World Organisation for Animal Health

IZS TERAMO

WOAH Reference Laboratory for CBPP

Regional Training Course on Abattoir Surveillance for Contagious Bovine Pleuropneumonia (CBPP)

24 – 27 March 2026, Nairobi, Kenya

# CBPP laboratory diagnosis: laboratory confirmation and differential diagnosis

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COMESA SPS Project

# Importance e application of CBPP laboratory diagnosis Direct tests: culture and PCR

*Table 1. Laboratory methods currently used for diagnosis of CBPP and their purpose*

WOAH Terrestrial Manual Chapter 3.4.8. CBPP

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | Population freedom from infection | Individual animal freedom from infection prior to movement | Contribution to eradication policies | Confirmation of clinical cases | Prevalence of infection – surveillance | Immune status in individual animals or populations post-vaccination* |
| **Detection of the agent[(a)]** | | | | | | |
| *In-vitro* culture isolation (followed by species identification tests) | + | – | – | +++ | – | – |
| Direct molecular test (PCR) | – | – | – | ++ | – | – |

# Importance e application of CBPP laboratory diagnosis Indirect test (serology): CFT, c-ELISA, (IB)

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| **Detection of immune response** | | | | | | |
| CFT | +++ | ++ | +++ | ++ | +++ | – |
| Immunoblotting | ++ | ++ | ++ | ++ | ++ | – |
| C-ELISA | +++ | ++ | +++ | ++ | +++ | – |

# Direct test: culture and PCR – Good quality samples

**A key to isolation success relies on collecting good quality samples (impact on test Se)**

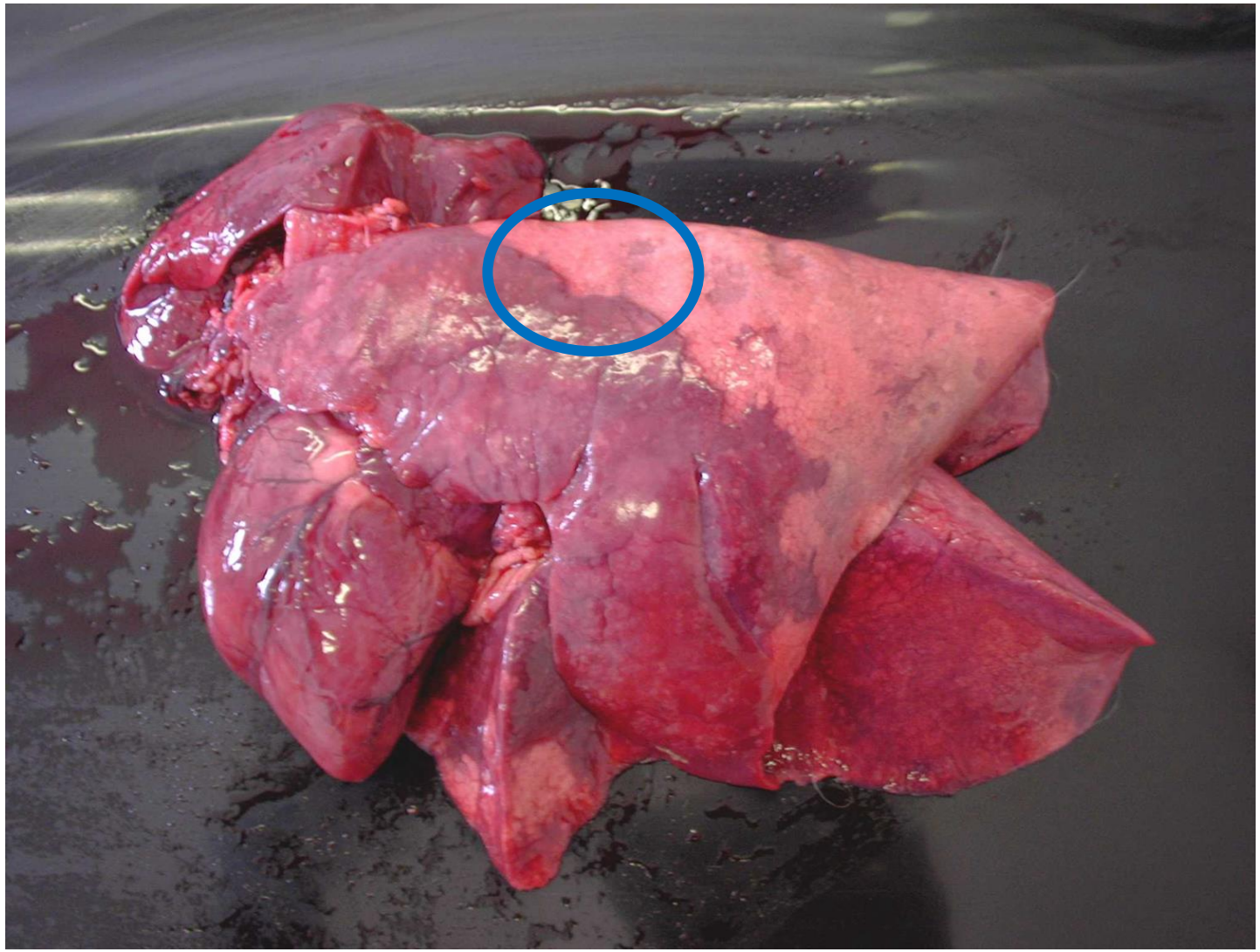
## Live animals (in theory)

- Nasal swabs
- Broncho alveolar lavages
- Pleural fluid (puncture with ¾ needle between 7th and 8th rib)
- Blood with anticoagulant (difficult detection of *Mmm*)

## Dead animals (in practice)

- Pleural fluid
- Lung (area with acute lesions)
- Respiratory lymph nodes (mediastinal and bronchial)
- Sinovial fluid

Lung samples should be collected from lesions at the **interface between diseased and normal tissue**.



