World Organisation for Animal Health
Founded as OIE

Reference Laboratory for CBPP

REPÚBLICA PORTUGUESA
AGRICULTURA E ALIMENTAÇÃO

Instituto Nacional de Investigação Agrária e Veterinária, I.P.

RL FAO Webinar

Serological diagnosis of CBPP: background, interpretation and troubleshooting

28th October 2022

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About Contagious Bovine PleuroPneumonia (CBPP)

Bos taurus

Bubalus bubalis

Bos indicus

CLINICAL SYMPTOMS

Acute form: polypnoea, fever, cough, etc.

Hyperacute form: *idem*

Subclinical or chronic form: no symptoms

LESIONS

Lung hepatization, fibrinous pleurisy

idem

Sequestra, pleura lesions

Recovery

Death

Asymptomatic animals

Mycoplasma mycoides Cluster

M. mycoides* subsp. *mycoides → CBPP

M. mycoides* subsp. *capri

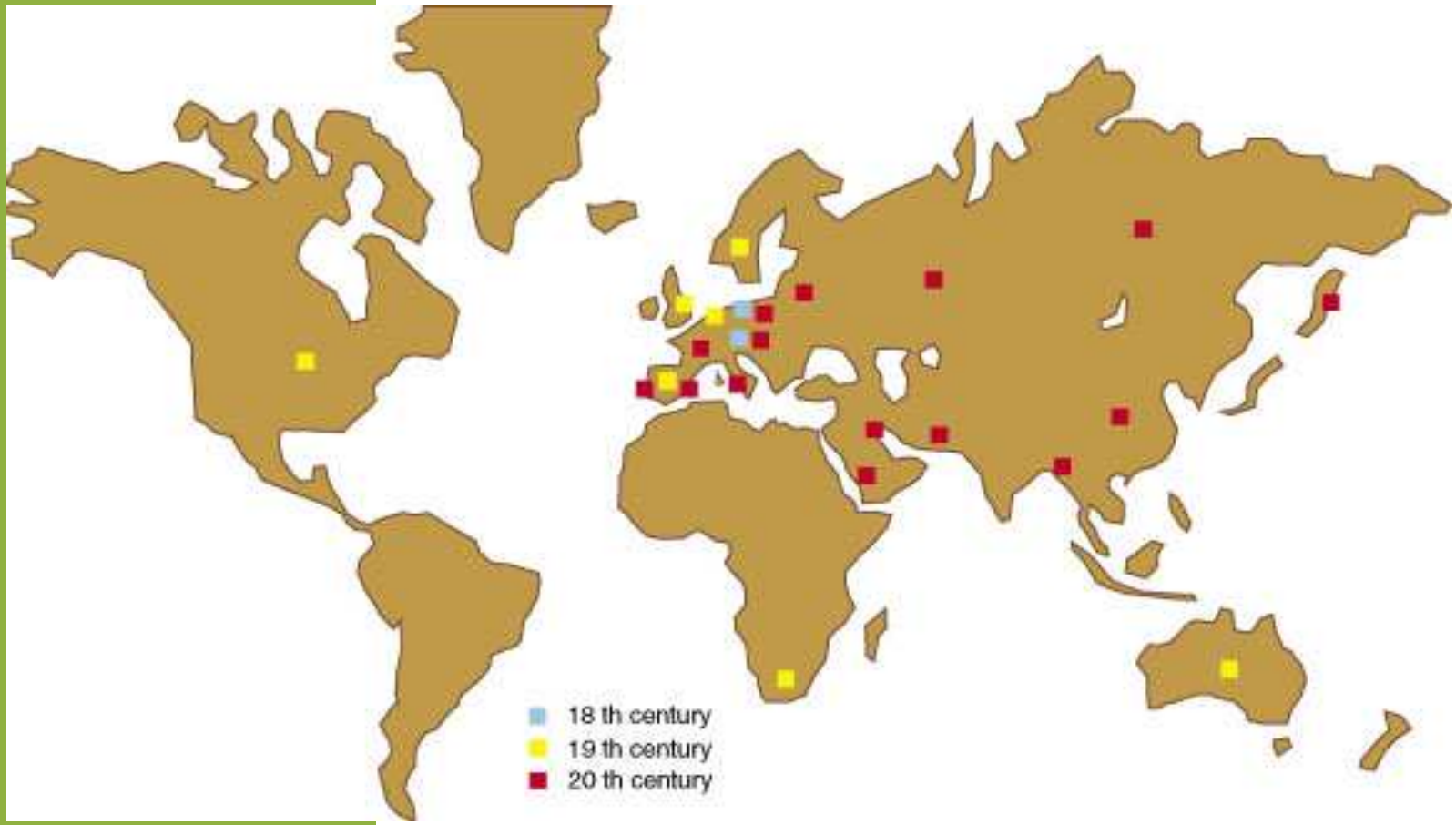
M. capricolum* subsp. *capricolum

M. capricolum* subsp. *capripneumonia → CCPP

***Mycoplasma leachii* sp. nov**

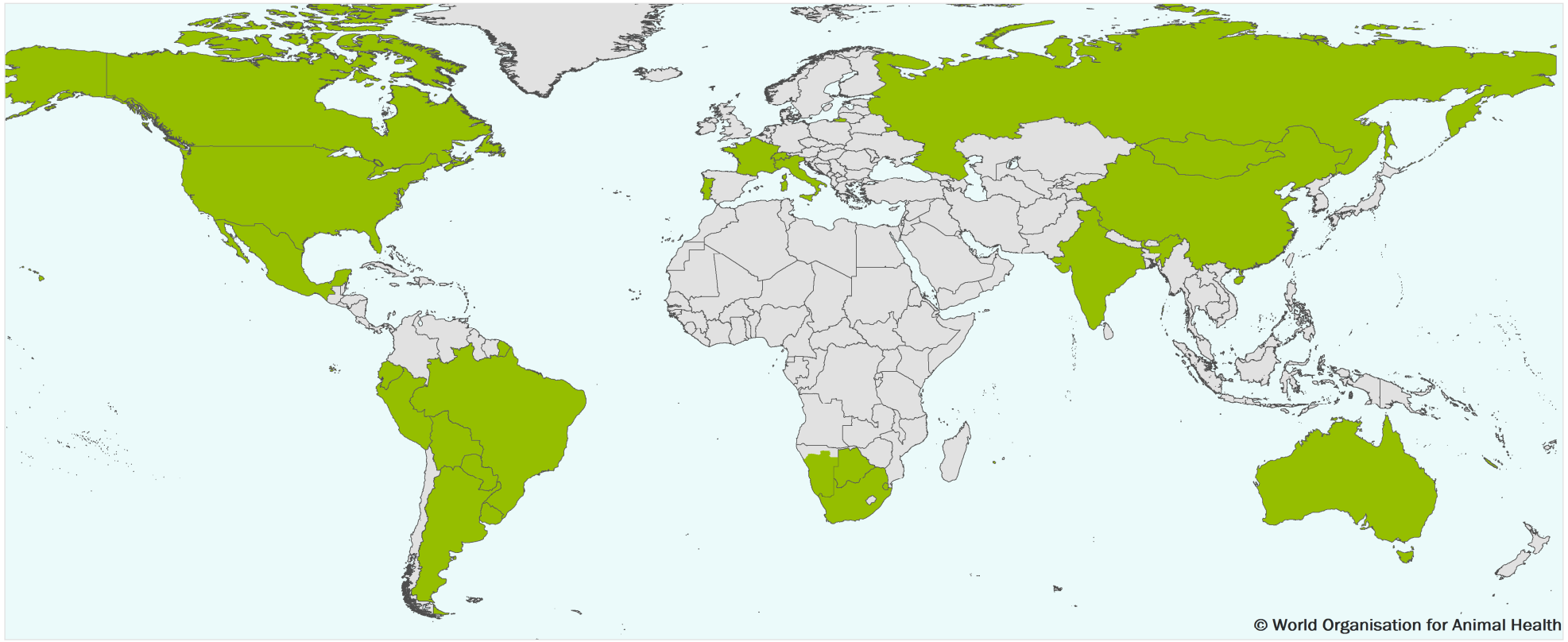
- first isolated, linked to respiratory infections of bovines, in 1898 (Nocard & Roux)
- lack of cell wall
- requirement for cholesterol
- causative agent of Contagious Bovine PleuroPneumonia (CBPP)

