box of the triple packing box (UN 3373 transport). Surround the tubes with as many ice packs as the transport box allows.
4. Bring the specimens ASAP to the next DHL or World Courier office and send them to:

Prof. Ignacio Moriyón
Instituto de Salud Tropical y Depto.
Microbiología y Parasitología
Universidad de Navarra
Edificio de Investigación
(Room 3150, office 3151)
c/Irunlarrea 1,
31008 Pamplona
Spain

phone number +34 608 170 594

Annex 7: Data entry information at reference laboratory

Fill out EpiData file containing following variables and email data file to both addresses:

- Cara.Kosack@amsterdam.msf.org
- Anne-Laure.Page@epicentre.msf.org

1. Study inclusion number
2. Results of RBT at ITH incl. last positive dilution
    - Non-reactive
    - Reactive, titer: _____
3. Results of SAT at ITH incl. last positive dilution
    - Non-reactive
    - Reactive, titer: _____
4. Result of Coombs test at ITH incl. last positive dilution
    - Non-reactive
    - Reactive, titer: _____
5. Result of Brucellacapt at ITH incl. last positive dilution
    - Non-reactive
    - Reactive, titer: _____
6. Results of RDT IgM (One Diagnostics/KIT)
    - Non-reactive
    - Reactive
7. Results of RDT IgG (One Diagnostics/KIT)
    - Non-reactive
    - Reactive

Annex 8: SOPs diagnostic tests at reference laboratory

- **Rose Bengal plate test (Spinreact, Sant Esteve de Bas, Spain; # 1200901)**

  According to package insert.

- **Rose Bengal test on serum dilutions**

Materials

- Flat glossy white ceramic tiles (these are optimal; glass plates can be used but readings are not so clear). They can be cleaned by rinsing/scrubbing in clean water after use.
- An automatic pipette delivering 25 to 200 µL (microliters) and plastic tips (cones). Tips can be rinsed in clean tap water, dried and reused many times.
- Antigen. Available commercially. Antigen should be stored at 4 °C (not frozen!).
- Tooth picks (or similar; a glass rod that is cleaned alter each mixing [see below] can also be used).
- Control serum. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of the test conditions. This serum should be stored frozen in small aliquots and brought to room temperature before use.

Procedure

1. Dispense four 25 µL drops of saline (0.85% NaCl) on the tile.
2. To the first saline drop, add 25 µL of the positive plain serum and mix thoroughly by aspirating and expulsing the mixture several times with the pipette.
3. Rinse the pipette tip with saline and transfer 25 µL of the first dilution to the second saline drop.
4. Mix again as in 3 and transfer 25 µL of the second dilution to the third drop.
5. Mix again as in 4 and transfer 25 µL of the second dilution to the fourth drop.
6. Mix again, take 25 µL and discard them.
7. Test each drop (serum dilution) with 25 µL of the RB reagent as described above for the plain serum. The RBT results are expressed as serum titers:

Last sample positive	RBT titer
Plain serum	1/2
First drop	1/4
Second drop	1/8
Third drop	1/16
Fourth drop	1/32

Titers equal to or higher than 1/8 indicate active brucellosis; titers 1/2 and 1/4 must be considered with care taking into account the presence/absence of clinical symptoms.

- **Serum agglutination test (BioSystems, Barcelona, Spain, # 33309)**

  According to package insert.

- **Coombs antiglobulin agglutination test (in-house procedure)**

  1. After recording the results of the serum agglutination, centrifuge the microtiter carrier plates using a centrifuge with a microtiter plate carrier head.
  2. Wash the bacteria in the negative wells three times with PBS (add 150 µL of the diluent to each well, shake gently and centrifuge the plate).
  3. To each well, add 50 µl of SAT-PBS, shake gently, and add 50 µL of previously titrated anti-human immunoglobulin serum.
  4. Mix by gently shaking and incubate the plates at 37°C for 24 hours.
  5. Read the results. A positive test is manifested by agglutination of the *Brucella* suspension in the wells in which previously appeared the blue button of cells (negative agglutination). The test is considered positive when the titer is at least 3 times higher than that of the serum agglutination.

- **Brucellacapt (Vircell, Granda, Spain; # Brucapt)**

  According to package insert.