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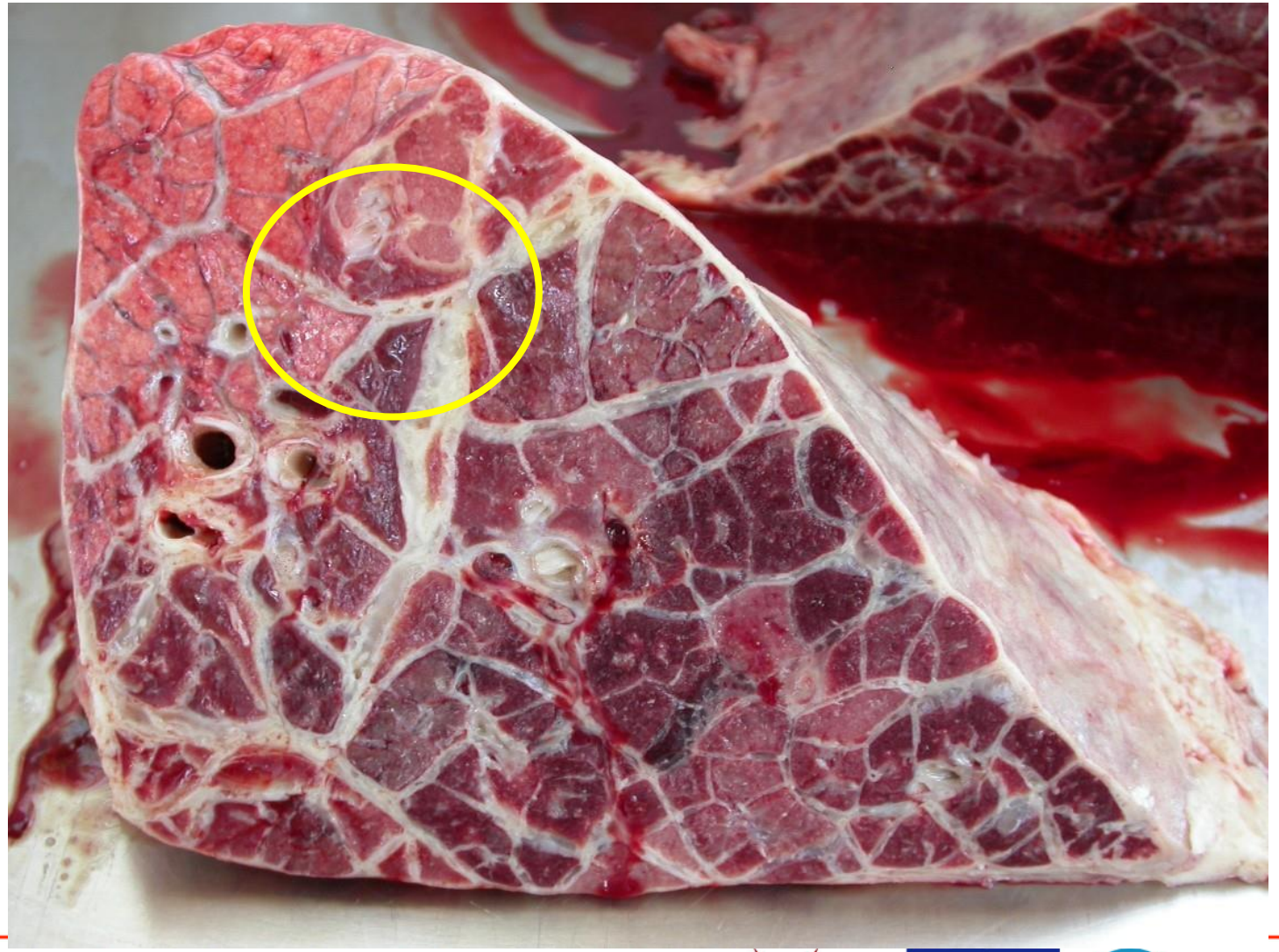
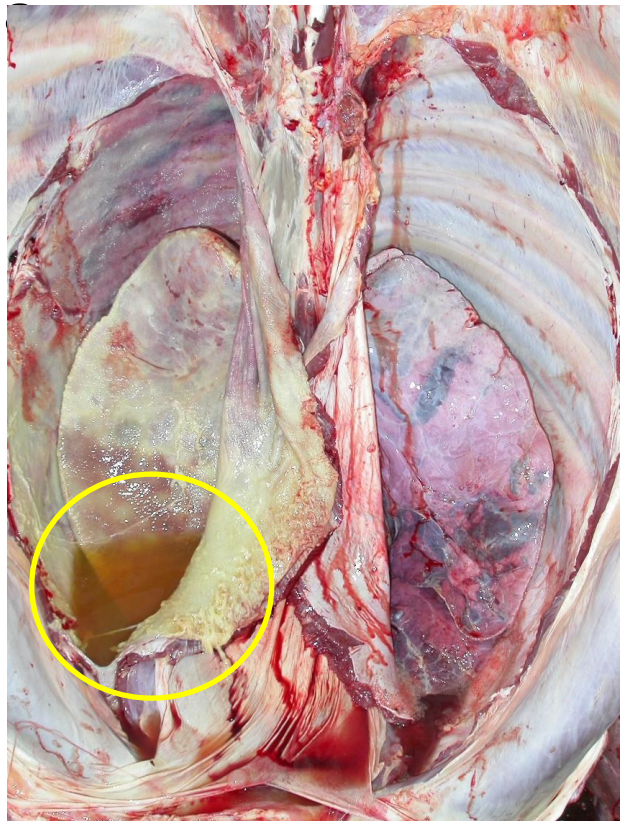
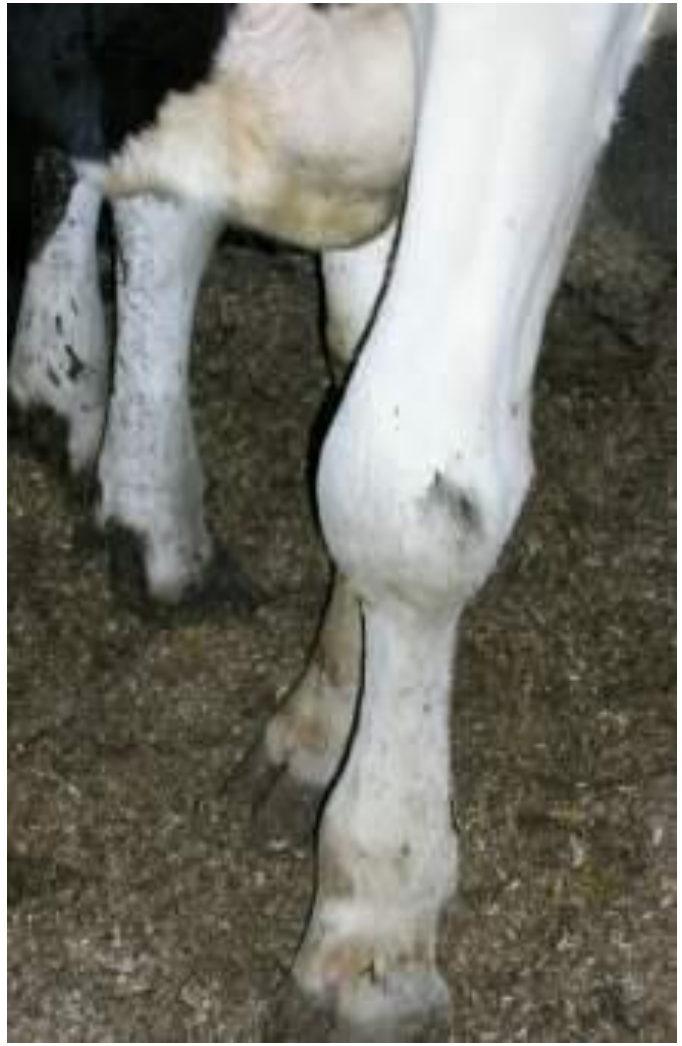


