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| --- | --- | --- |
|  | **DETECTION OF ANTIBODIES AGAINST BURKHOLDERIA**  **BY THE TECHNIQUE OF COMPLEMENT FIXATION**  **(CFT GLANDERS)** | |
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This protocol is an OIE-based method used at the EU-RL, all OIE-CFT based methods validated and used successfully in the proficiency tests can be used for this assay.

1. Topic and scope

This document describes the method for the detection of antibodies specific to *Burkholderia mallei*, the agent of glanders, by the microtitre complement fixation test (CFT) according to the world organisation for animal health (OIE) international standard: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Chapter 3.5.11, glanders and melioidosis (<https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.11_GLANDERS.pdf>).

It is applicable to the serological diagnosis of glanders from serum samples of any equid (horse, donkey, mule...).

A specific antigen is added to the serum to be tested. If specific antibodies against this antigen are present, immune complexes are formed. Heterologous complement is added. Once the specific antibody-antigen immune complexes are formed, the heterologous complement fixes to these complexes. Indigenous complement naturally present in the serum to be tested is prior destroyed by heat inactivation.

This reaction is revealed by adding a second immune system: erythrocytes-hemolysin (sensitised-Red Blood Cells (RBC)). The heterologous complement that was not fixed to the first complexes, will fix to the sensitised-RBC, thus causing the lysis of RBC to an extent that depends on the quantity of the complement that was not used on the first stage. The degree of hemolysis, observed through the colouring of the reaction medium (after centrifugation or sedimentation), is inversely proportional to the titre of specific antibodies originally present in the serum.

1. material to be examined

**2.1. Serum**

The serological diagnosis of glanders by complement fixation test is performed on equid sera.

Upon reception, the sample tubes must not have been opened or damaged. A volume of serum greater than 100 µL must be provided. The serum must not be hemolyzed or coagulated. Before testing, a serum sample should be kept refrigerated (5 ± 3°C).

**2.2. Transport of samples**

Sera prepared from the whole blood samples received at the lab must be stored at 5 ± 3°C. It is advisable for all blood samples to be centrifuged and stored whenever possible without the clot. For long-lasting storage, it is advisable to freeze sera without clot at ≤ -16°C.

1. Diluents, reagents and other products

**Notice**: Trade names or suppliers may be mentioned in the description of the products required for the implementation of this method. This information is provided for the benefit of the users of the method and does not mean that the EU-RL recommends the exclusive use of these products. Equivalent products may be used if it is demonstrated that they lead to the same results. The conditions for aliquoting the reagents described in this procedure are given for the benefit of the users of the method and each other laboratory is free to choose its own aliquoting conditions. All reagents should be stored according to the conditions described by the suppliers.

**3.1 Diluents**

3.1.1 Veronal buffer calcium magnesium ph 7.2 (vb)

This may be prepared from tablets available commercially.

*Example:*

*NaCl 8.500 g*

*Barbital 0.575 g*

*Diethylmalonylurea sodium 0.185 g*

*MgCl2, 6 H2O 0.168 g*

*CaCl2 0.028 g*

*Distilled or equivalent quality water up to 1 000 mL*

**3.2 Reagents**

3.2.1 antigen (Ag)

The antigen is a protein suspension of *B. mallei*. The antigen should be stored and prepared according to the supplier’s instructions (see **Annex B**), at a rate of 25 µL per well.

Potential suppliers:

|  |  |
| --- | --- |
| **Company** | **Antigen reference** |
| Bioveta | Antigen *Burkholderia mallei* RVK 1x10 mL t320 (VABUR002981) |
| Ccpro | Malleus-KBR Antigen (1x10 mL)(VD-0013-N) |
| WUR | Glanders Antigen (2 mL) |

3.2.2 control sera: positive and negative

Negative and Positive control serum could be prepared by the lab or commercially acquired (in that case, always used the positive control provided by the company from which the antigen was issued).