History of occurrence of CBPP in the World



World Organisation for Animal Health
Members´official CBPP status map

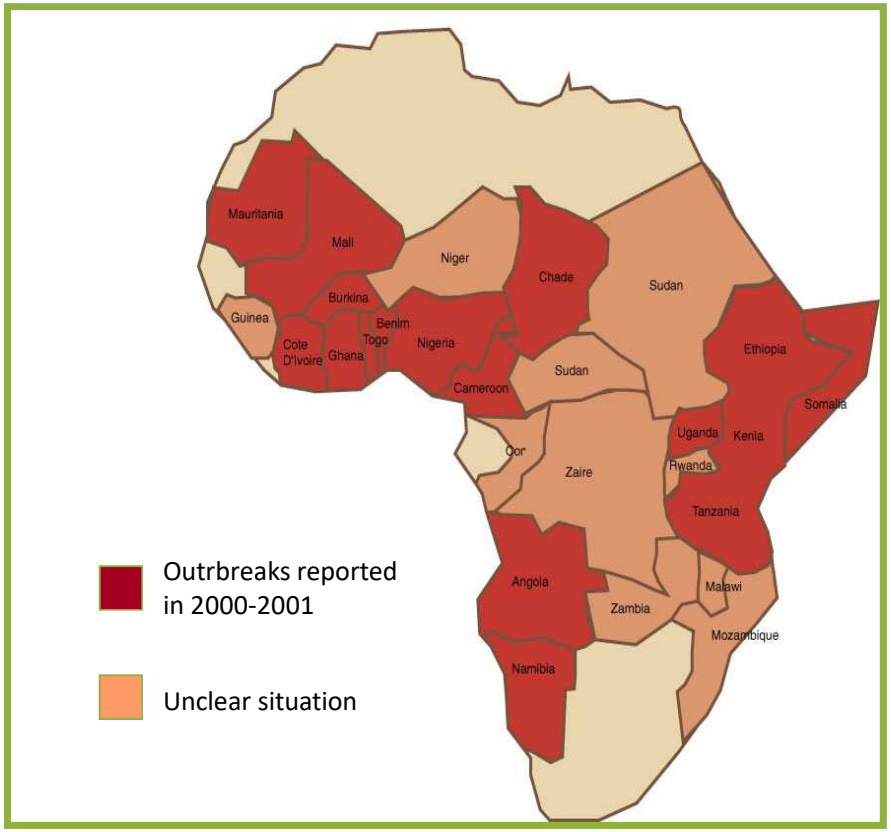
Last update May 2022

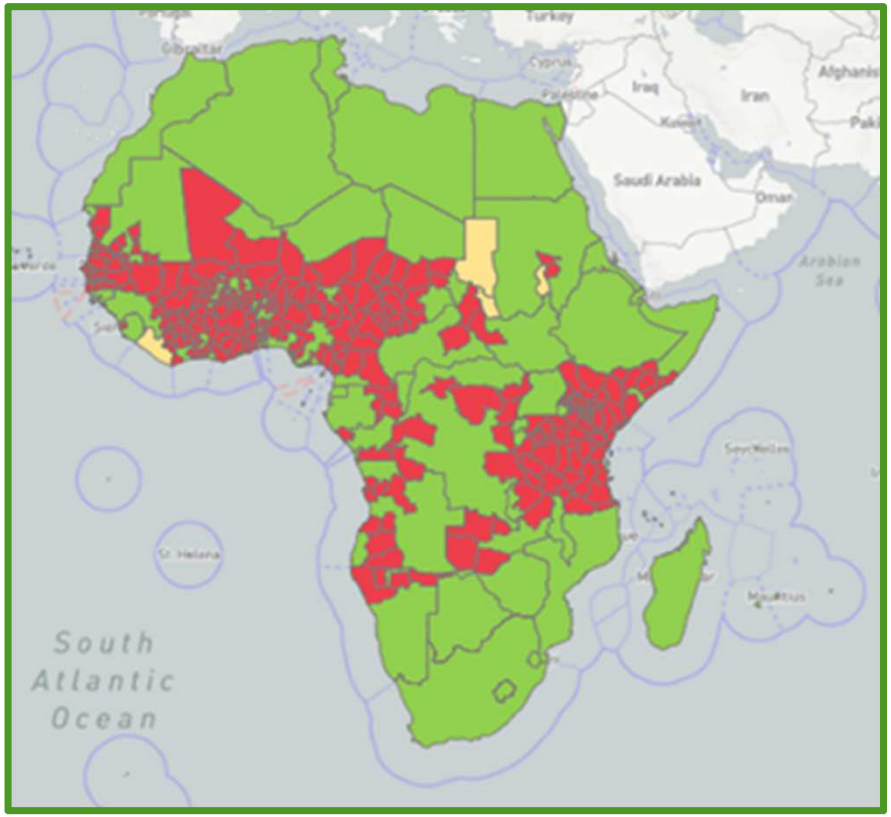


Members and zone recognised as free from CBPP

Countries and zone without an official status for CBPP

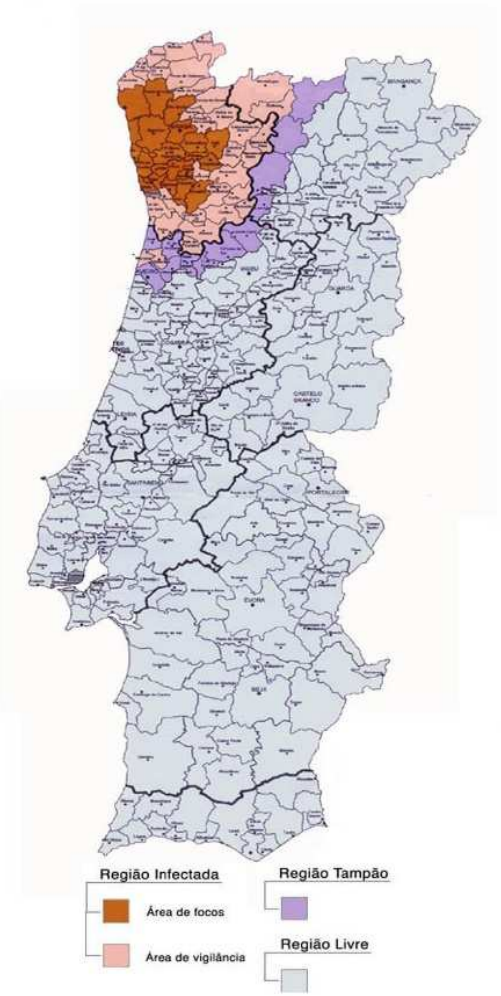
Occurrence of CBPP in Africa

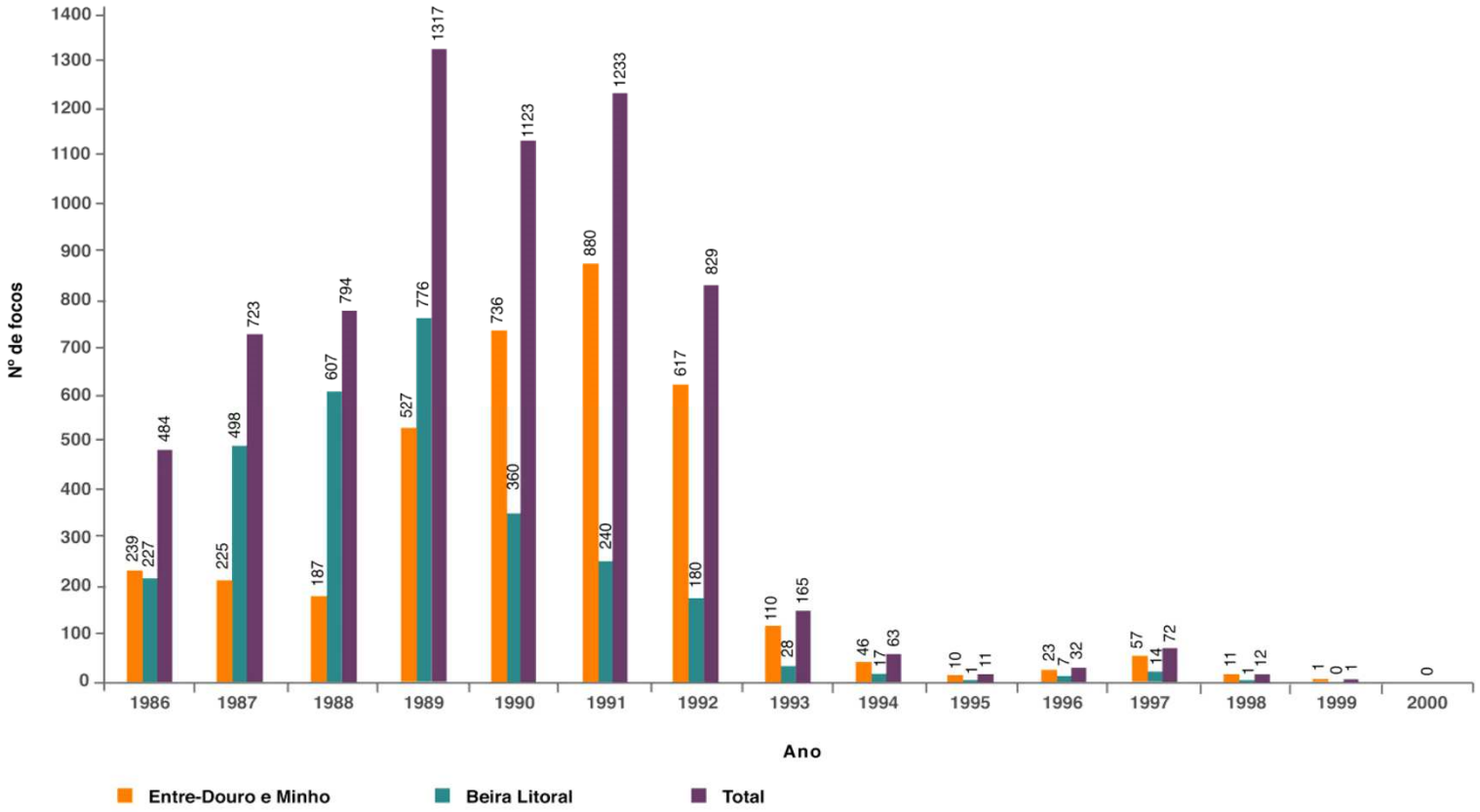




WOAH-WAHIS composite map (2005 – 2019) of the distribution of CBPP in Africa (red = present; yellow = suspected). Map generated on 5 April 2022.

CBPP in Portugal





Milestones in CBPP Control & Erradication in Portugal