**Samples must be collected as aseptically as possible to avoid risk of overgrowth by contaminating bacteri**

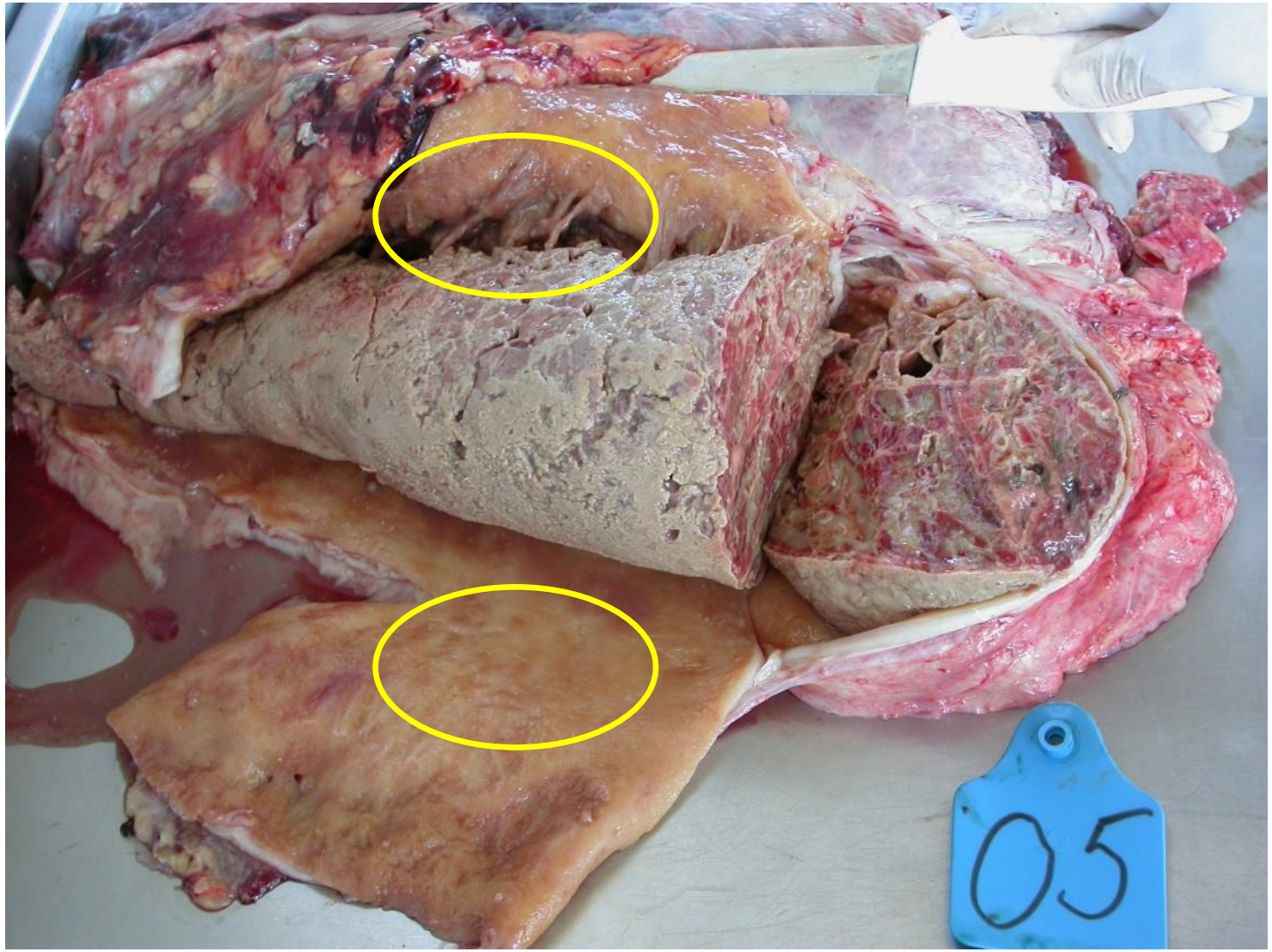




**The pleural fluid is almost a pure culture of *Mmm*.**

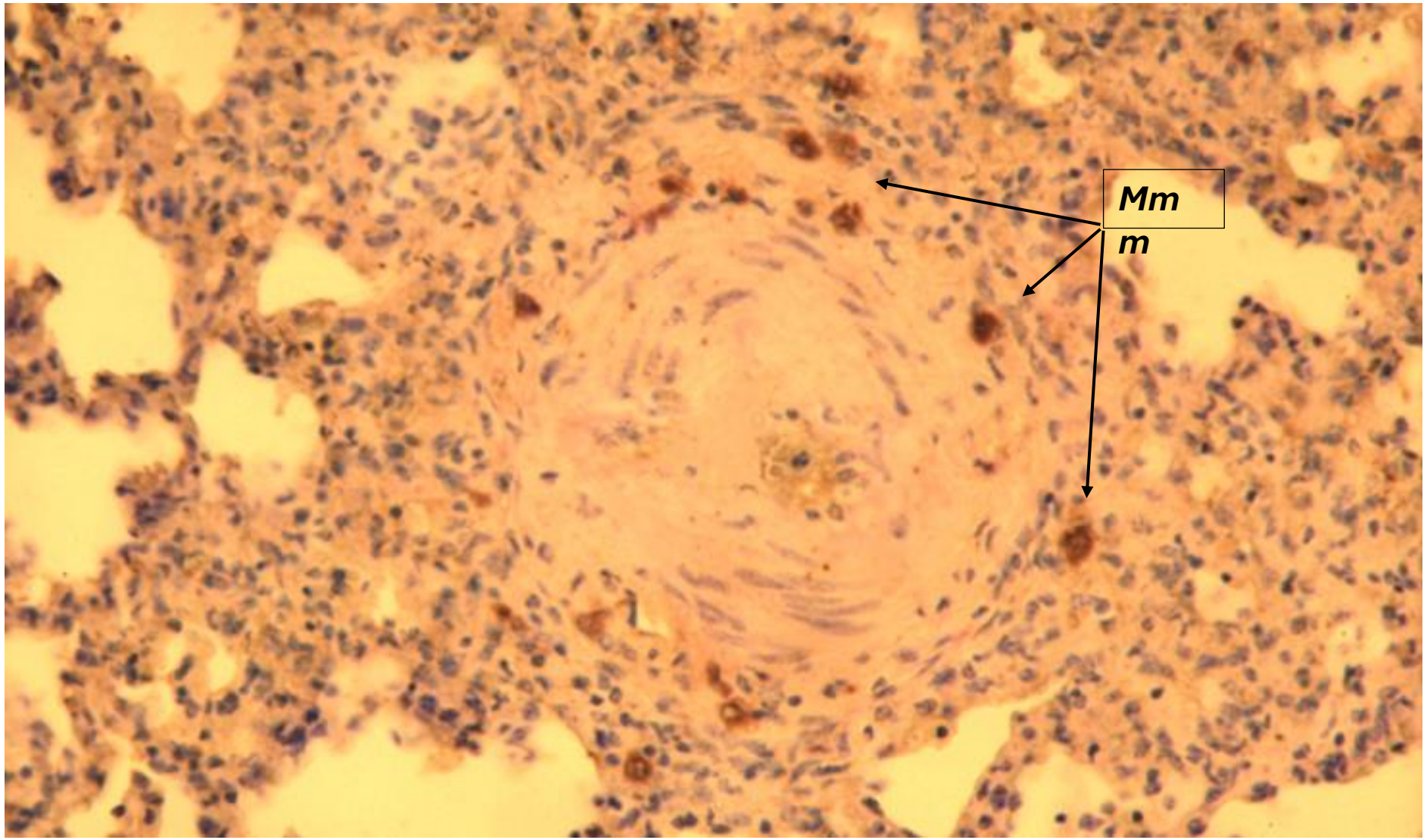
Joint fluid can be collected from animals (young) with arthritis.

Target area for sampling of encaplulated sequestra

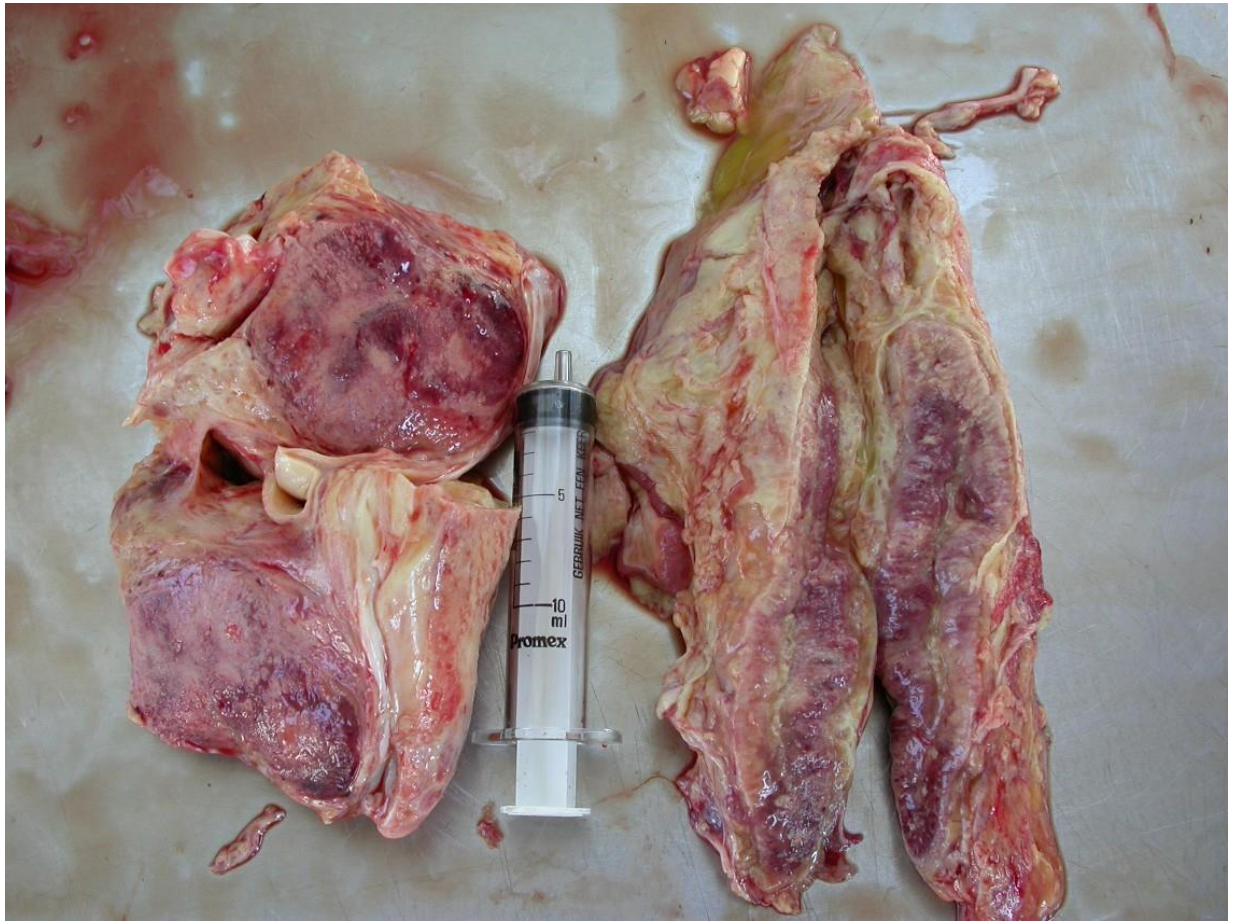


**IHC of a small lung sequestra.**

*Mmm* is detected at the periphery of sequestra



# Mediastinal and bronchial lymph nodes

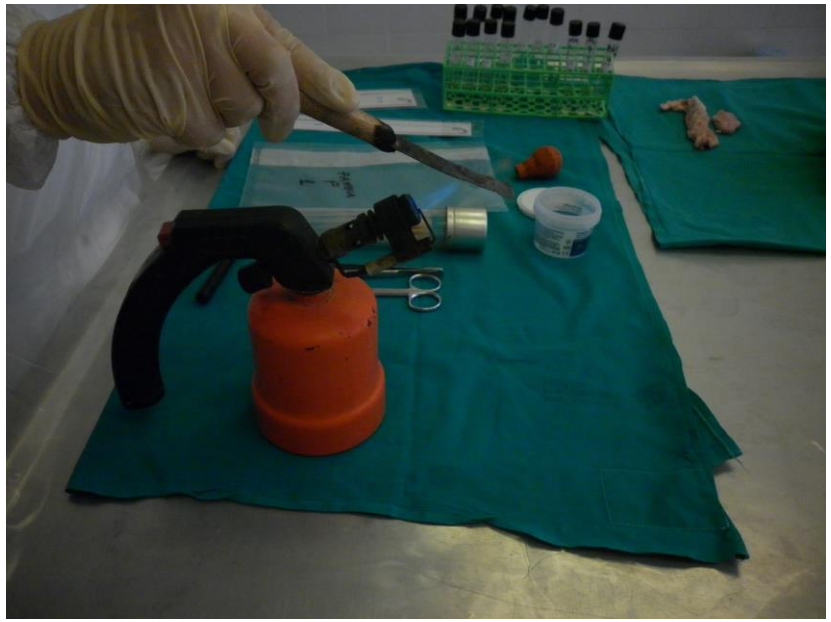


# Sample collection

- At the necropsy room, all the material necessary for the sampling must first be prepared

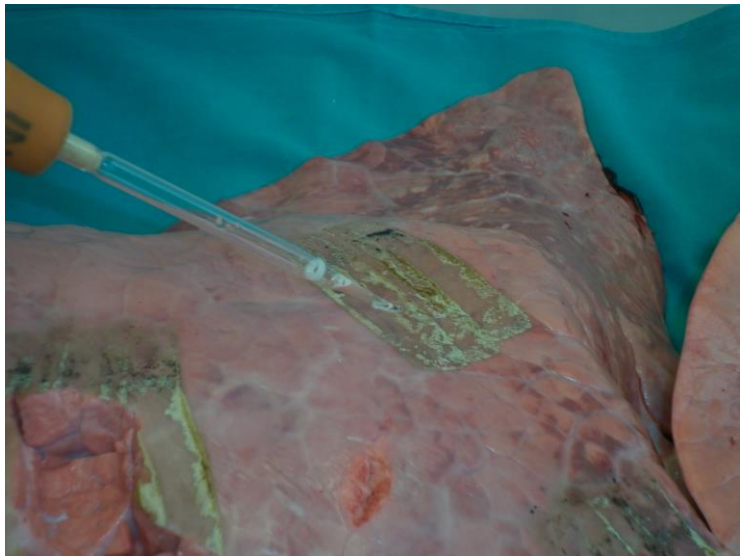
# Sample collection

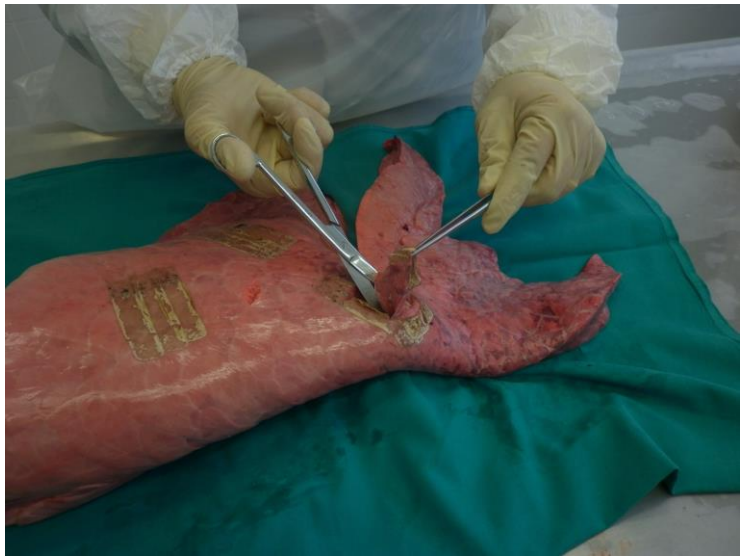
- Samples must be collected as aseptically as possible to avoid risk of overgrowth by contaminating bacteria.
- Identify the lesions and sterilize the external surface of the organ with hot instrument.