- **ELISA (Svanova Biotech, Uppsala, Sweden; # 10-2701-10)**

  According to package insert.

- **The Test-it Brucella IgM/IgG Lateral Flow Assay (One Diagnostics, Amsterdam, Netherlands or other manufacturer)**

  According to package insert.

Annex 9: Brucellosis treatment protocol MSF-OCA South-Sudan (April 2012)

Due to the in-availability of Streptomycin (global shortage) in the projects, GENTAMYCIN is being used as an ALTERNATIVE. See below the updated protocol in April 2012; all projects will use this protocol for the management of Brucellosis.

Target group	Treatment	Inpatient / Out-patient
Child under 8 years	**GENTAMICIN IM:** 5 mg/kg once daily (or in 2 divided doses) for **7 to 14 days** depending clinical response. + **Cotrimoxazole PO**: 40 mg SMX + 8 mg TMP/kg/day in 2 divided doses for **6 weeks**.	**ADMIT at least 2-3 days**. IF clinically stable, and able to visit hospital for IM injections on DAILY basis, then continue Tx on OPD basis. If SICK and from far distance to hospital Continue admission to complete 7 to 14 days of treatment for the **INJECTION PHASE**
Children over 8 years and Adults **(except in Pregnant and Lactating Women)**	**Gentamicin IM**: 5 mg/kg once daily for **14 days** + **Doxycycline PO:** **Children:** 100 or 200 mg once daily or in 2 divided doses for 6 weeks **Adults:** 200 mg once daily or in 2 divided dosed for 6 weeks	**ADMIT to IPD for at least 2-3 days**. IF clinically stable, and able to visit hospital for IM injections on DAILY basis, then continue Tx on OPD basis. If SICK and from far distance to hospital Continue admission to complete 7 to 14 days of treatment for the **INJECTION PHASE**
	Gentamicin IM: 5 mg/kg once daily for **14 days**	**ADMIT to IPD / Maternity for at least 2-3 days**. IF clinically stable, and able to visit hospital for IM injections on DAILY basis, then continue Tx on OPD basis.

Pregnant and lactating women	+ **Cotrimoxazol PO:** 1600 mg SMX + 320 mg TMP/day in 2 divided doses for **6 weeks**	If SICK and from far distance to hospital Continue admission to complete 7 to 14 days of treatment for the **INJECTION PHASE** **Refer to Maternity for regular ANC checks.**

NB: Gentamicin: 5 mg/kg/day once daily for 7 to 14 days. If no clinical improvement, increase dose up to 7mg/kg ONCE daily. Monitor ELDERLY and VERY SICK patients CLOSELY for renal impairment, ADJUST or STOP Gentamicin if SUSPECTED. CHECK for Sr. CREATININE.

Annex 10: Flow chart for clinical decision making – brucellosis study

1. Patient assessment

Assess patients for symptoms

- History of fever in past 7 days
- Sweating, presence of chills, weakness, rigor, malaise, headache
- Lack of appetite, weight loss
- Constipation, abdominal pain, vomiting, diarrhea, abdominal tenderness
- Arthralgia/joint pain, aches (muscles), back pain
- Enlarged liver, enlarged spleen
- Cough, sore throat, bronchitis, epistaxis, hemoptysis
- Orchitis, adenitis/lymphadenitis,
- Rash
- Visual disturbances, photophobia
- Depression, insomnia, irritability

Assess exposure risk

- Occupation
- Cattle living at the compound where patient lives
- Contact to cattle outside compound (assisting in birthing of cattle, milking cattle)
- Drinking blood of animals
- Drinking milk of animals.

If a patient has any symptoms and at least one exposure risk factor, test for brucellosis.

V

2. Test for brucellosis

Test for brucellosis using the Rose Bengal test.

V

3. Treatment and alternative diagnosis

Positive Rose Bengal test

a) Treat patient for brucellosis according to treatment protocol (annex 9).
b) Counsel the patient to return to clinic/hospital if symptoms remain after completion of treatment course.

Negative Rose Bengal test

- Consider and test for alternative diagnosis: malaria, kala azar etc.
- If no diagnosis can be found, consider discussing the case with local colleagues and/or on the MSF telemedicine platform.