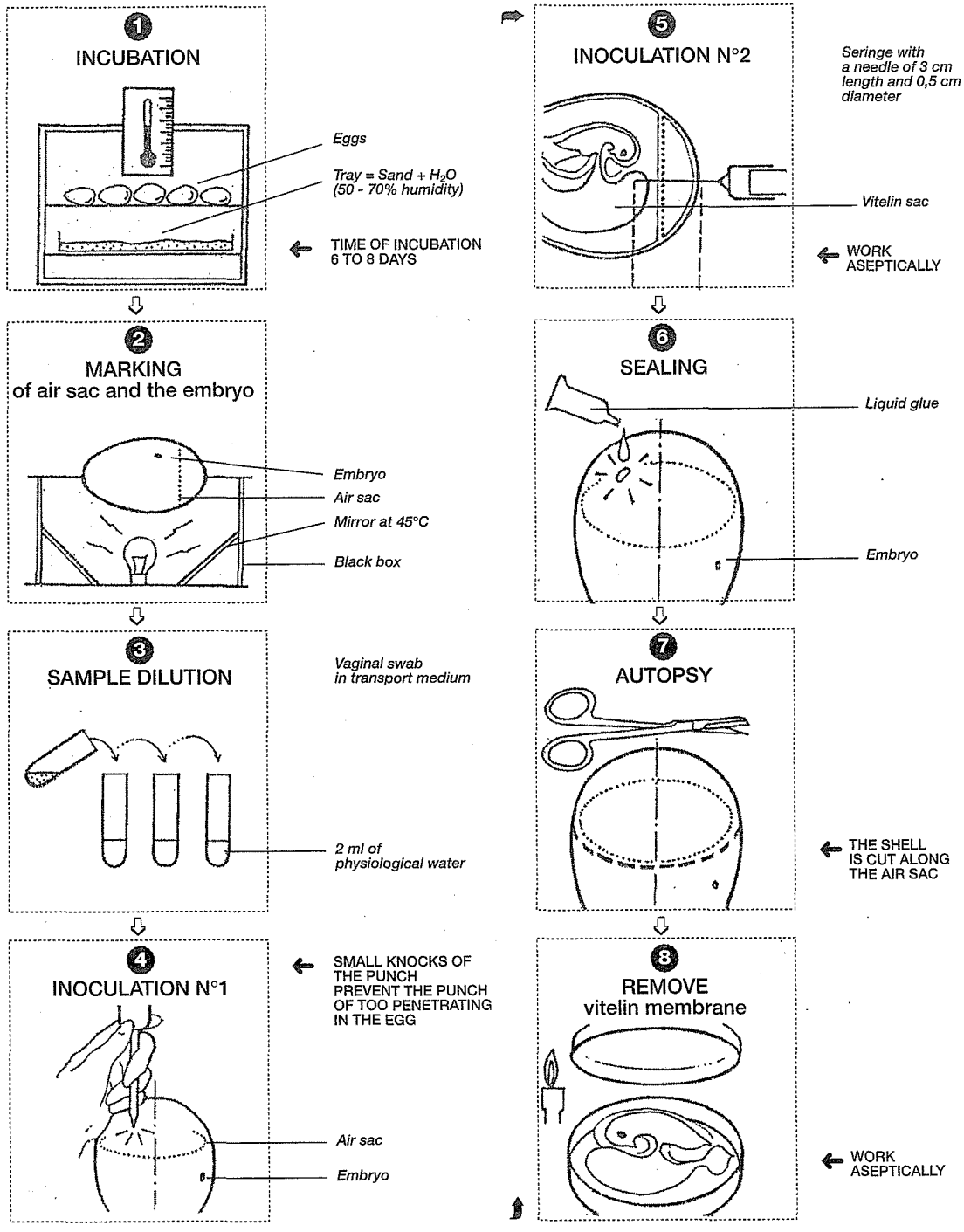


5.3 : Isolation of Chlamydia in embryonated hen's egg. Egg inoculation protocol

- Acridine orange stain :
  - Acridine orange
  - Absolute alcohol
  - Glacial acetic acid
  - Citric acid
  - Na_2HPO_4.