# Sample collection

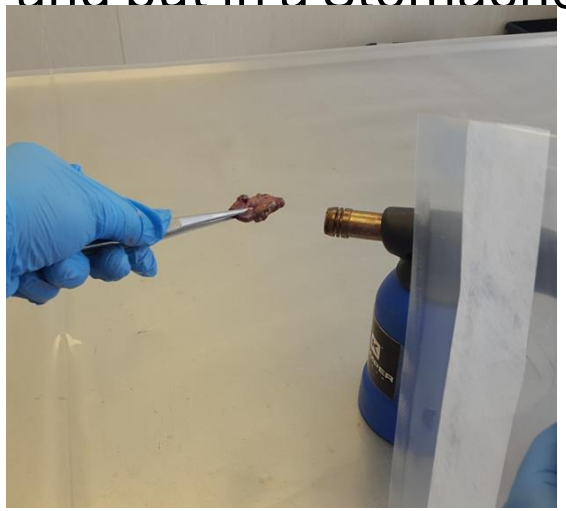
- For **biological fluids**, collect **at least 1,5 ml** of fluid with a Pasteur pipette or a syringe.
- For organs, take **at least 2 cm$^3$** of tissue

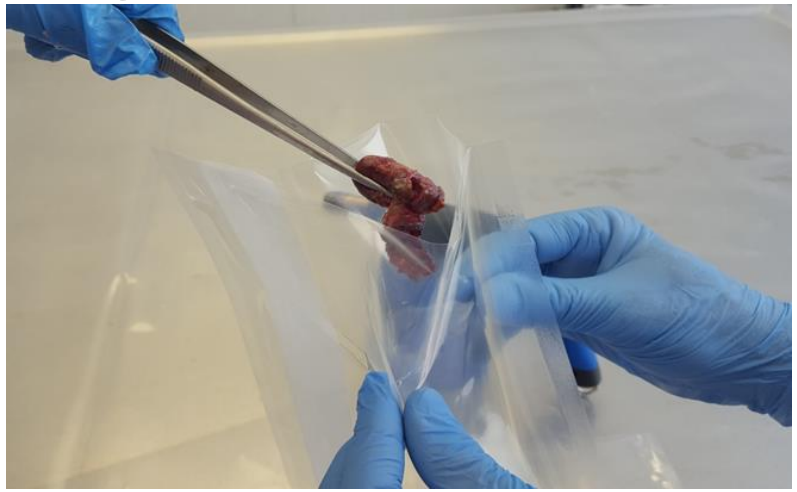




# Lymph nodes sampling collection

- Identify and isolate with sterile instruments the lymph node to be collected
- Remove extraneous material (e.g. fat)
- Sterilize the outside of the organ with a flame
- Cut into small pieces and put in a Stomacher bag





# To preserve the sample

- When collecting nasal swab samples, a transport medium should be used to protect the mycoplasmas and prevent proliferation of cell-walled bacteria and fungi (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% heat-treated serum (horse or pig), 0.3% agar, 500 International Units [IU]/ml penicillin, 0.2 g/litre thallium acetate). WOAH Manual

- **After collection, all samples must be kept refrigerated at 4°C and sent to the laboratory within 24 hours.**
- **For longer periods they should be frozen at or below –20°C.**

# Biosafety

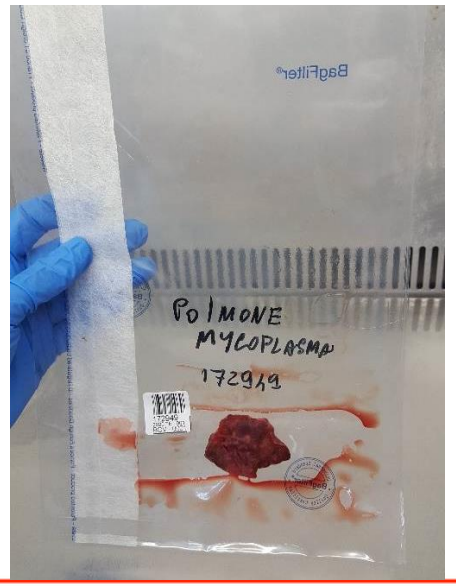
- There is no known risk of human infection with *Mmm*. Biocontainment measures should be determined by risk analysis.
- In CBPP-free areas, it is advisable to manipulate *Mmm* in biosafety level (BSL) 2 laboratories, while BSL1 would be sufficient in enzootic zones.

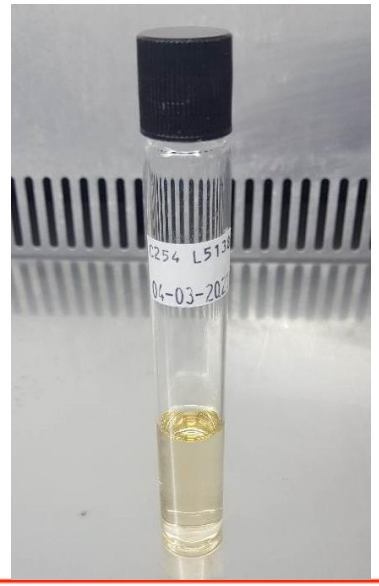
# *Mmm* and *Mycoplasma* spp. isolation

- ***Mmm* needs selective media to grow, but it is not considered a fastidious mycoplasma.**
- There are several media compositions used in different reference laboratories but, essentially, they should contain: a basic medium such as heart-infusion broth or PPLO broth (pleuropneumonia-like organisms), 1–2.5% yeast extract, 10–20% inactivated horse serum, 0.1% glucose, 1% tryptose, and 0.0024% DNA.
- **To avoid growth of other bacteria**, the media can also contain an **antibiotic of the penicillin family** (for example, 500 IU/ml penicillin G) as mycoplasmas are naturally resistant. The media should be used both as broth and solid.
- All culture media prepared should be subjected to quality control. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are performed correctly. WOAH Manual

# *Mmm* and *Mycoplasma* spp. first isolation

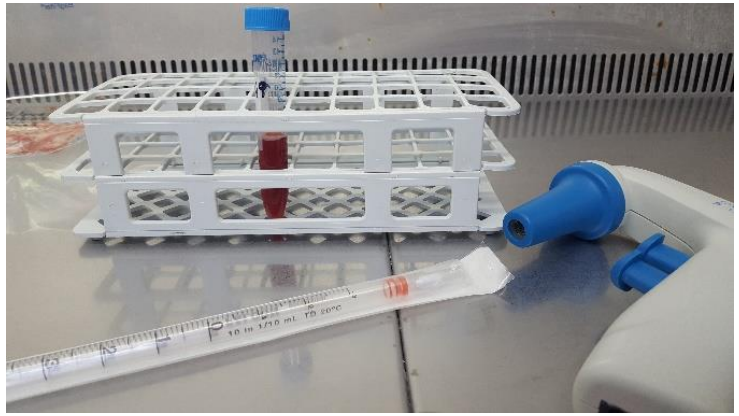
- Add 10 ml of Tryptose broth to the 2 $cm^3$ sample and homogenize the sample until the rupture of the parenchyma

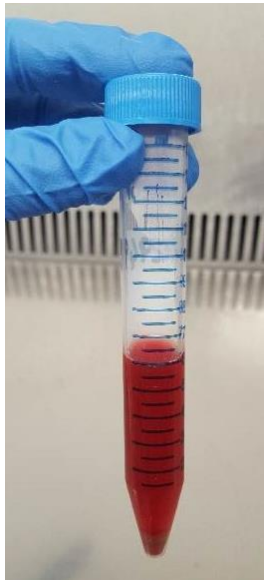



# *Mmm* and *Mycoplasma* spp. first isolation

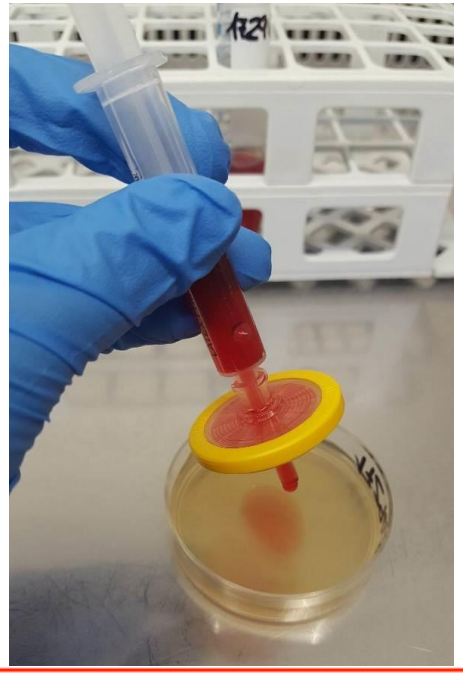
- Collect the homogenate with a sterile pipette and transfer it to a V-bottom tube
- Then centrifuge at 1400g at 4°C for 15 minutes