Potential suppliers:

|  |  |
| --- | --- |
| **Company** | **Positive control reference** |
| Bioveta | Serum *Burkholderia mallei* POZ 1x5 mL (VSEBU001973) |
| Ccpro | Malleus-KBR Positivserum (1x5 mL) (VD-0012-R) |
| WUR | No more available |

3.2.3 freeze-dried guinea-pig complement (C)

The guinea-pig complement is a serum molecular complex, with some components of which may fix themselves to specific antigen-antibody immune complex. Guinea pig complement is used at 25 µL per test and at 5 H50 units. For the CFT, the complement should be diluted according to the result of the complement titration (**Annex A**). If the complement is not stabilised, it should be titrated at each series of tests. If the complement is stabilised the titration should be performed for each new batch, or, ideally, each new vial. In any case, complement titration at each series of tests provides the best reliability of the results.

The reconstituted complement, if not used immediately, must be stored at 5 ± 3°C, until the performance of the series of tests on the same day, and for any volume left, at ≤ – 16°C or 5 ± 3°C according to the supplier’s instructions.

The complement is available commercially and should be reconstituted according to the supplier’s instructions.

Potential suppliers:

|  |  |
| --- | --- |
| **Company** | **Complement reference** |
| Innovative Diagnostics | CPLT-2x5 mL |

3.2.4 sheep erythrocytes at 50 % (RBC)

Sheep erythrocytes (RBC) at 50 % are to be diluted to 1/25 (final concentration: 2 %) in VB.

Potential suppliers:

|  |  |
| --- | --- |
| **Company** | **RBC reference** |
| Orgentec | VSE-410 |

3.2.5 rabbit haemolytic anti-rbc serum (haemolysin (H))

Serum from a hyper-immunised animal against heterologous red blood cells (RBC), with a high titre of anti-red blood cells antibodies and causing *in vitro* the lysis of specific corresponding erythrocytes whenever the complement is present. Hemolytic serum is rabbit anti-sheep red blood cell serum. It is used at 2 units (see **Annex C**)

Potential suppliers:

|  |  |
| --- | --- |
| **Company** | **Haemolysin reference** |
| Innovative Diagnostics | HS |

3.2.6 sensitised-sheep red blood cells (sensitised-RBC)

The sensitised-RBC used at a volume of 50 µL, are a mixture of equal volumes of:

- a sheep red blood cells (RBC) suspension at 2 %, and of

- a rabbit haemolysin dilution at a titre of twice the minimum concentration required to produce 100 % lysis of sensitised-RBC in the presence of a titrated solution of guinea-pig complement (two 100%-haemolytic-units).

**3.3 Other products**

3.3.1 water

The chemical and bacteriological quality of the water used to prepare the different reagents must be verified. It must meet the requirements of the supplier and/or those imposed by the laboratory. These requirements enable a satisfactory implementation of the technique described in the present document.

1. CONTROLS

**VARIOUS CONTROLS MUST BE INCLUDED IN THE ANALYSIS BEFORE READING THE SAMPLE RESULTS.**

**4.1 antigen control**

The absence of the anti-complementary activity of the antigen used is checked in an “antigen-control well”. In this well, the test is performed without serum, the latter being replaced by the same volume of diluent. This control is performed once for each series of tests.

**4.2 complement control**

The activity of the complement on the sensitised-RBC is checked in a “complement-control well”. In this well, the test is performed with the complement and the sensitised-RBC only, the antigen and the serum being both replaced by identical volumes of diluent. This control is performed once for each series of tests.

**4.3 sensitised rbc control**

The sensitised-RBC’ quality is checked in a “sensitised-RBC-control well”. In this well, the test is performed with the sensitised-RBC only, the complement, the antigen, and the serum being all replaced by identical volumes of diluent. This control is performed once for each series of tests.

**4.4 control positive and negative sera**

Positive and negative control sera are both included in each series of tests.

**4.5 serum to test control**

The absence of the anti-complementary activity of the tested serum is checked in a corresponding “serum-control well”. In this well, the test is performed without the antigen, its volume being replaced by the same volume of the diluent. This control is performed for each tested serum. In this technique, control sera are established for the 1/5 and 1/10 dilutions.