1984 - 1986

Standardization of CFT

Improvement of CFT as the official serological screening test for CBPP

1992 - 1995

Evaluation and implementation of PCR systems for specific detection and identification of *Mmm*

1995 - Routine use of PCR in the detection and identification of *Mmm*

1994 - 1997

Immunological recognition of 5 peptides (p110, p98, p62/60 and p48) in CBPP positive sera

1998 - Routine use of Immunobloting (IBT) as CBPP positive confirmatory test

2004 - Approval by OIE of immunobloting as a confirmation of CFT positive animals

Laboratory diagnosis

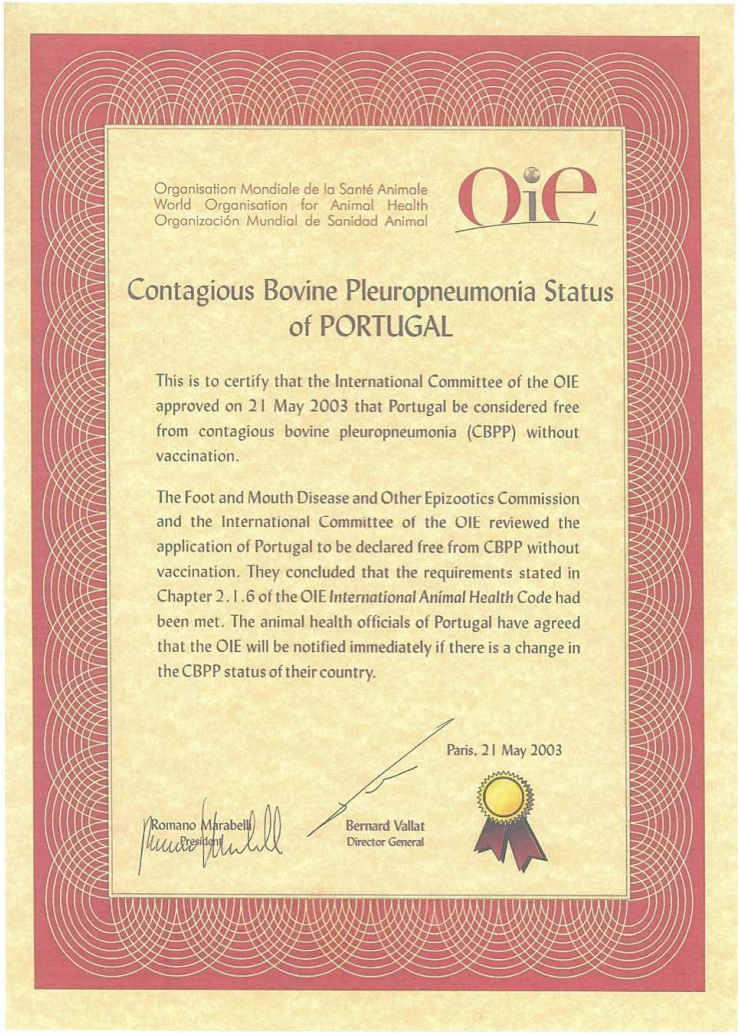
LIVE ANIMAL

- Nasal swabs, bronqueal wash, pleural fluid → Isolation and identification of *Mmm*
- Blood/Sera → Serological tests (CFT & Immunobloting)

POST MORTEM - NECROPSY

- Lung lesions, pleural fluids, lymph nodes → Isolation and identification of *Mmm*

Contagious Bovine Pleuropneumonia Status of PORTUGAL



Organisation Mondiale de la Santé Animale
World Organisation for Animal Health
Organización Mundial de Sanidad Animal

OIE

Contagious Bovine Pleuropneumonia Status of PORTUGAL

This is to certify that the International Committee of the OIE approved on 21 May 2003 that Portugal be considered free from contagious bovine pleuropneumonia (CBPP) without vaccination.