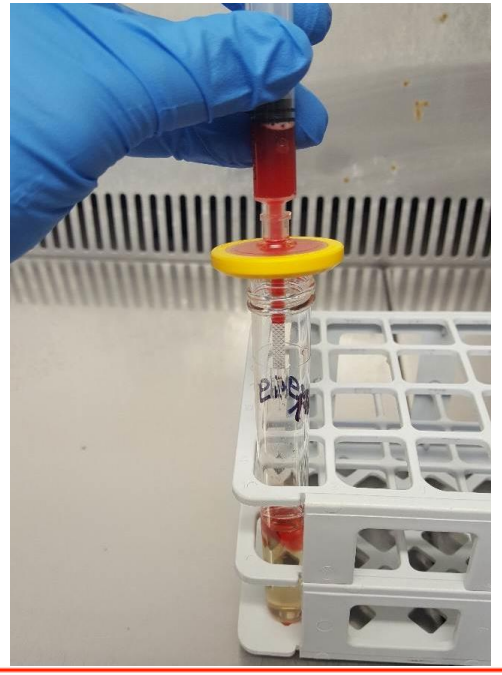



# *Mmm* and *Mycoplasma* spp. first isolation

- With a syringe and a **0.45 µm filter** pour 6-7 drops of the supernatant both on agar and in broth media.



1 week
37°C
5% $CO_2$

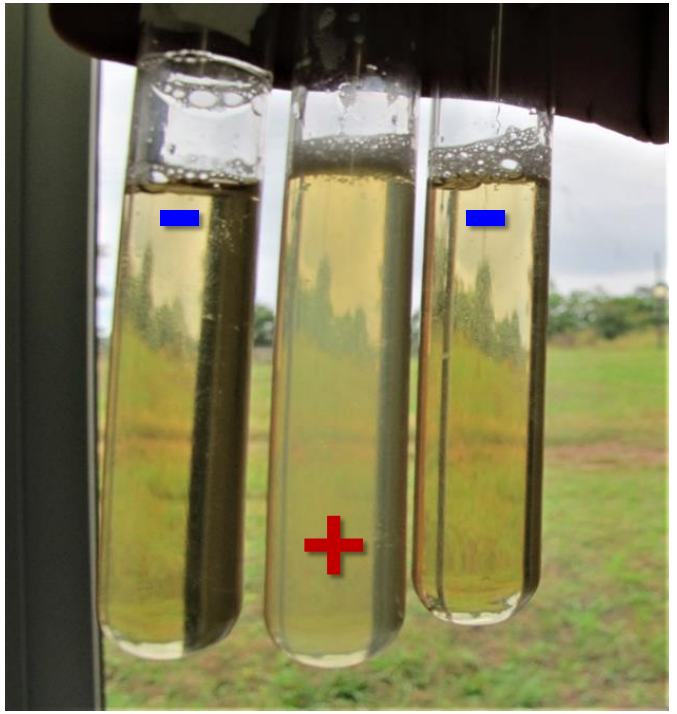



# *Mmm* and *Mycoplasma* spp. first isolation

- The tissue samples are diluted tenfold in medium containing antimicrobials, to minimize contaminating bacteria.
- To avoid contaminating bacteria and to reduce the number of tubes and plates per sample, the supernatant of the ground sample may be filtered through a 25-mm filter with 0.45 µm pore size. Most bacteria size range from 0.5 to 5 µm
- The pleural fluid can be inoculated directly without prior dilution or filtration as, when infected, it is almost a pure culture of *Mmm*.

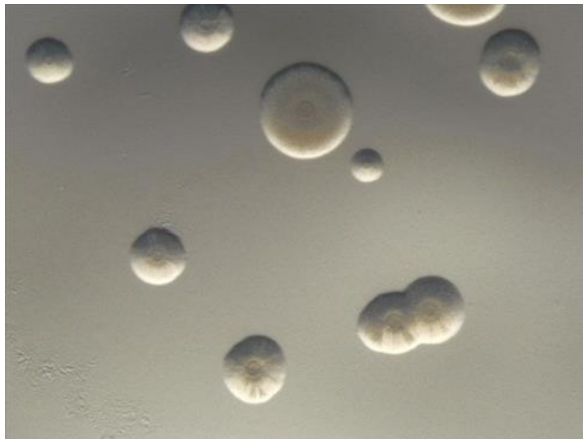
# *Mmm* and *Mycoplasma* spp. first isolation

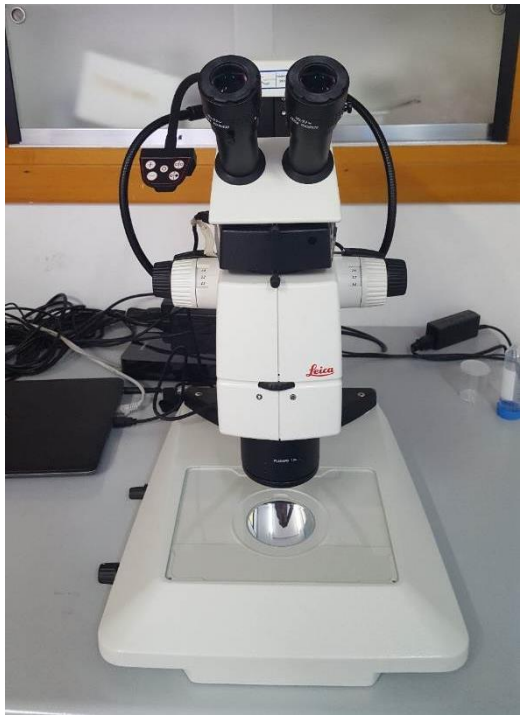
- Check daily, **up to 7-10 days**, the growth of *Mycoplasma* spp. both in the liquid medium and on the agar
- Positive samples **in liquid medium** show a homogeneous **cloudiness**, usually within 2–4 days. They frequently present a silky, fragile filament called a **'comet'**. During the following days a uniform opacity develops which forms swirls when shaken
- If excess turbidity / contamination occurs filter approx. 1ml through a 0.45µm filter so a few drops are collected in a fresh broth or onto agar.



# *Mmm* and *Mycoplasma* spp. first isolation

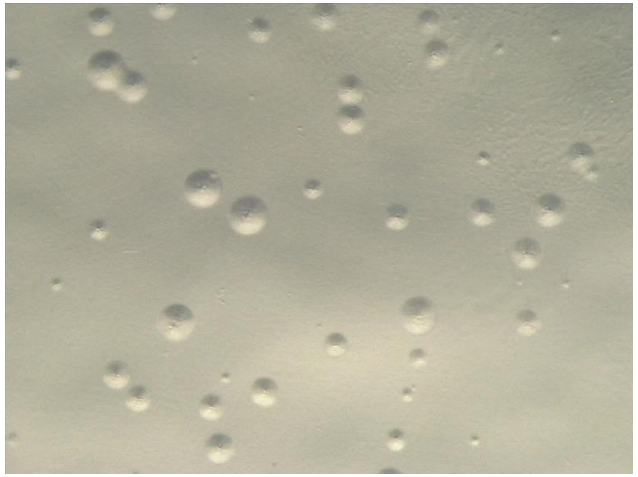
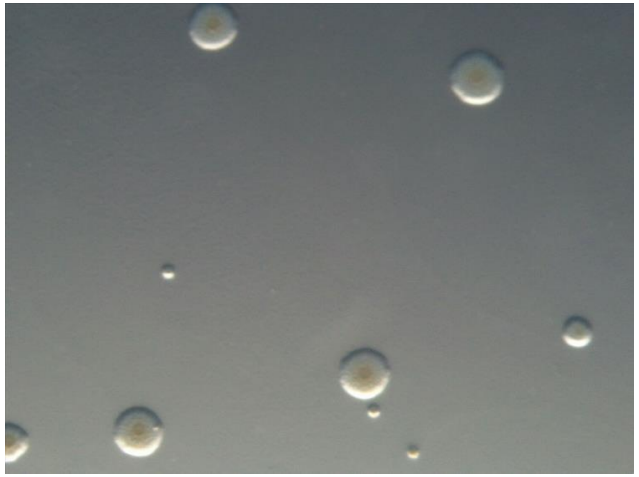
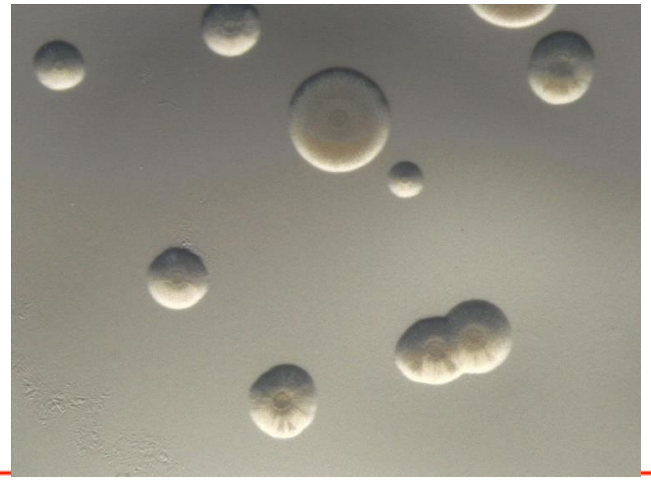
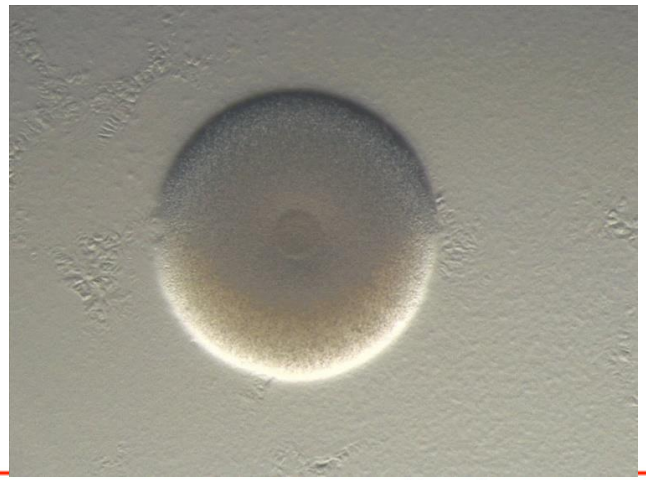
- Check daily, **up to 7-10 days**, the growth of *Mycoplasma* spp. both in the liquid medium and on the agar
- **<u>On agar</u>**, the colonies are small (1 mm in diameter) with the classical appearance of **'fried eggs'** with a dense centre.



