Using ELISA for the detection of *Brucella* antibodies in milk

TECHNICAL BRIEF

Zoonoses & Emerging Livestock Systems
ZELS-BRUCELLOSIS PROJECT IN WEST AND CENTRAL AFRICA

Zoonoses & Emerging Livestock Systems (ZELS) is a joint research initiative between the Department for International Development (DFID) and:

- Biotechnology and Biological Sciences Research Council (BBSRC)
- Economic and Social Sciences Research Council (ESRC)
- Medical Research Council (MRC)
- Natural Environment Research Council (NERC)
- Defence Science and Technology Laboratory (DSTL)

The ZELS brucellosis research project in West and Central Africa focuses on dairy farms in the main peri-urban dairy production areas of West and Central African countries, which are members of the Interstate School of Veterinary Science and Medicine of Dakar (EISMV).

One of the project objectives is to obtain estimates of the prevalence of brucellosis in peri-urban dairy systems in West and Central Africa. These estimates are needed to design control programs that are appropriate for the existing baseline level of infection. In partnership with diagnostic laboratories from different countries, cross-sectional studies of dairy herds are conducted using an indirect ELISA for the detection of antibodies in milk provided by the OIE Reference Laboratory for Brucellosis at the UK’s Animal and Plant Health Agency (APHA). The high sensitivity of the assay allows identification of infected unvaccinated herds by testing of bulk milk samples, making the milk ELISA a useful tool for brucellosis surveillance in disease-free countries such as the UK or as part of baseline surveys to generate initial prevalence estimates in areas where they are lacking, such as many West and Central Africa dairy production zones.

In this technical brief, we summarize lessons learned from the use of the indirect ELISA assay as part of cross-sectional studies of brucellosis in dairy farms carried out across 10 dairy production zones of 7 West and Central African countries (Figure 1). We highlight the problems encountered and how to avoid and overcome them in order to facilitate the adoption of this technique as a diagnostic tool in other low and middle-income countries, where the facilities are not optimal.
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Figure 1. Laboratories from six West and Central African countries involved in bulk milk sample testing by indirect ELISA as part of the ZELS-brucellosis project in West and Central Africa.
WHAT IS ELISA?

The Enzyme-linked immunosorbent assay (ELISA) is a test used to detect and quantify specific antibodies in humans and animals. The test consists of capturing antigens or antibodies present in a sample using an enzyme-labelled immunoglobulin, which drives a colour change when a specific chromogenic substrate is added\(^2\). This change is detected and quantified by measuring the optical density. Different types of ELISA have been developed for different purposes, among them the indirect ELISA. In this type of ELISA, the corresponding antigens are coated on the surface of the plate and used to capture the specific antibodies present in the sample. To detect the bound antibodies, secondary antibodies that are conjugated to an enzyme such as peroxidase or alkaline phosphatase are added. After an incubation period, the unbound secondary antibodies are washed off. When a suitable substrate is added, the enzyme reacts with it to produce a colour. This colour produced is measurable as a function or quantity of antibodies present in the given sample (Figure 2).

![Figure 2. Illustration of the different steps of the indirect ELISA method](image-url)
INDIRECT ELISA

Indirect ELISA has been developed for the detection of antibodies against *Brucella* spp. and is recommended by the OIE for screening of cattle against *Brucella* spp. infection. In the UK, quarterly bulk milk testing of dairy herds by indirect ELISA is one of the key elements of the currently applied brucellosis surveillance program. This technique has the potential to be used as an initial step to ascertain the status of unvaccinated dairy herds with regard to brucellosis and can be used to produce baseline prevalence estimates in areas where this information is lacking. It is with this objective that the assay has been used, so far, in 10 dairy production areas of Burkina Faso, Burundi, Cameroon, Mali, Niger and Togo.

INDIRECT ELISA PROTOCOL

To perform the assay, laboratories should all be provided with: pre-coated *Brucella abortus* S99 smooth lipopolysaccharide antigen plates with a lid; conjugate stored at -20°C; chromogen substrate; diluting buffer; stopping solution; and wash solution. Additionally, the following laboratory equipment is also required: microtitre plate reader with 405nm filter; single and multichannel pipettes along with tips and reagent troughs; containers, bottles, tubes and beakers for distilled/deionised water, sera and/or reagent storage; refrigerators and freezers; rotary and microtitre plate shaker; and absorbent towels. Collected milk samples must be immediately placed in ice, transported to the lab as soon as possible and kept in the fridge until testing. Curdled samples must not be used because this will affect the test result. On the day of the test, milk samples should be centrifuged at 10,000 x g for 2 min at 4°C and the cream layer should be removed. It is highly recommended to divide the supernatant into different aliquots and stored at -80°C. However, repeated thawing and freezing should be avoided as it will compromise the sample integrity and thus, the accuracy of the test result. Finally, it is also highly recommended to test each sample in duplicate in order to obtain reliable results.
The general protocol of the indirect ELISA assay includes the following main steps:\(^4\):