Procedure

1. Culture the McCoy cells in monolayers in Medium 1.
2. Trypsinise once cells are confluent.
3. Suspend the cells in Medium 1 at a concentration of 3.5 x 10^5 cells/ml.
4. Place 200µl aliquots of this suspension in 96 wells of a microtitre plate and incubate overnight at 37°C in a humid atmosphere containing 5% CO_2.
5. Remove the Medium 1 and replace with the vaginal swab samples diluted 1 in 2, 1 in 20 or 1 in 200 in PBS-DEAE (two wells per dilution).
6. Incubate the plates for 1 hour at 37°C.
7. Remove the inoculum and replace with 200µl of Medium 2.
8. Incubate the plates for 30-48 hours at 37°C in a humid atmosphere containing 5% CO2.
9. Fix and stain the plates by:

• May-Grunwald Giemsa (Figure 5.5, page 94)

• or acridine orange:

1. Fix the cultures without drying for 10 minutes in a 2:1 (v/v) mixture of ethanol:acetic acid. The fixed cultures can be stored if necessary in 80% alcohol.
2. Wash the cultures twice for 2 minutes each wash in McIlvain buffer.
3. Stain for 4 minutes with 0.01% acridine orange.
4. Wash the cultures twice for 2 minutes each wash in McIlvain buffer as before.

Reading of results

Examine the slides by microscope in McIlvain buffer by UV light. Chlamydial inclusion bodies, rich in DNA, appear orange at the side of the cell nucleus which is stained green (Figure 5.6, page 94).

• Either by immunofluoresence as described in 5.5.2.1 except the cultures are not air-dried but are fixed in ethanol for 10 minutes before reaction with antibodies. The *Chlamydia* than appear as yellow- green cytoplasmic inclusion bodies (Figure 5.7, page 94).

• Or by ELISA according to the same immunofluorescence method but using a conjugate coupled to alkaline phosphatase and a mixed 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate (Figure 5.8, page 95).

Note

• Centrifugation of the samples on the cells at 1400 xg for one hour helps chlamydial penetration of the cells and therefore increases the number of visible cytoplasmic inclusion bodies.

• Addition of 1µg/ml of cyclohexamide to Medium 2 blocks the division of eukaryotic cells and helps the reading of the results: the cells and chlamydial inclusion bodies appear larger (Figure 5.7, page 94).

Preparation of reagents

Cell Maintenance Medium (Medium 1) : Eagles Minimal Essential Medium (MEM) supplemented with 1% (v/v) of vitamins (100x concentrate vitamins for Eagles MEM), 1% (v/v) 200mM glutamine, 2g/l $NaHCO_3$ and 5% foetal calf-serum.

Cell Growth Medium (Medium 2): Same composition as Medium 1 but containing 10% foetal calf serum and antibiotics : 20µg/ml gentamycin, 25µg/ml vancomycin and 5µg/ml amphotericin B.

Fixative for Acridine Orange Stain :
Prepare immediately before use :
absolute alcohol 2 volumes
glacial acetic acid 1 volume
Acridine Orange :
Stock solution: 1% in distilled water. This can be kept for several months at 4°C in the dark. The solution should be discarded if heavy precipitation occurs.
Working solution: dilute the stock solution to 1% with McIlvain buffer.
McIlvain buffer.
Solution 1 : 0.1M citric acid
Solution 2 : 0.2M Na_2HPO_4
Mix 12.9ml of solution 1 with 7.1ml of solution 2 to obtain 20ml of buffer, pH 3.8.

5.5.4.3 General notes about the isolation of Chlamydia

Certain strains of *Chlamydia* are sensitive to penicillin and it should therefore not be used. Gentamycin is more expensive than streptomycin but it remains active for longer.