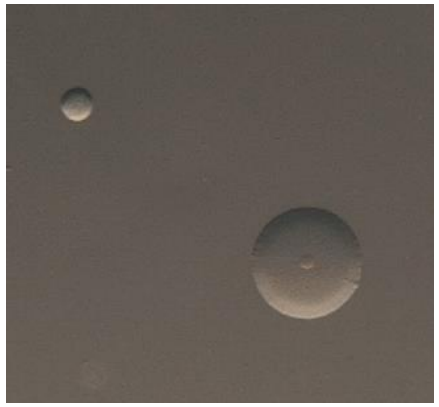


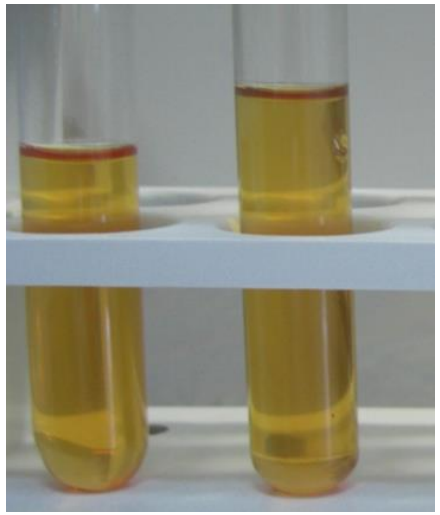
Stereomicroscope 10X

# *Mycoplasma* spp. colonies

# *Mmm* and others *Mycoplasma* spp. typing






# *Mycoplasma* spp. typing

- In the field, CBPP might be confused with other diseases causing respiratory problems such as pasteurellosis or other mycoplasmosis.
- It is essential that isolation is followed by species identification tests
- Biochemical tests alone do not allow identification of a precise *Mycoplasma* species because of overlapping of the few phenotypic traits that can be evaluated. Therefore, molecular tests such as PCR are recommended for identification of isolates.
- PCR has become the method of choice for the rapid and specific identification of *Mmm* when the organism is isolated from a clinical sample.

# Be careful

- The presence of the pathogen varies greatly with the stage of development of the lesions and a negative result is not conclusive, particularly if the animal was treated with an antibiotic.

# Direct diagnosis / identification by PCR - Real time PCR

- Directly from tissues or biological fluids (reduction of sensitivity – matrix effect?)
- From enriched cultures
- PCR-rflp: Use of restriction enzymes (Es. *Mmm* / *Mm capri* and mycoides cluster) (Bashiruddin et al. 1994)
- Mmm T1- specific PCR (Lorenzon et al. 2000)
- Real time PCR (Gorton et al, 2005; Fitzmaurice et al., 2008; Lorenzon et al, 2008; Schnee et al, 2011)
- Primers are species specific, more than one PCR or multiplex PCR are required for identification/differential diagnosis

# Indirect diagnostic tests (serology)

- CFT and c-ELISA (international trade) are recommended as screening tests and used in serological surveillance plans for disease eradication.
- Doubtful cases should be tested in Immunoblotting characterized by higher specificity

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| **Detection of immune response** | | | | | | |
| CFT | +++ | ++ | +++ | ++ | +++ | - |
| Immunoblotting | ++ | ++ | ++ | ++ | ++ | - |
| C-ELISA | +++ | ++ | +++ | ++ | +++ | - |

# CBPP CFT

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
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| **Detection of immune response** | | | | | | |
| CFT | +++ | ++ | +++ | ++ | +++ | - |
| Immunoblotting | ++ | ++ | ++ | ++ | ++ | - |
| C-ELISA | +++ | ++ | +++ | ++ | +++ | - |

- One of the serologial test prescribed by WOAH for serological diagnosis and for international trade
- Sensitivity of 63.8%