1. Equipment and plastic/glass ware

Conventional serology laboratory equipment and in particular:

- Temperature-controlled incubator set at 37 ± 2°C.

- Water bath (circulating water bath if possible) set at 37 ± 1°C.

- Water bath (circulating water bath if possible) set at 59 ± 1°C or 63 ± 1°C

- Temperature-controlled refrigerator at 5 ± 3°C.

- Temperature-controlled freezer at ≤ -16°C.

- Centrifuge, refrigerated if possible (allowing adequate acceleration).

- Distribution and dilution device having a suitable volume range and accuracy.

- Disposable microplates (96 well round U bottomed) with lid or cover (plastic or adhesive).

- Test tubes and racks.

- Light table (eventually).

- Timer or chronometer.

1. Preparation of the reagents

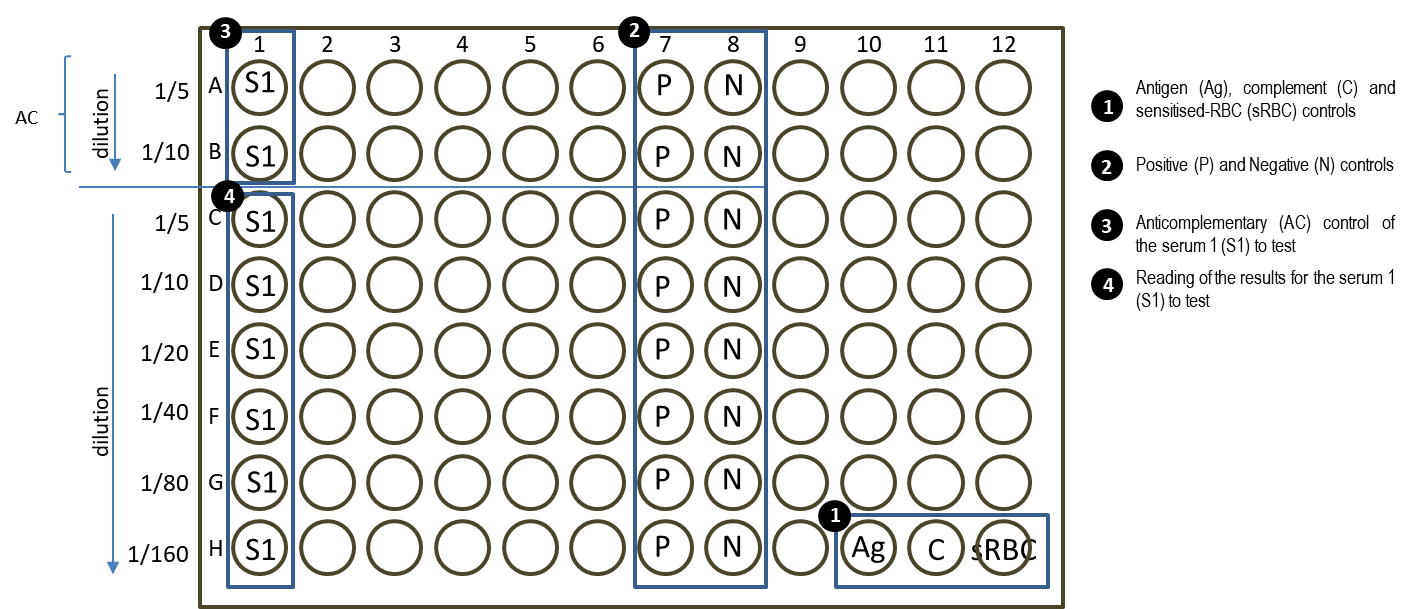
In order to proceed with the analysis of sera by CFT it is necessary to have previously: titrated: the sensitised-RBC, the complement C and the antigen Ag. The procedures for the preparation and titration of the reagents are described in the Annexes:

- **Annex A**: Titration of complement

- **Annex B**: Titration of antigen

- **Annex C**: Titration of hemolytic serum

1. DESCRIPTION OF THE CFT PROCEDURE



**7.1 Dilution of test sera and of control sera**

Prepare the 1/5 dilution in VB of each test and control (positive and negative) sera

*Example: 40 µL of each test and control sera in 160 µL of VB.*

**7.2 Heat-inactivation of test and of control sera**

Diluted test and control sera are decomplemented for 30 min. in the water bath at 59 ± 1°C (for sera from horses) or at 63 ± 1°C (for sera from donkeys or mules).