5.5.5 Comparison of the different direct diagnostic techniques

The different methods of direct diagnosis are compared in a large number of publications concerned with the diagnosis of human infections with *C.trachomatis* and in a much smaller number of reports concerned with the diagnosis of *C.psittaci* infections in ruminants. However, in all accounts PCR and LCR have been found to be the most sensitive methods allowing detection of the greatest numbers of infected individuals. On the other hand, differences in sensitivity have been noted in both human and veterinary medicine when comparing the isolation of chlamydia in cell culture with immunofluorescent detection or ELISA: isolation in cell culture is more sensitive than the other techniques in human medicine but less sensitive in veterinary medicine. This is probably due to the fragility of the *Chlamydia* and differences in the sampling conditions. In human medicine the patients go directly to the laboratory for sampling or samples are sent very quickly to the analytical laboratory under the best conditions which maximises the survival chances of the *Chlamydia.*

5.6 INDIRECT DIAGNOSIS

The diagnosis of abortive chlamydiosis is usually undertaken by detecting chlamydial antibodies in the blood of the animal.

The complement fixation reaction is usually used. It is an easy method to perform but does present some drawbacks :

The antigen used may also detect antibodies raised against intestinal *Chlamydia* present in most ruminants. This has resulted in standardisation of the method and fixing of a threshold value (1/80) to separate animals considered as positives.

Even taking this threshold into account, the complement fixation reaction cannot be used for individual diagnosis and it does not allow detection of infection in the young or in males. It should preferably be carried out 3 weeks to one month after abortion or time of low production (time of maximum antibody response).

Other techniques (ELISA, immunofluorescence) can be used. They present the same drawbacks as complement fixation as they use the same type of antigen although problems with anti-complementary sera do not occur. They are more expensive methods and require specific pieces of equipment.

5.6.1 Complement fixation

Breakdown in the supply of commercial complement fixation antigens for the diagnosis of abortigenic *Chlamydia* frequently occurs. Antigens sold for the diagnosis of avian

Chlamydia can be used but it is also possible to prepare a stock antigen by the following method :

5.6.1.1 Antigen preparation (Figure 5.9)

Antigen can be prepared from infected yolk sac membranes, placental material or cell cultures.

Principle

The antigen used is a lipopolysaccharide antigen extracted from bacterial bodies using ether.

Materials and reagents

- Diethyl ether.
- Sterile physiological saline.

Procedure

1. Place the preparations of cell culture derived *Chlamydia* or ground samples of yolk sac membrane or placental material in a boiling water bath for 30 minutes.
2. Allow to cool and add 10 volumes of cold diethyl ether.
3. Mix vigorously, then leave overnight at 4°C with gentle shaking with the flask positioned to avoid any evaporation of the ether.
4. The cellular debris will settle in the bottom of the flask. The ether will become clear yellow.
5. Remove the ether with a pipette and evaporate in a water bath at 37°C or under vacuum.
6. Resuspend the residue in a small volume of physiological saline.
7. Determine the antigen titre by the complement fixation reaction method, using a positive control serum at a constant dilution and various dilutions of the antigen. One unit of antigen is the highest dilution of antigen which gives 100% complement fixation.

Note

• The volume of physiological saline in the initial chlamydial suspension must be sufficient otherwise coagulation may occur during boiling at 100°C.

• The temperature during extraction in ether can be higher than 4°C but if the temperature rises too high evaporation may extend beyond the ether phase and reduce the yield.