  **(Consider herd testing: ↑↑Se)**
- Specificity of 98%

# Flow chart for CBPP CFT

Washing Sheep Red Blood Cells and preparation of **6% SRBC suspension**

**Titration of haemolysins (H)**

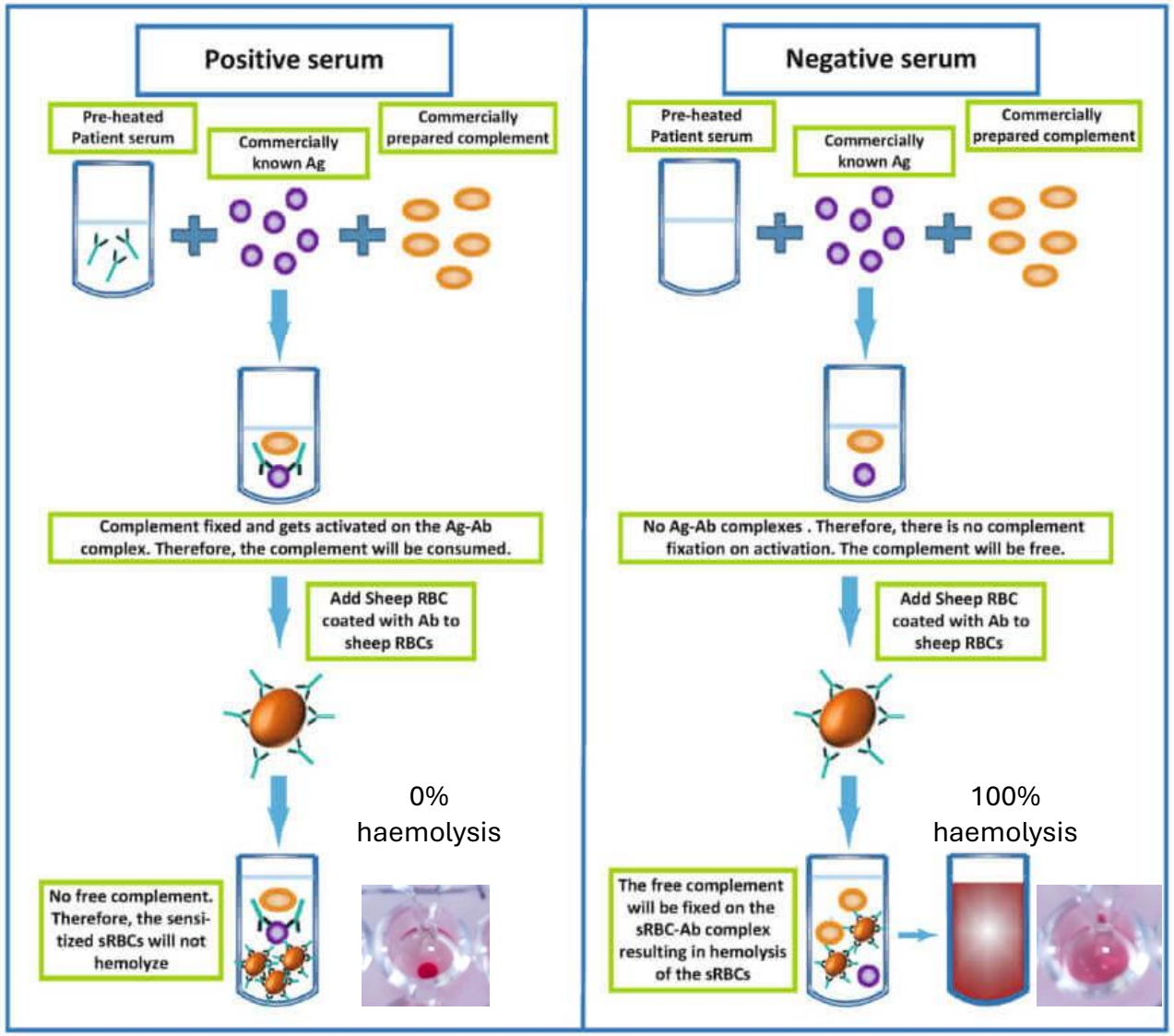
**Titration of Complement (C')**

Titration of Mmm CFT Ag

CBPP CFT diagnosis
**SCREENING dilution 1:10 – 1:40**

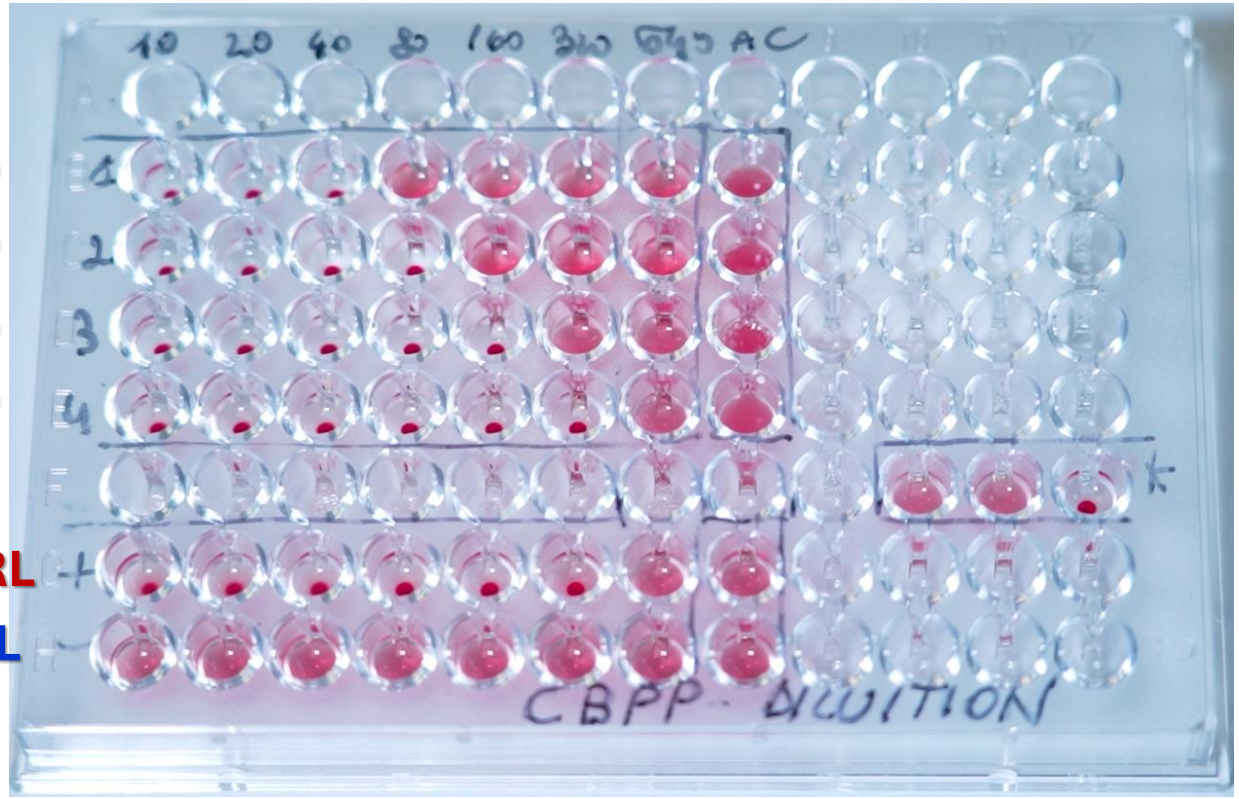
CBPP CFT diagnosis
**TITRATION dilution 1:10 – 1:320**

# CFT principle




Modified from https://microbenotes.com/complement-fixation-test/

# CFT results interpretation

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- 100% fixation at 1:10 → titer 1:10 (+ve)
- 25–75% fixation at 1:10 → doubtful.
- <25% at 1:10 → negative (<1:10).
- Anticomplementary → reported as such.

For dilutions after the first (1:10), the serum titre is expressed as the highest serum dilution, among those tested, that produces 100% complement fixation (e.g.: 1:20, 1:80, 1:320….)

# CFT: strengths and weaknesses

- can detect nearly **all sick animals with acute lesions**, but a rather smaller proportion of animals in the early stages* of the disease or of animals with chronic lesions.
- * The nature of the pathogenesis of the disease is such that the incubation period, during which antibodies are undetectable by the CF test, may last for several months

# CFT: strengths and weaknesses

- **For groups of animals (herd or epidemiological unit) the CF test is capable of detecting practically 100% of infected groups.**
- Despite the high specificity of the CF test, false-positive results can occur. (serological cross-reactions with other mycoplasmas: *M. Bovis* and mycoides cluster).
- Results have to be confirmed by post-mortem and bacteriological examination. (Test and slaughter)

# CFT: strengths and weaknesses

- Provide titers of antibody response (not only positive/negative)
- Possibility of self production of reagents (reduced costs and no dependency from availability of commercial
- Require highly trained laboratory technicians

**Next CBPP laboratory training in Senegal in August 2026**

**CBPP CFT and CFT Ag production Training 2023 - NVL Gaborone (Botswana)**
Angola, Mauritania, Etiopia, Senegal, Tanzania e Zambia

# CBPP c-ELISA


Contagious bovine pleuropneumonia (CBPP)

## ID Screen® CBPP Competition

**ELISA**

**Competitive ELISA for the detection of antibodies against *Mycoplasma mycoides* subsp. *mycoides* (Mmm) in bovine serum or plasma**

- Commercially available as ready-make kit
- Kit production was discontinued from January 2023 to late 2025. No possibility of serological surveillance for more than 2 years in Countries were no CFT was available

# CBPP c-ELISA

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|---|---|---|---|---|---|---|
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| **Detection of immune response** | | | | | | |
| CFT | +++ | ++ | +++ | ++ | +++ | - |
| Immunoblotting | ++ | ++ | ++ | ++ | ++ | - |
| C-ELISA | +++ | ++ | +++ | ++ | +++ | - |

- The 2nd serologial test prescribed by WOAH for serological diagnosis and for international trade
- Sensitivity similar to CFT (63.8%)

**(Consider herd testing: Se)**

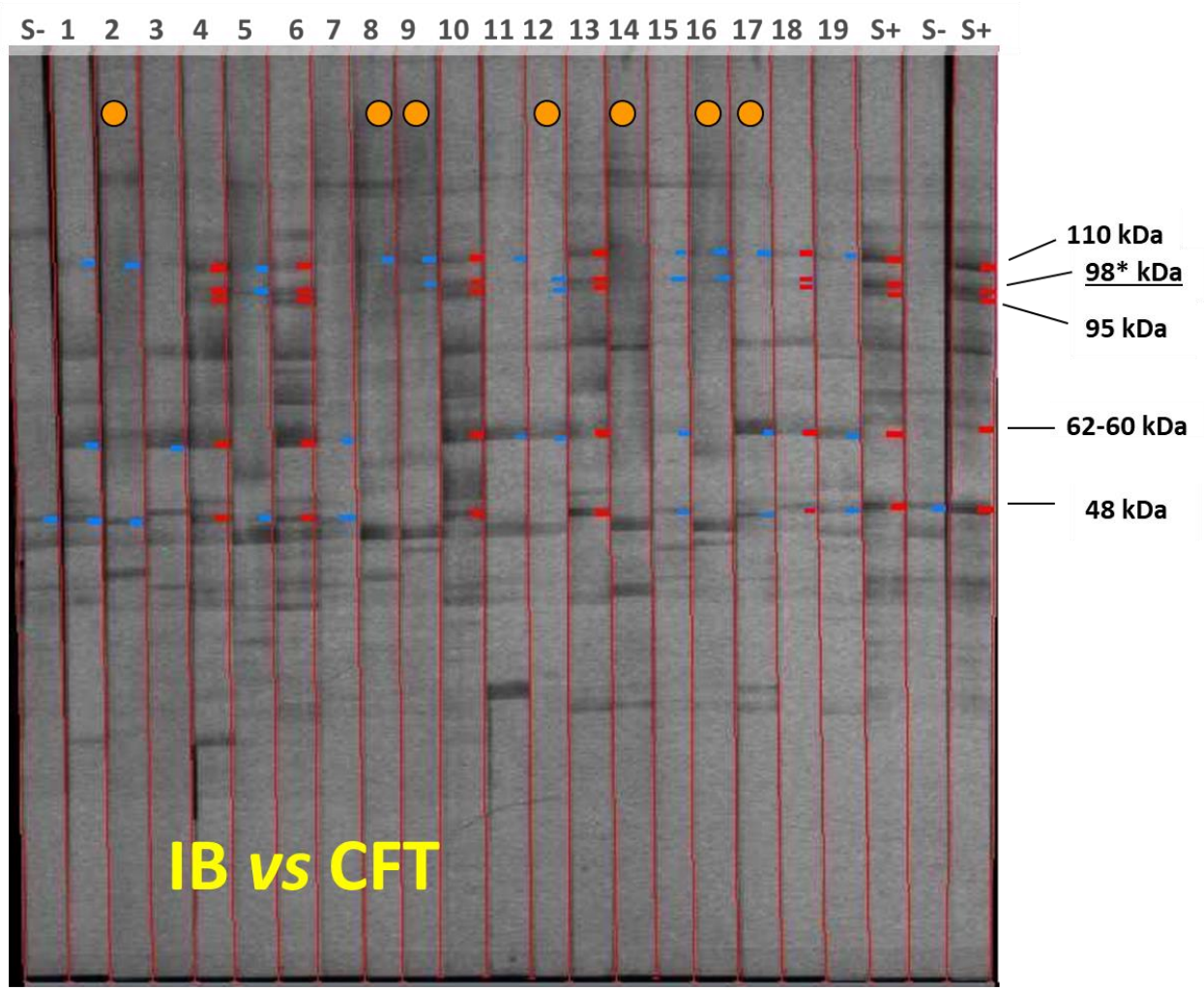
- Specificity of 99.9% (CFT 98%)