**7.3 Dilution of inactivated test and control sera**

* For each test sera, 25 µL of this inactivated 1/5 dilution are placed in the well of the first, second, third and fourth rows of a plate. The first and the second row are an anti-complementary control for each serum.
* Volumes of 25 µL of VB are added to the wells of the first and second row (anti-complementary controls) to compensate for lack of antigen.
* Volumes of 25 µL of VB are added to the wells of the second row then of the fourth to the eighth row.
* Serial doubling dilutions are then made by transferring 25 µL volumes of serum from the fourth row onwards; 25 µL of the resulting mixture in the second and last row are discarded.
* Same procedure for the positive and negative control sera

**7.4 Distribution of the antigen, the buffer and of the complement**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Diluted serum** | **VB** | **Diluted antigen** | **C** (5 H50) |
| **Anti-complementary control wells** (dilutions 1/5 and 1/10) | 25 µL | 25 µL | — | 25 µL |
| **Test wells**  (dilutions 1/5 to 1/160) | 25 µL | — | 25 µL | 25 µL |

**7.1.3 Preparation of controls**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **VB** | **Diluted antigen** | **C** (5 H50) |
| **Antigen control** | 25 µL | 25 µL | 25 µL |
| **Complement control** | 50 µL | — | 25 µL |
| **Sensitised-RBC control** | 75 µL | — | — |

**7.1.4 Reaction**

Shake the plates, cover them and place them at 5 ± 3°C overnight (16 to 20 hours).

**7.1.5 2nd step (haemolysis / haemolysis inhibition) (2nd day)**

7.1.5.1 Preparation of the sensitised RBC: mix equal quantities of the sheep red blood cells (RBC) suspension at 2 % and the rabbit haemolysin dilution (two 100%-haemolytic-units) that have been prepared beforehand and stored separately at 5 ± 3°C.

7.1.5.2 Leave the mixture at room temperature for 10 min.

7.1.5.3 Take the plates out of the refrigerator and place them for 10 min. in the incubator at 37 ± 2°C, if possible, without stacking them. The sensitized-RBC stay then 20 min at room temperature.

7.1.5.4 Add 50 µL of the sensitized-RBC in each well, shake the plates, cover them and place them in the incubator at 37 ± 2°C for 45 min, if possible, without stacking them.

7.1.5.5 Centrifuge plates in order to obtain the sensitised-RBC sedimentation, 600 *g* for 10 min. Otherwise, they should be placed at 5 ± 3°C for 2–3 hours to allow unlysed cells to settle.

1. READING AND VALIDATION

**8.1 Reading of the plate**

Read the results by looking the plate from above with a light source beneath it. Notation is done by reading the colour of the supernatant.

The % of hemolysis for each of the wells is determined by comparison with a gradiant made as follows:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Haemolysis control (%) | **100** | **75** | **50** | **25** | **0** |
| Notation | 0 | + | ++ | +++ | ++++ |
| VB (µL) | 0 | 25 | 50 | 75 | 100 |
| Total haemolysis supernatant (µL) **a** | 100 | 75 | 50 | 25 | 0 |
| *a prepared from 100 % haemolysis wells.* | | | | | |

If needed, read the results using a plate-reading mirror.