1. Prepare the plate by adding 50 μl of diluting buffer to all wells.
2. Add 50 μl of the samples and controls (positive, medium and negative) to the designated wells (Figure 3).
3. Incubate at room temperature for 30 minutes on a rotary shaker (or at 37°C for 1 hour without shaking).
4. Wash with wash solution and dry by tapping with absorbent paper towel.
5. Add 100 μl of conjugate solution and incubate the plate as mentioned is step 3.
7. Add 100 μl of well mixed substrate solution to all wells and incubate at room temperature for 10 to 15 minutes.
8. Add 100 μl of stopping solution and read the plate at 405nm (blanked at the blank well).

![Figure 3. Designated control wells; positive controls should be placed in wells A12 and B12 (red), intermediate controls in column 11 (yellow), negative controls on wells C12, D12 and E12, and no sample (blanks) should be added in wells F12, G12 and H12.](image)

Plate acceptance requires the compliance to certain criteria, which include:

- Binding ratio (mean of 8 positive control wells/mean of 3 negative control wells) ≥ 10
- Optical density (OD) of the blank well and the mean of the negative ODs < 0.100
- Mean OD of the 8 positive control wells > 0.7000 (optimal = 1.000)
- Mean OD of 3 medium control wells >10% or <30% of the mean positive OD (optimum = 20%)

If a microtitre plate is unavailable, visual inspection is possible. A strongly positive sample will cause a green colour appearance, while a negative sample will show clear (Figure 4).
Figure 4. Example of an acceptable plate where samples display different reactions and the controls display as expected; strong colour in positive wells (A12 and B12), intermediate colour in the medium controls (column 11) and clear negative wells in negative wells (C12, D12, E12) and blank wells (F12, G12, H12).
ISSUES IDENTIFIED DURING PRACTICAL USE OF THE TEST

Despite its apparent simplicity and the precautions taken in the shipment and storage of assay reagents, which were directly provided by the laboratory in charge of their production, inspection of initial test results revealed a number of issues. We summarize below the problems encountered and make recommendations to detect and avoid these issues, which may be useful for other laboratories interested in making use of this or similar assays, in particular in low-resource settings:

1. Problems with the pH of the diluting buffer

Some sites reported discolouration of the plates, which can be explained by the misuse of the diluting buffer where the pH is either too high or too low. When the pH of the diluting buffer is > 8.2, phenol red indicator will turn bright pink (Figure 5.A). On the other hand, when the pH of the diluting buffer is below 7.2, the colour of phenol red indicator turns yellow (Figure 4.B). If this occurs the buffer should be discarded. In order to avoid this problem, the diluting buffer should be prepared following the manufacturer’s instructions and the pH should be adjusted between 7.2 and 7.6. This issue was resolved in the study sites when the test was repeated ensuring the pH of the diluting buffer was within the recommended range.

2. Problems with the controls

Occasionally, control wells did not develop the expected results and thus, compromised plate acceptance (see above for plate acceptance criteria). In figure 6 there is a clear visible lack of colour in the positive control wells marked by red arrows (A12 and B12). This could be due to; (a) using the wrong concentration of some reagents, (b) not dispensing the positive control in the designated wells or (c) pipetting errors.

Figure 5. Invalid plate due to the diluting buffer being (A) > 8.2, whereby phenol red indicator turns bright pink or (B) < 7.2, whereby phenol red indicator turns yellow.

Figure 6. ELISA plates with medium control wells not developing colour.
3. Problems with reading the ELISA plate

In one instance, there was an OD reading that was completely different to the expected range values due to the use of an inappropriate filter. After carrying out all test procedures, the ELISA plate should be read by using a microtitre plate reader with 405nm filter. Using a filter with a different wavelength results in incorrect OD values and will cause misinterpretation of the results.

SUMMARY

Indirect ELISA in milk is a simple and practical tool for the serological diagnosis of brucellosis at herd level. Validation studies carried out to assess its potential use as part of brucellosis surveillance programs in disease-free settings suggest the assay has a high sensitivity allowing the detection of very low proportion of seropositive animals in the milking herd. Despite its apparent simplicity, the use of the test, in particular in laboratories with limited resources or where ELISA testing is not carried out routinely poses some challenges. Adherence to the instructions is critical and caution should be taken at all stages – from sample collection and storage to interpretation of test results. Most importantly, samples must not curdle; the pH of the buffer must be adjusted; the inclusion of controls is mandatory; optical density readings must be carried out using the appropriate filter and overall validity of the test requires that the results of control are within the expected ranges.

We hope that this technical brief may be of assistance to laboratory technicians in low and middle-income countries who are interested in using the indirect ELISA for testing bulk milk samples.
REFERENCES


FURTHER INFORMATION

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https://zelsbrucellosis.wordpress.com/