The Foot and Mouth Disease and Other Epizootics Commission and the International Committee of the OIE reviewed the application of Portugal to be declared free from CBPP without vaccination. They concluded that the requirements stated in Chapter 2.1.6 of the OIE *International Animal Health Code* had been met. The animal health officials of Portugal have agreed that the OIE will be notified immediately if there is a change in the CBPP status of their country.

Paris, 21 May 2003

Romano Marabelli
President

Bernard Vallat
Director General

> This is to certify that the International Committee of the OIE approved on **21 May 2003** that Portugal be considered free from contagious bovine pleuropneumonia (CBPP) without vaccination.

CBPP – Control and Eradication

MAIN OBSTACLES

- Occurrence of subacute or asymptomatic infections
- Persistence of chronic carriers - spread of the disease
- Uncontrolled cattle movement
- Sensitivity of serological diagnostic tests
- Absence of effective vaccines
- Tracing the source and routes of infection
- Cross-reactions with closely related species

- ✓ Serological tests (CFT)

CFT & IMMUNOBLOTING

- ✓ Slaughter of all animals of the herd - stamping out
- ✓ Control of cattle movements
- ✓ Extensive vaccination with T1-44 or T1-44Sr strains (Africa)

Serological diagnosis of CBPP: Complement Fixation (CFT), ELISA & Immunoblotting tests

Laboratory methods currently used for diagnosis of CBPP and their purpose

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[3]						
***In-vitro* culture isolation (followed by species identification tests)**	+	–	–	+++	–	–
Direct molecular test (PCR)	–	–	–	++	–	–
Detection of immune response						
CFT	+++	++	+++	++	+++	–
Immunoblotting	++	++	++	++	++	–
C-ELISA	+++	++	+++	++	+++	–

*NB: at present, there is no test described in the table that allows evaluation of the immune status of an animal after vaccination, with the current T1 strains.
Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test;
C-ELISA = competitive enzyme-linked immunosorbent assay.

Serological tests for CBPP diagnosis

Diagnosis is based on clinical signs and the characteristic gross pathologic lesions of the lungs, and confirmation of the disease is only achieved by the isolation and identification of the aetiological agent – ***Mycoplasma mycoides mycoides***.

Complement Fixation Test (CFT) and **Competitive Enzyme-linked immunosorbent assays (C-ELISA)** are the recommended serological tests for screening the disease in a herd.

CFT can detect nearly all sick animals with acute lesions in a herd and those in the early stages or with chronic lesions.

CFT or ELISA positive reactions should be confirmed by **Immunoblotting test**.

CFT still a very useful tool to understand the extent of the infection in a herd or to confirm a free status maintenance of a region

Principles of Good Laboratory Practice

The CF test can be a qualitative or semi-quantitative test (serial dilutions).

The characteristics of all reagents must be taken into account, as they have an impact on the interpretation of the test result. **It is essential to control the origin and validation dates of all reagents**.

The quality control and standardization of all reagents is a critical point in CF test harmonization. Thus, it is important to use appropriate controls, which should be requested from the OIE Reference Laboratories for the CBPP.

The quality of the water must be controlled.
Preferably use ultrapure water, with pH 6.5 ± 0.2, resistivity of 18.2 MΩ / cm and maximum conductivity of 0.1 µS / cm at 25ºC.

Principles of Good Laboratory Practice

Room temperature should be between 18 and 26ºC.

Inactivation of serum samples (free from erythrocytes) is important to reduce bacterial contamination, to destroy the natural complement of the serum itself, to reduce most non-specific IgM. Inactivation should be carried out on the same day of the test.

Samples and reagents must be at room temperature (22 ± 4ºC) at the beginning of the test.

Inactivation according to:

- ✓ Diluted sera - inactivation at 58±2 ºC for 30-50 min.
- ✓ Undiluted sera - inactivation at 60±2 ºC for 30 min.

Complement Fixation Test (CFT) for CBPP diagnosis

Doc. Ref.: PE – 006 – PSA/BM

- CFT is a screening test. All serum samples positive or doubtful to this test must be subjected to a complementary test to confirm the infection.
- CFT is based on the detection and quantification of antibodies fixing the complement anti-*Mycoplasma mycoides* subsp. *mycoides* (*Mmm*).

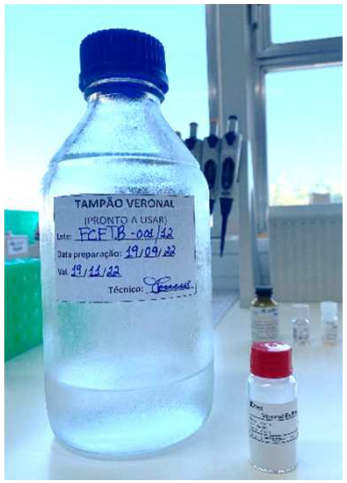
The CFT test consists of two phases:

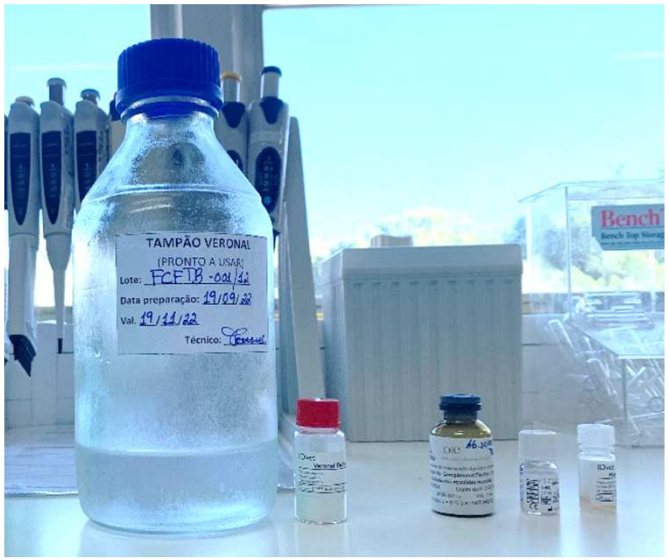
- ✓ **1st Phase – Fixation**
  Fixation of complement to anti-Mmm Ag-Ac complex, if formed.
- ✓ **2nd Phase – Hemolysis**
  Addition of hemolytic system (SH).