**8.2 Validation of the CFT**

The CFT is validated only if:

* Complement control: complete haemolysis (100%) and,
* Antigen control: complete haemolysis (100%) and,
* Sensitised-RBC control: absence of haemolysis (0%) and,
* Negative control: complete haemolysis (100%) and,
* Positive control: absence of haemolyis (0%) and expected titre ± one dilution and,
* For each tested serum, no anticomplementarity: complete haemolysis (100%)

In summary:

|  |  |  |
| --- | --- | --- |
|  | **Haemolysis (%)** | **Notation** |
| Antigen control | 100 | 0 |
| Complement control | 100 | 0 |
| Sensitised-RBC control | 0 | ++++ (or 4+) |
| Negative serum control | 100 | 0 |
| Positive serum control | expected titre ± one dilution | To determine |
| Sera to test, Anticomplementarity at 1/5 and 1/10 | 100 | 0 |

**8.3 Results analysis**

Once the CFT validated, read the results for each sample at dilution 1/5:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Anticomplementarity | | yes | no | no | no | no | no |
| Haemolysis (%) | | **/** | **100** | **75** | **50** | **25** | **0** |
| Notation | | AC | 0 | + | ++ | +++ | ++++ |
|  | |  |  |  |  |  |  |
| **Interpretation** | | anticomplementarity | Negative | Suspicious | Suspicious | Suspicious | Positivea |
|  | *adefine the titer of the positive sample, by reading the % haemolysis for all dilutions* | | | | | | |

8.3.1 In case of anticomplementarity serum

Carry out a new decomplementation and perform a second CFT.

8.3.2 In case of Suspicious serum (1+ to 3+ at dilution 1/5)

Carry out a new decomplementation and perform a second CFT.

8.3.3 In case of positive serum (4+ at dilution 1/5)

According to the terrestrial animal health code (Chapter 12.10 Infection with *Burkholderia mallei* (glanders) <https://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_glanders.htm>)

The following defines the occurrence of [infection](https://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm#terme_infection) with *B. mallei*:

1. *B. mallei* has been isolated from a sample from an equid; or
2. antigen or genetic material specific to *B. mallei* has been identified in a sample from an equid showing clinical or pathological signs consistent with glanders, or epidemiologically linked to a confirmed or suspected [case](https://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm#terme_cas) of [infection](https://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm#terme_infection) with *B. mallei*, or giving cause for suspicion of previous contact with *B. mallei*; or
3. antibodies specific to *B. mallei* have been detected by a testing regime appropriate to the species in a sample from an equid showing clinical or pathological signs consistent with glanders, or epidemiologically linked to a confirmed or suspected [case](https://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm#terme_cas) of [infection](https://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm#terme_infection) with *B. mallei*, or giving cause for suspicion of previous contact with *B. mallei*.

Therefore, we recommend to:

* isolate as much as possible the animal for other equids,
* repeat the CFT test on this sample and perform other alternative tests (Western blot, iELISA, recELISA..),
* ask for more blood samplings, each with 14-21 days interval,
* control the clinical evolution of the animal during its quarantine,
* conduct an epidemiological study to determine if the animal is likely to have an epidemiological link to a confirmed case of glanders,
* contact the person in charge of glanders activity in the EU-RL for equine diseases ([Karine.laroucau@anses.fr](mailto:Karine.laroucau@anses.fr)).

1. Summary

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample** | **Result of the CFT** | **Conclusion** | **Result of the 2nd CFT** | | **Conclusion** |
| Hemolized or coagulated serum, insufficient volume or degraded sample | Sample rejected | Request for a new sample |  |  |  |
| Compliant serum | Anti-complementary | Carry out a new decomplementation and perform a second CFT | No-anticomplementarity effect | Negative | Negative sample |
| Positive | Positive sample  (*see 8.3.3*) |
| Suspicious | Suspicious sample (*request for a new sample*) |
| Anti-complementary still shown after treatment | Uninterpretable | Uninterpretable sample (*request for a new sample*) |
| Negative  (100% haemolysis at 1/5) | Negative |  |  |  |
| Suspicious  (25-75% haemolysis at 1/5) | Carry out a new decomplementation and perform a second CFT | Negative | Negative | Negative sample |
| Suspicious | Suspicious | Suspicious sample (*request for a new sample*) |
| Positive | Positive | Positive sample  (*see 8.3.3*) |
| Positive  (0% haemolysis at 1/5) | Positive sample (*see 8.3.3*) | - | Positive | no |

Annex A: Titration of the complement

Complement titration must be performed for **each test series**. This titre is determined by micromethod under the conditions of the complement fixation test.