5.6.1.2 Complement fixation reaction [9]

Antibody titre is determined according to the cold incubation micromethod of Kolmer, following the technique described in the chapter on Brucellosis with the following differences:

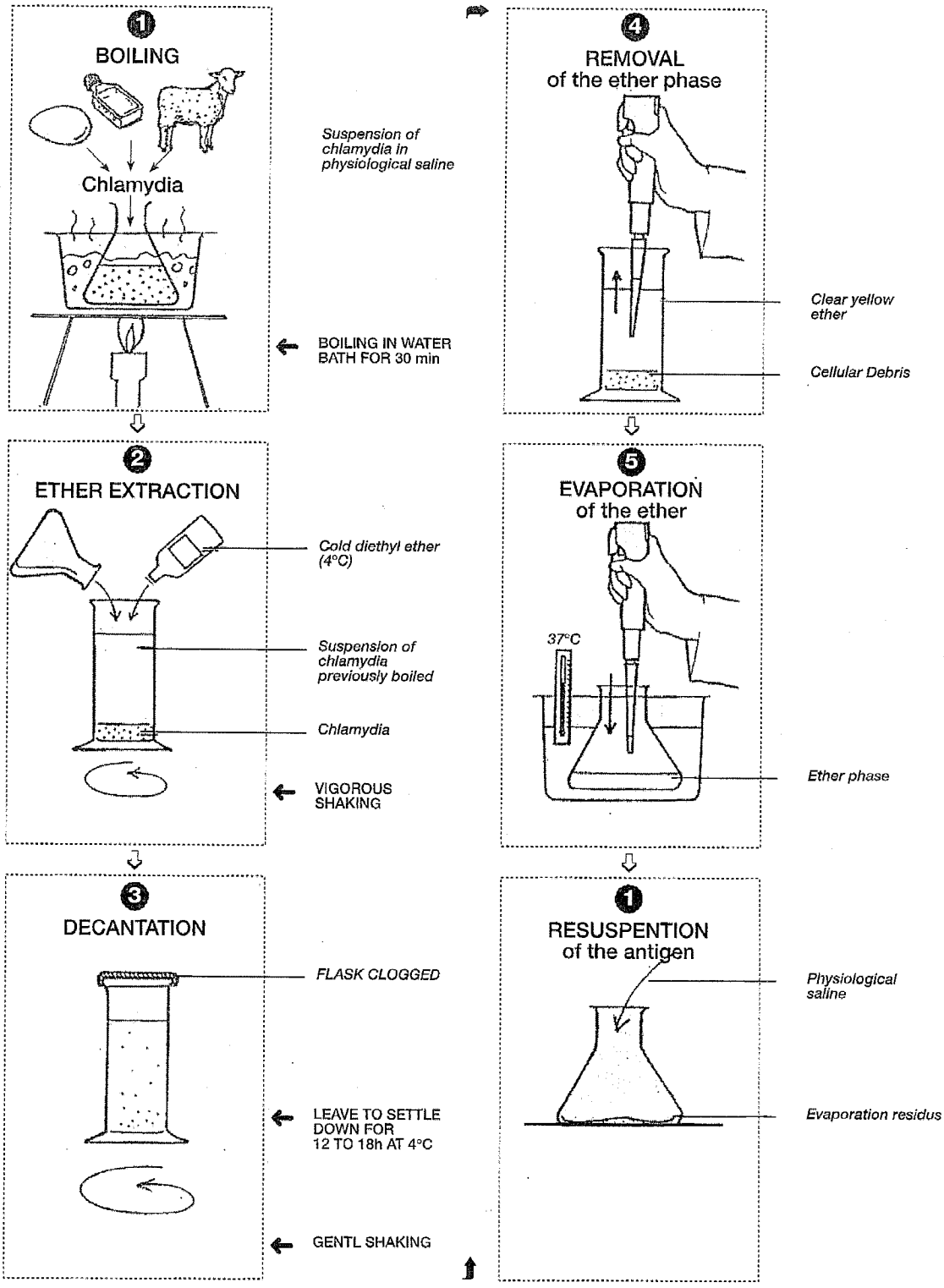
- Dilute test sera 1/5 in veronal buffer and decomplement in tubes at 58.5°C for 30 minutes.
- Decomplement positive and negative control sera in the same way.
- Chlamydial antigen contains 4 units.
- Guinea-pig complement is diluted in veronal buffer to contain 2 units at 100% haemolysis.