Relevant Equipment

- Calibrated centrifuge, preferably refrigerated (tubes and microplates rotor)
- Incubator at 37ºC
- Plate and tube shaker (Vortex type)
- Refrigerator (5±3ºC) and freezer (-16ºC)
- Laboratory water distiller (ultrapure and/or distilled water)
- Variable volume single and multichannel micropipettes

Reagents





- **Veronal buffer** (VB) pH 7.3
- **Antigen** (Ag) - is a suspension of *M. mycoides* subs. *mycoides,* used at a dose of 2 complement fixing units (2 CFU). It must be kept at 4ºC and not frozen.
- **Complement** (C') - It has to be titrated (checkerboard titration) and the highest dilution with complete haemolysis of the sheep red blood cells (SRBC), equals 1 C' unit, from which the working dilution of 2.5 C' units can be calculated.
- **Haemolysin** (H) - is a hyper immune rabbit serum. It is used in 6 haemolytic units, titration read at 50% end-point.

Reagents

CFT procedure

Sheep Red Blood Cells (SRBC)

Is obtained by aseptic puncture of the sheep jugular vein. Preserved in Alsever's solution or citrate solution, and used in a 6% suspension, after 3 washings in VB.

Haemolytic System (HS)

Is prepared by diluting haemolysin in VB to give $12HD_{50,}$ in an equal volume of 6% SRBC. The system is sensitised in a water bath at 37ºC for 30 minutes with periodic shaking.

Control sera → OIE Standards

PRS (4+/160; 2+/320); **NRS** ; and **IC806** (4+/20; 2-3+/40).

Complement Fixation Test Procedure

CFT procedure

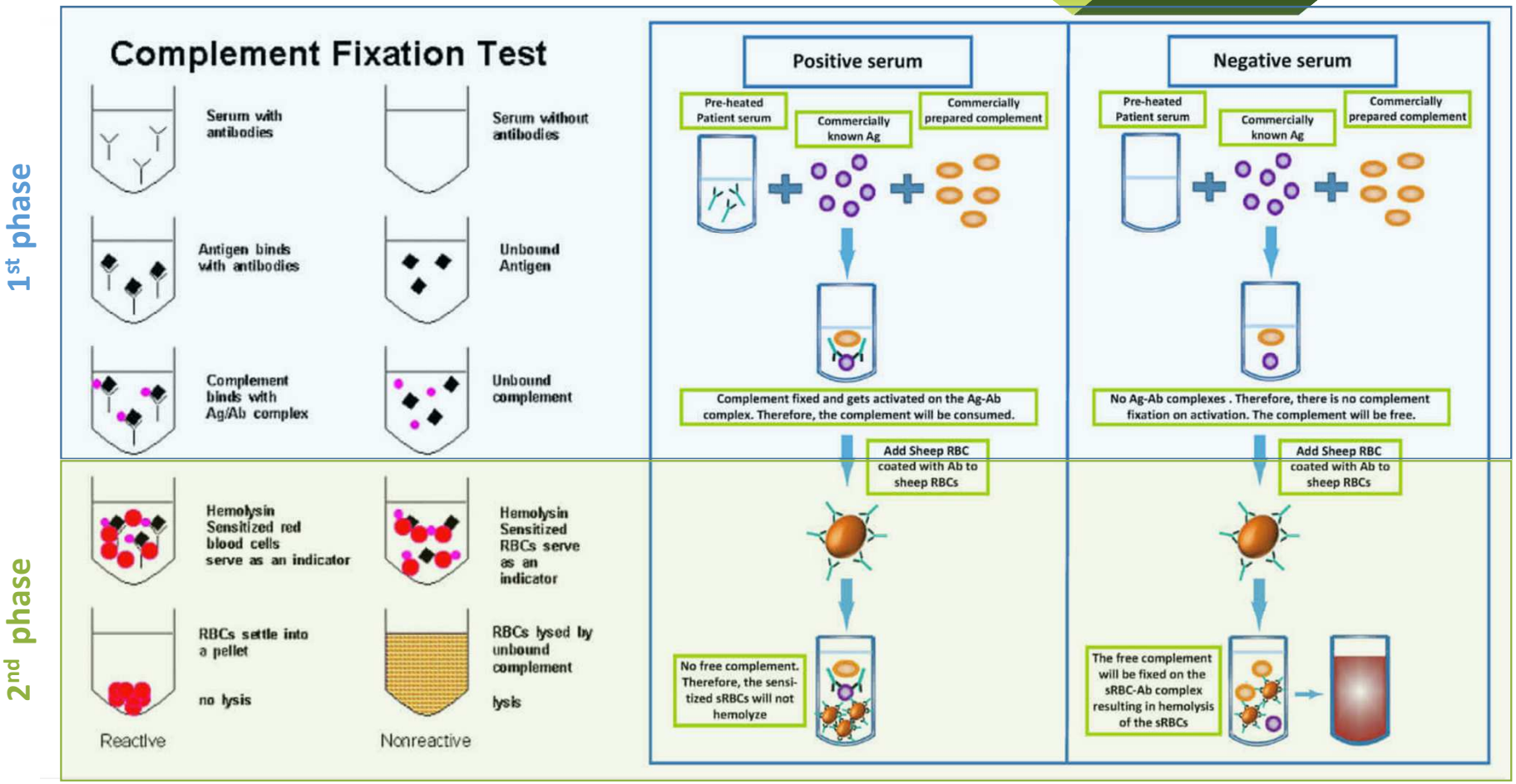


Image Source: Texas Department of State Health Services (DSHS) and Rowa Yousef Alhabbab.