All dilutions are performed in TV and all solutions must be prepared in sufficient quantity for the titration and test series.

A.1 Turn on the water baths.

A.2 Dilute the complement 1:100 in TV (for 5 mL of complement to be prepared 1:100, dispense 5 mL of TV into a pillbox, remove 50 µL of TV and add 50 µL of complement). Do not dip the tip into the TV diluent, vortex it.

A.3 Dilute the antigen according to the results of the antigen titration (see **Annex B**). Prepare this suspension in sufficient quantity for the titration and the test series (For 10 mL of antigen to be prepared, dispense 10 mL of TV into a pillbox and if the antigen is to be diluted 1:160, remove 62.5 µL of TV and add 62.5 µL of antigen). Do not dip the tip into the TV diluent, homogenise by vortexing.

A.4 Prepare 15 haemolysis tubes and dispense the complement, TV and antigen into the tubes as follows:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Tube Ident.** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **Haemolysis controls** | |
| **H100** | **H0** |
| C 1/100 (µL) | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 | 160 | 400 | 0 |
| TV (µL) | 360 | 350 | 340 | 330 | 320 | 310 | 300 | 290 | 280 | 270 | 260 | 250 | 240 | 0 | 400 |
| Diluted antigen (µL) | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |

A.5 Shake the tubes and incubate them in a water bath at 37 ± 2°C for 30 min.

A.6 Meanwhile, prepare:

* 2% red cell suspension in TV (for 10 mL of 2% red cells, dispense 10 mL of TV into a pillbox, and if the red cells are 50%, remove 0.4 mL of TV and add 0.4 mL of 50% red cells, without dipping the cone into the TV diluent). Gently homogenise.
* The haemolytic serum at the dilution defined during the titration of the batch (see Paragraph 8) (for 10 mL of haemolytic serum to be prepared, distribute 10 mL of TV in a pillbox, and if the titration of the haemolytic serum gave a titre of 1/2000, and knowing that double the amount is being used, it will be necessary to dilute it to 1/1000 and therefore to remove 10 µL of TV and to add 10 µL of haemolytic serum, dipping the cone into the TV in order to be in excess). Homogenise with a vortex.
* The haemolytic system is prepared 20 min prior to use by gently mixing equal parts of the amount required for the complement titration (e.g. 4 mL of red cells and 4 mL of haemolytic serum). The remaining reagents (red blood cells and haemolytic serum) are stored separately at 5 ± 3°C overnight.

A.7 After 20 min of contact for the haemolytic system and after 30 min of incubation of the tubes at 37°C, add 400 µL of haemolytic system to each of the tubes still incubating in the water bath.

A.8 Shake the tubes out of the water and place them back in a water bath at 37 ± 2°C for 30 min.

A.9 Immediately after removal from the water bath, centrifuge the tubes at 600 g for 5 min.

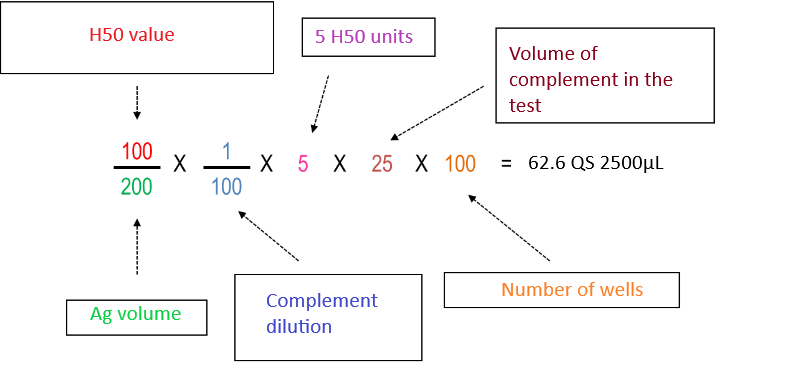
A.10 Determine the H50 unit by identifying the tube with 50% inhibition of haemolysis (compare to an H50 control tube, prepared by mixing, for example, 500 µL of 0% haemolysis supernatant with 500 µL of 100% haemolysis supernatant).

