Contagious Bovine Pleuropneumonia (CBPP)
Doc.Ref.: PE – 006 – PSA/BM

Complement Fixation Test (CFT)

Principle of complement fixation test

The complement fixation test (CFT) is used as a screening test; all serum samples positive or doubtful to this test must be subjected to a complementary test to confirm the infection. This test 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 - Fixation and Hemolysis. In the 1st phase (Fixation), the Mmm antigen (Ag) and the serum sample to be tested (SS) are mixed with guinea pig serum that contains complement (C). If the ST contains specific antibodies (Ab) anti-Mmm an Ag-Ac complex is formed and the complement is activated and fixed, not being available to react in the 2nd phase. In the 2nd phase (Hemolysis), the hemolytic system (SH) is added. When the ST has antibodies specific to the Ag, the GV are not lysed by the complement because it was fixed in the 1st phase of the test and the reaction is positive. When the ST does not have specific Ab, there is no formation of the Ag-Ac complex in the 1st phase of the test, the C is not activated and remains free to smooth the GV of the hemolytic system, the reaction being negative. The degree of hemolysis, a consequence of globular lysis, is the basis for the interpretation and quantification of the results.

In cattle, the main complement-fixing antibody is IgG1; IgG2 does not fix the guinea pig complement and has the ability to prevent complement fixation by other immunoglobulins, thus producing a prozone phenomenon. It occurs when the amount of antibodies present in the serum sample is disproportionate to the amount of test antigen (i.e. highly positive sera) generating false-negative results. The problem is easily solved by testing the sample at higher dilutions.

1. Reagents:

1.1. **Veronal buffer (VB)** pH 7.3

1.2. **Serum samples (SS)** free from erythrocytes must be inactivated at 56-58ºC for 30 minutes and diluted 1/10 in VB.

1.3. **Antigen (Ag) -** is a suspension of *Mmm* and used at a dose of 2 complement fixing units (2CFU). It must be kept at 4ºC and not frozen.

1.4. **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.

1.5. **Haemolysin (H) -** is a hyper immune rabbit serum. It is used in 12 haemolytic units, titration read at 50% end-point.

1.6. **Sheep Red Blood Cells (SRBC) -** is obtained by aseptic puncture of the sheep jugular vein. It is preserved in Alsever's solution (or citrate solution) and used in a 6% suspension, after 3 washings in VB.

1.7. **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.

1.8. **Positive Reference Serum (PRS)** – positive serum from a CBPP infected animal, with a titre of 4+/160 and 2+/320. Available at OIE Reference Laboratory for CBPP (Portugal)

1.9. **Negative Reference Serum (NRS)** – negative serum from a CBPP free animal. Available at OIE Reference Laboratory for CBPP (Portugal)

2. Test Procedure (Micromethod):

2.1. Dispense 25µl of the test serum samples, already diluted at $^1/_{10}$ and inactivated, and perform the following serum dilutions (see scheme presented in Table 1).

2.2. Add 25µl of Ag at a dose of 2 CFU.

2.3. Add 25µl of C' at a dose of 2.5 units. Shake vigorously in a microplate shaker and incubate at 37ºC for 30 minutes with periodic shaking.

2.4. Add 25µl of HS. Shake vigorously in a microplate shaker and incubate at 37ºC for 30 minutes with periodic shaking.

2.5. It is necessary to set up the following controls:

Ag → 25µl VB + 25µl Ag + 25µl C' + 25µl SH

C' → ½ unit, 1 unit and 2.5 units (50µl VB + 25µl C' + 25µl SH)

HS → 75µl VB + 25µl HS

PC → 25µl PC $^1/_{10}$ + 25µl Ag + 25µl C' 2.5 units + 25µl SH

NC → 25µl NC $^1/_{10}$ + 25µl Ag + 25µl C' 2.5 units + 25µl SH

ACA (anti-complementary activity of SS) → 25µl VB + 25µl SS + 25µl C' + 25µl HS

Note: The PC and NC are always used in each microplate or in a series of microplates where the same batches of reagents are used.

2.6. After centrifugation of the microplates at 125g for 2 minutes, the reading is carried out based on the percentage of complement fixation observed.

2.7. Reading of the results:

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

2.8. <u>Expected control readings</u>:

Ag → 100% haemolysis

C' ½ units → 50% haemolysis; C' 1 unit → 100% haemolysis; C' 2,5 units → 100% haemolysis

HS → 100% inhibition of haemolysis

PRS → ++++ $^1/_{160}$ and ++ $^1/_{320}$

NRS → 100% haemolysis

Table 1. 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
SS6	ACA	$^1/_{10}$	$^1/_{20}$	$^1/_{40}$	$^1/_{80}$	$^1/_{160}$	$^1/_{320}$	$^1/_{640}$					F
PRS	$^1/_{10}$	$^1/_{20}$	$^1/_{40}$	$^1/_{80}$	$^1/_{160}$	$^1/_{320}$	$^1/_{640}$						G
NRS	$^1/_{10}$	$^1/_{20}$	$^1/_{40}$	$^1/_{80}$	$^1/_{160}$	$^1/_{320}$	$^1/_{640}$	AgC	C'$^1/_2$C	C'1C	C'2,5C	HSC	H

SS → serum sample; AgC → Ag control; C'$^1/_2$C → C'$^1/_2$ unit control; C'1C → C'1 unit control; C'2,5C → C'2,5 units control; HSC → HS control