1st phase:

Inactivated and diluted serum samples (SS) are added to a microplate, and mixed with well standardized *M. mycoides* subs. *mycoides* antigen and complement at working dilution.

Controls for Ag, C', HS, ACA should always be included in the test.

CFT scheme

	1	2	3	4	5	6	7	8	9	10	11	12	
SS1	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					A
SS2	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					B
SS3	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					C
SS4	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					D
SS5	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					E
PRS	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					F
CI806	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640					G
NRS	ACA	1/10	1/20	1/40	1/80	1/160	1/320	1/640		AgC	C'2,5C	HSC	H

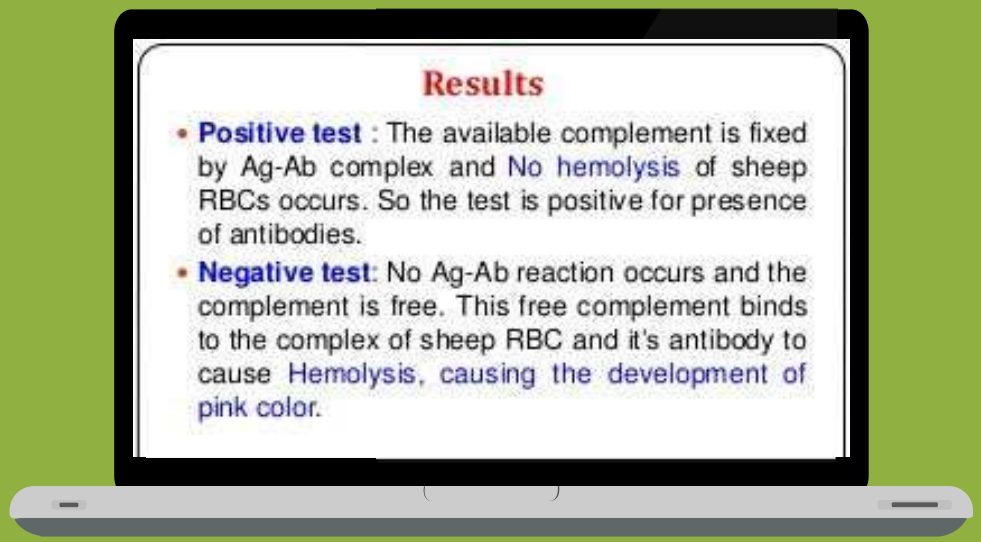
Test Procedure (Micromethod)

2nd phase:

Addition of the SRBCs which have been pre-bound to anti-SRBC antibodies.

After centrifugation of the microplates, the **reading is carried out based on the percentage of complement fixation observed**.

- **Positive result →** 100% inhibition of haemolysis at $^{1}/_{10}$ or (++++ $^{1}/_{10}$)
- **Doubtful result →** 25, 50 or 75% inhibition of haemolysis at $^{1}/_{10}$ or (+, ++, +++ $^{1}/_{10}$)
- **Negative result →** 100% haemolysis
- **Anti-complementary action (ACA) result →** 100% inhibition of haemolysis at $^{1}/_{10}$ in the control well without antigen