The result of the titration is written on the bench sheet.

A.11 For the complement fixation technique, 5 H50 units are used.

A.12 Calculation of the complement dilution for the test to be performed.

For one plate (equivalent to 100 wells), 25 µL of diluted complement should be dispensed per well, therefore 25 µL x 100 = 2500 µL should be prepared. If the 50% haemolytic unit (H50) was found for tube 7, this corresponds to 100 µL of 1/100 complement. The assay uses 5 H50 or 62.5 µL of pure complement diluted in 2437.5 µL of TV to give a total volume of 2500 µL.



In the reduced formula, for 10 mL of supplement to be prepared (corresponding to 4 plates):

Volume of complement for 10 mL = Volume of 1:100 diluted complement (µL) found on titration (H50) x 2.5.

For example: for an H50 value found in tube 7, this corresponds to 100 µL x 2.5 or 250 µL of complement in 10 mL of TV. So dispense 10 mL of TV into a pillbox, remove 250 µL of TV and add 250 µL of complement. (Discard the old pillbox with the 1:100 dilution of complement).

The result of the titration and the calculation for 10 mL are written on bench sheet.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Tube Ident.** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
|
| C (µL) to be added | 100 | 125 | 150 | 175 | 200 | 225 | 250 | 275 | 300 | 325 | 350 | 375 | 400 |

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Annex B: Titration of the antigen

Each new batch of antigen must be titrated in parallel with the batch currently in use. The titre is determined in micro-method in the conditions of the complement fixation test. All dilutions are done in Veronal Buffer (VB).

B.1 Perform the complement titration procedure:

B.2 Prepare 180 µL of the current batch positive serum, at the pre-dilution prior determined.

B.3 Decomplement the positive serum at 59 ±1 °C for 30 min.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Positive serum dilution | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 |
| Positive serum (µL) | 80 | 40 | 20 | 10 | 10 | 10 |
| VB (µL) | 320 | 360 | 380 | 390 | 790 | 1590 |

B.4 Prepare six positive serum dilutions: 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 as follow:

B.5 Prepare the new batch antigen dilutions as follow (including the dilution recommended by the supplier):

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Ag Dilution | 1/20 | 1/40 | 1/60 | 1/80 | 1/100 | 1/120 | 1/140 | 1/160 | 1/180 | 1/200 | 1/220 | 1/240 |
| Ag (µL) | 20 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| TV (µL) | 380 | 390 | 590 | 790 | 990 | 1190 | 1390 | 1590 | 1790 | 1990 | 2190 | 2390 |

B.6 Prepare 300 µL of the current batch antigen at the predetermined titer (NB: the antigen should have been prepared during the complement titration procedure and can be used here instead of making another solution)

B.7 Distribute the positive serum dilution vertically, 25 µL per dilution and per well, as follows:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **Positive serum dilutions** (25 µL) | | | | | |
|  | Dilutions | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 |
| **Ag dilutions**  (25 µL)  New batch | 1/20 |  |  |  |  |  |  |
| 1/40 |  |  |  |  |  |  |
| 1/60 |  |  |  |  |  |  |
| 1/80 |  |  |  |  |  |  |
| 1/100 |  |  |  |  |  |  |
| 1/120 |  |  |  |  |  |  |
| 1/140 |  |  |  |  |  |  |
| 1/160 |  |  |  |  |  |  |
| 1/180 |  |  |  |  |  |  |
| 1/200 |  |  |  |  |  |  |
| 1/220 |  |  |  |  |  |  |
| 1/240 |  |  |  |  |  |  |
| **Current batch Ag**  (25 µL)  Batch N°: | Pre-determined titer |  |  |  |  |  |  |

B.8 Distribute the new batch antigen, 25 µL per dilution and per well.

B.9 Distribute the current batch antigen, 25 µL per well.