# c-ELISA: strengths and weaknesses

- Antibodies are detected by the c-ELISA in an infected herd earlier than CFT, and for a longer period of time (chronic animals)
- Costly when used for mass screening during surveillance campaigns

# Immunoblotting

- Highly specific
- 2nd level test
- Useful to resolve CFT or c-Elisa (doubtful) cases
- Time consuming
- Only for limited number of samples
- Performed in very few Lab

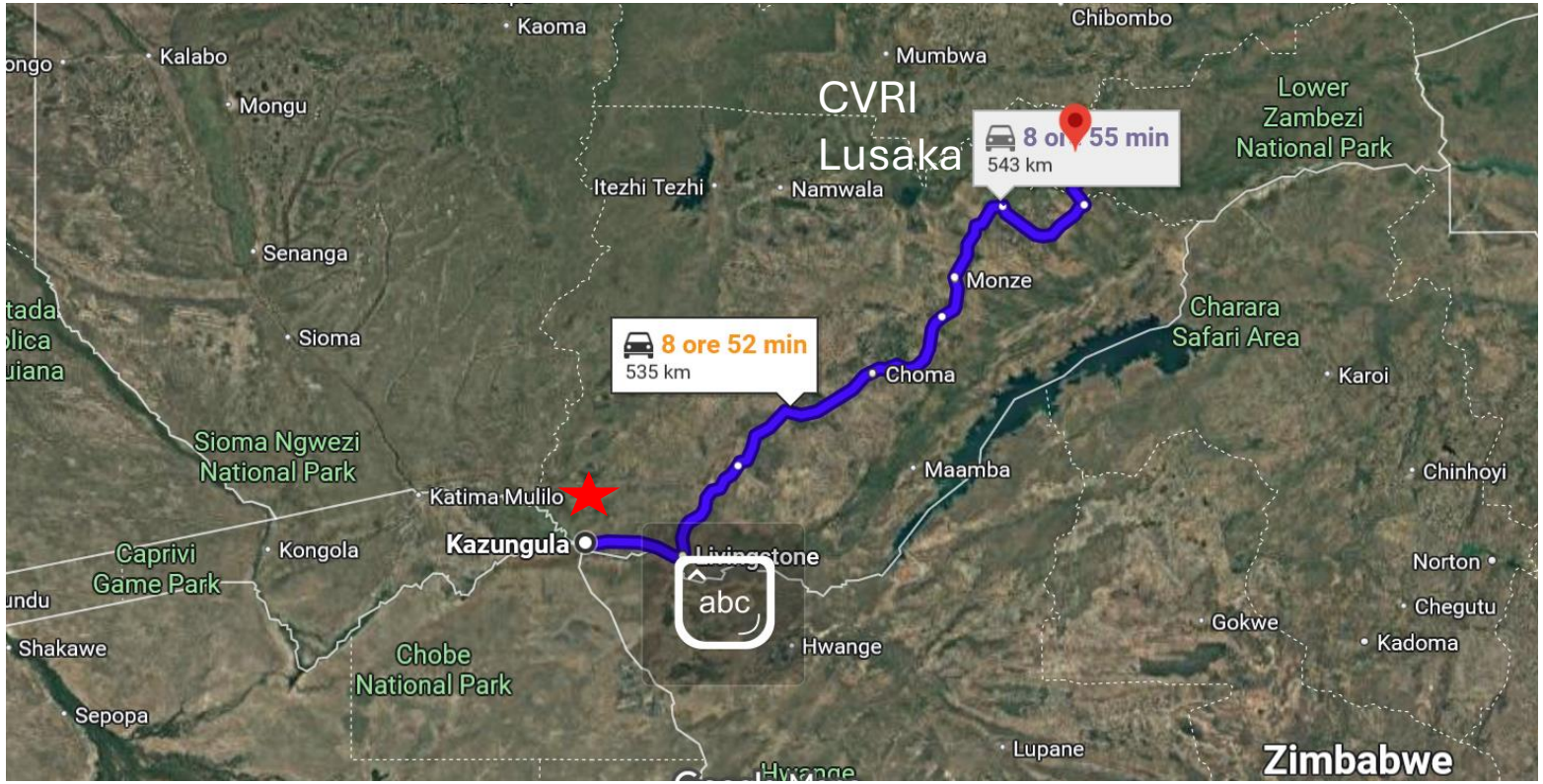


# Possible challenges and solutions for CBPP Lab support during CBPP surveillance and control

- *Mmm* detection and identification is important at first outbreak confirmation in a herd or in village.
- After confirming the presence of CBPP in the area, pathological lesions detected at slaughtering or positivity to serological tests is sufficient to confirm the infection.
- If no CBPP laboratory support is available in the Country, post mortem surveillance at slaughterhouses and in the field remains the only possibility to reveal CBPP **(Evidence Based Surveillance)**.
- Training of animals health technician or selected people in remote areas would increase disease surveillance.
- Vet Lab of neighbouring Countries may support *Mmm* detection from tissue samples or DNA extracted from clinical cases to confirm outbreaks

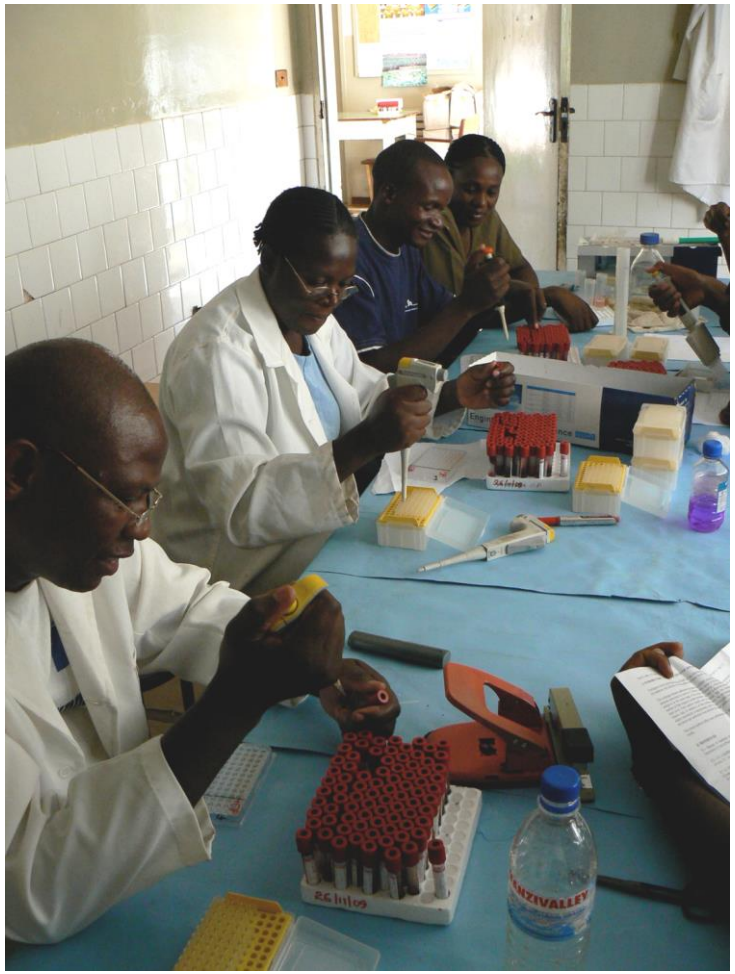
# Possible challenges and solutions during CBPP surveillance – The Zambia example

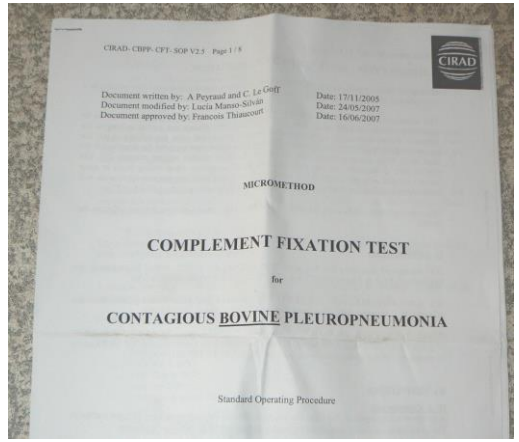
- CBPP outbreaks in Kazungula district (SW province, close to Namibia and Botswana)
- ~9 hrs distance from CVRI in Lusaka
- Need to test several herds in a short period of time

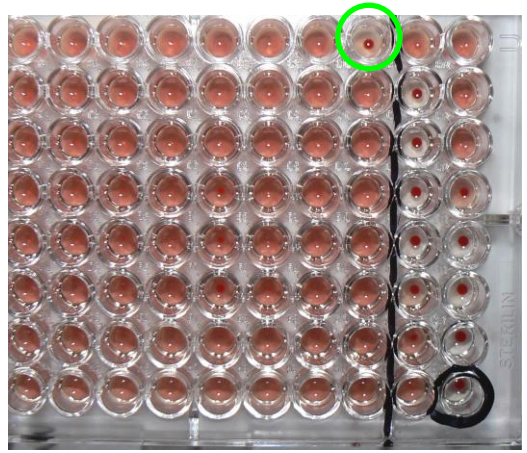


# Solution: moving the Lab into the field

- No peripheral Lab available but veterinary offices with suitable rooms for lab testing
- Personnel, equipment and reagents moved from CVRI for 2-3 weeks
- Time delivery of samples and results
- Rapid knowledge of disease sero- prevalence and faster application of sanitary measures





# Thank you for your attention!