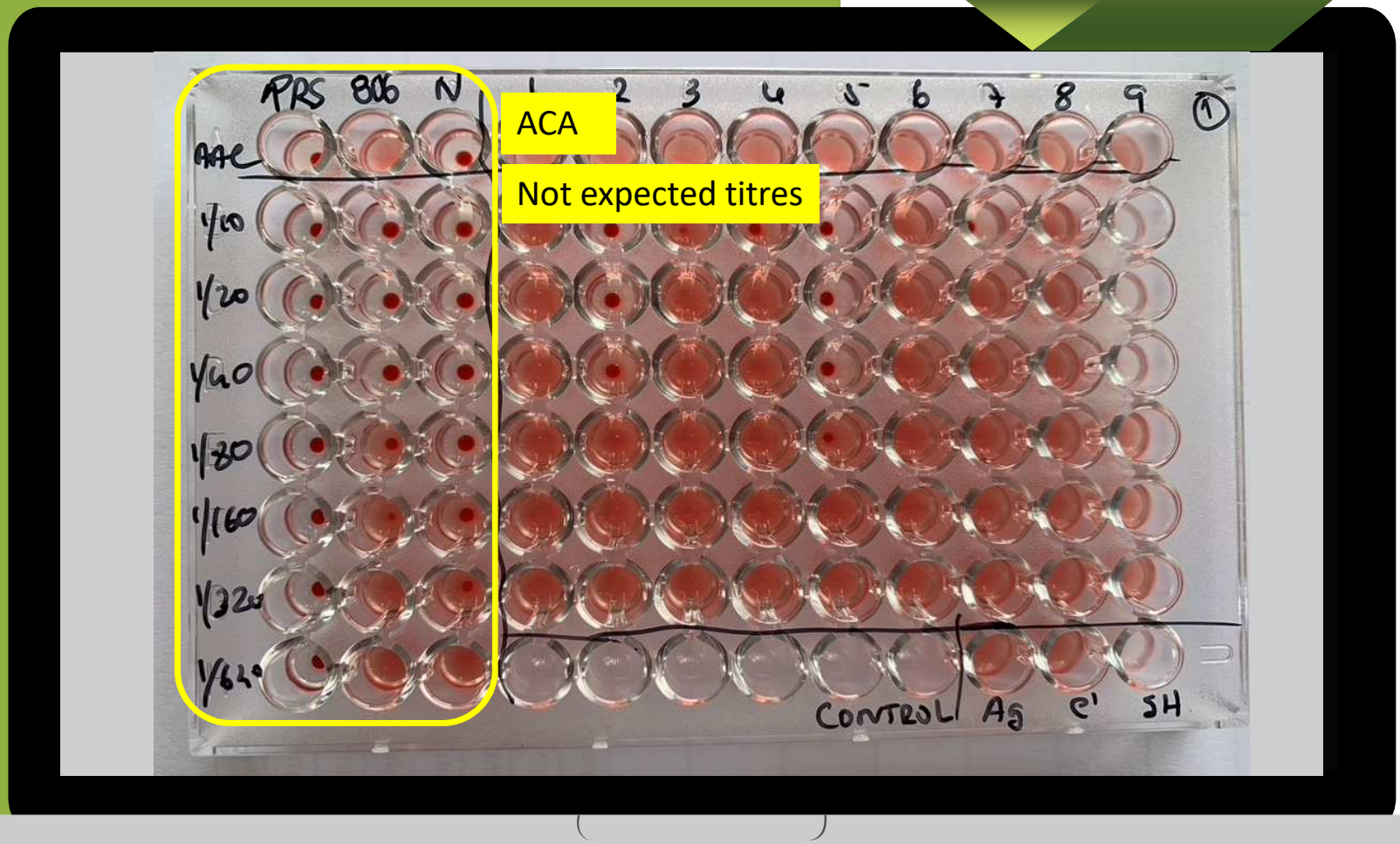
Results Interpretation

CFT procedure

Results Interpretation

CFT procedure

Analysis of Results - Critical points



Complement Fixation Test

ADVANTAGES

- large variety of test antigens can be used
- Reading is easy (lysis, no lysis)
- More specific (98%) than other serological tests

DISADVANTAGES

- Demand on equipment and reagents is large
- Some of components need to be fresh(SRBC's, Complement)
- Less sensitive (~63%) than ELISA tests

Available ELISA tests

IDEXX CBPP Ab
Test competitive ELISA (cELISA)

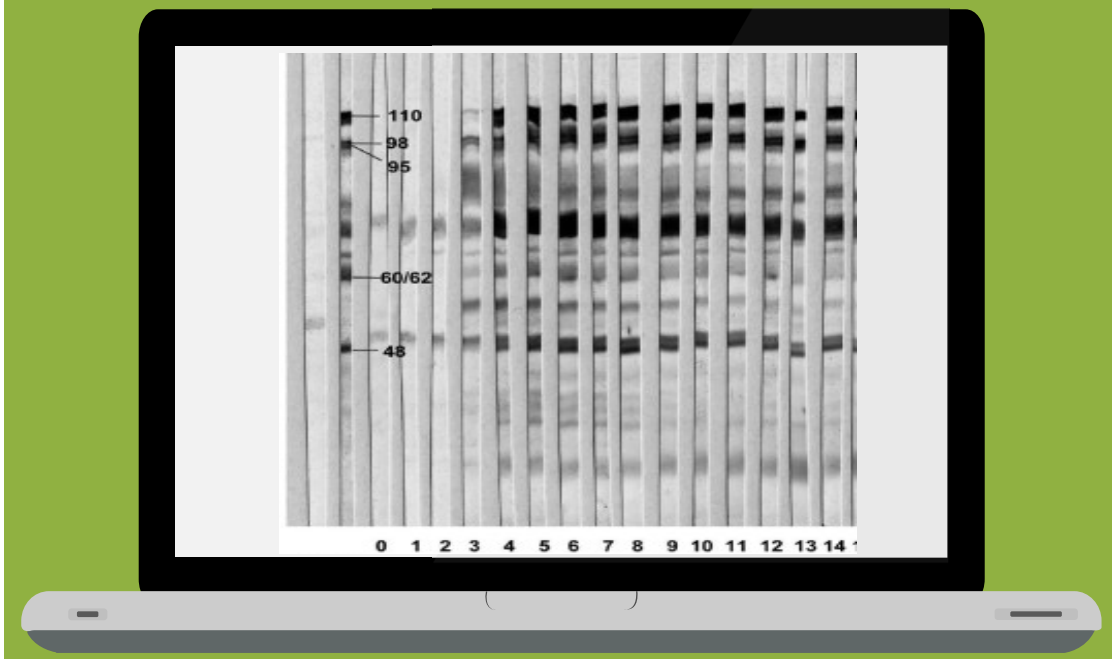
The IDEXX CBPP Ab Test is a cELISA based on a monoclonal anti-MmmSC antibody (named 117/5). Developed by the CIRAD-EMVT (FAO world reference center for CBPP), as an alternative to the CFT for the OIE, and can be used for official CBPP control. This test is under evaluation by the Joint Division FAO/AIEA within the framework of a Coordinated Research Project (CRP).

ABBEXA – CBPP ELISA Kit
(Sandwich ELISA)

Is based on sandwich ELISA technology. An antibody is pre-coated onto a 96-well plate. Controls, test samples and HRP-conjugated reagent are added to the wells and incubated.
Not validated. For research use only

Immunoblotting Test (IBT)

- Is an immunoenzymatic test that presents a higher specificity than the CFT, enabling the detection of false positives reactions *(Gonçalves et al. 1998)*.
- IBT is used to confirm doubtful CFT or C-ELISA results.
- IBT is difficult to standardise. The strain used to prepare the antigen is a critical factor. Antigen should be prepared from a *M. mycoides* subs. *mycoides* strain that must present five specific antigenic bands of 110, 98, 95, 62/60 and 48 kDa.
    - The CBPP WOAH Reference Laboratory in Portugal can provide strips as well as the positive and negative control sera, upon request.



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