B.10 Add 25 µL of complement (at the concentration determined in step 1) to each well.

B.11 Add the plate controls:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | VB (µL) | Ag (µL) | C (µL) | Expected result |
| Current batch Ag Control (pre-determined titer) | 25 | 25 | 25 | 100% Haemolysis |
| New batch Ag Control (1/100 dilution) | 25 | 25 | 25 | 100% Haemolysis |
| C Control | 50 | 0 | 25 | 100% Haemolysis |
| Sensitised-RBC Control | 75 | 0 | 0 | 0% Haemolysis |

B.12 Cover the plate and homogenize by gently tapping on each side. Incubate 16 to 20 hr at 5 ± 3°C.

*The following day*

B.13 Prepare the sensitised-RBC 20 min before use. Mix equal parts of hemolytic serum, diluted according to its titer, and 2% sheep red blood cells in solution.

B.14 After 10 min, take the plate out from the refrigerated cabinet and incubate it at 37 ± 2°C for 10 min.

B.15 After 10 min, take the plate out and add 50 µL of HS to each well. Cover it and homogenize by gently tapping on each side.

B.16 Place the plate back at 37 ± 2°C for 45 min.

B.17 After 45 min, centrifuge the plate at 600 *g* for 5 min.

B.18 Read the controls first. If the results are within the expected margin, determine the concentration of the new antigen batch by comparing it to the previous batch. Choose the titer closest to the current batch titer.

Annex C: hemolytic serum titration

The hemolytic serum titration must be performed **for each new batch**. The titre is determined by micromethod under the conditions of the complement fixation test. All dilutions are performed in VB.

Hemolytic serum is titrated according to the following protocol:

C1 Perform an initial dilution (in tube) of the hemolytic serum (hs) to 1:250.

|  |  |
| --- | --- |
| **Initial dilution of hs** | |
| hs (µL) | 10 |
| TV (µL) | 2490 |

C2 From this dilution, make the following successive dilutions (in tubes):

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Dilution of the**  **hs 1/250** | **1/2** | **1/4** | **1/8** | **1/12** | **1/16** | **1/20** | **1/24** | **1/28** | **1/32** | **1/36** |
| hs diluted 1/250 (µL) | 100 | 100 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| TV (µL) | 100 | 300 | 350 | 550 | 750 | 950 | 1150 | 1350 | 1550 | 1750 |
| **Final dilution** | **1/500** | **1/1000** | **1/2000** | **1/3000** | **1/4000** | **1/5000** | **1/6000** | **1/7000** | **1/8000** | **1/9000** |

C3 Prepare a 1:10 dilution of the complement to be in excess of the complement. For a volume of 5 mL, remove 4.5 mL of TV and add 500 µL of complement.

C4 Prepare a 2% RBC suspension. For a volume of 10 mL, take 9.6 mL of TV and add 0.4 mL of 50% RBC.

C5 Distribute the reagents as follows and in duplicate (line A and B) in a plate:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Well** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| **Dilution hs** (µL) | **1/250** | **1/250** | **1/500** | **1/1000** | **1/2000** | **1/3000** | **1/4000** | **1/5000** | **1/6000** | **1/7000** | **1/8000** | **1/9000** |
| hs diluted (µl) | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Red blood cells 2% (µL) | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| TV (µL) | 75 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| C to 1/10 (µL) | 0 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |

Well 1 is the haemolytic serum control.

C6 Shake the plate gently by tapping, cover it and place it in the oven at 37 ± 2°C for 30 min.

C7 Centrifuge the plate at 600 g for 5 min.

C8 The reading is taken by comparison with haemolysis controls made in the same way as in the complement fixation test. The haemolytic serum control (well 1) must not show any haemolysis. The highest dilution of haemolytic serum resulting in total haemolysis determines the 100% haemolytic serum unit.

In the complement fixation test, excess haemolytic serum is used, i.e. twice the amount determined by the titration.

For example, if the highest dilution resulting in total haemolysis is well 5 (corresponding to the 1:2000 dilution), the 1:1000 diluted sh should be used in the assay.

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