Procedure

1. Add 50µl of the decomplemented serum to 50µl of veronal buffer in the first row of wells on the microtitre plate then make doubling dilutions in successive rows. Dilutions will range from 1 in 10 to 1 in 640 over seven rows of wells.
2. Add 25µl of antigen and 25µl of complement.
3. Seal the plates, agitate and incubate overnight at 4°C.
4. Add 25µl of sensitised red blood cells (RBC) and agitate the plates. Read after 30 minutes incubation at 37°C.

Note

• To avoid anticomplementary activity



5.9 : Complement fixation. Antigen preparation protocol

the blood cells must be separated from the sera as quickly as possible.

• The following standards should be included in each batch of tests :

- for each serum, an anticomplement standard prepared by a 1 in 10 dilution of the serum sample plus the complement but without the antigen, in the 8th well of the plate ;
- a positive serum ;
- a negative serum ;
- an antigen standard without addition of serum ;
- a complement standard without addition of serum or antigen ;
- a RBC standard without addition of RBC in veronal buffer.

Replace any reagents omitted from the wells with the same volume of veronal buffer.

The results are highly reproducible when the same source of RBC is used. It is best to use cell samples from the same sheep between 3 and 10 days after sampling.

• Decomplementation of the serum:

- at 60°C decreases the sensitivity of the reaction;
- at 56°C increases the number of anticomplementary sera.

5.6.2 ELISA

Principle

Chlamydial antibodies are detected in test sera by means of sheep or goat immunoglobulin conjugates labelled with alkaline phosphatase or peroxide, which react with the antibodies when they are bound to the chlamydial antigens which coat the bottom of the wells of the ELISA plates.

Kits are available for the diagnosis of human chlamydial infections. Although the antigens can be used to detect antibodies raised against other types of antigens, the human immunoglobulin conjugates do not react with ovine and caprine immunoglobulins. Until the commercialisation of specific kits, the following protocol can be used :

5.6.2.1 Antigen preparation

Semi-purified elementary bodies or antigen for complement fixation can be used but the best results are obtained using lysed semi-purified *Chlamydia.*

Materials and reagents

- Centrifuge suitable for Eppendorf tubes.
- Tris KCl buffer, pH 7.5.
- Renografine (Radioselectan, Schering, France).
- Extraction buffer, pH 8.8.
- Triton X100.

Procedure

1. Freeze and thaw *Chlamydia* (approximately 5 x 10^8 pfu/ml) recovered from cell culture or yolk sac membrane preparations three times at -70°C, then sonicate for 15 second (optional) to free the maximum number of EB's.
2. Centrifuge the suspension for 10 minutes at 300 xg to precipitate the cellular debris.
3. Prepare cushions of 1.2ml 40% Renografine in Tris KCl in Eppendorf tubes.
4. Place a 200µl volume of sample on the surface of the cushion of Renografine in each Eppendorf tube.
5. Centrifuge the tubes for 2 hours at 17600 xg at 4°C.
6. The precipitated pellet containing the semi-purified *Chlamydia* can be stored at -20°C or processed to obtain lysed *Chlamydia.*
7. In the latter case, resuspend the pellet in each tube in 200µl of extraction buffer. Homogenise then sonicate to help

resuspension of the plug.

8. Incubate the tubes for 2 hours at 37°C to ensure good solubilisation of the proteins.

9. Add Triton X100 to a final concentration of 1% (v/v).

10. Incubate the suspension for 15 minutes at 37°C to stop the action of the SDS.

11. Determine the antigen titre in an ELISA using a positive serum.

Preparation of reagents

- Tris KCl Buffer, pH 7.5

Tris hydroxymethyl aminomethane	2.42g
KCl	11.2g
Deionised water	1000ml

Adjust to pH 7.5 with 1M HCl.

- Extraction Buffer, pH 8.8

NaCl	0.2mM
Tris-HCl	0.1M
EDTA	4 mM
SDS	0.2% (v/v)
β-mercaptoethanol	0.5% (v/v)

5.6.2.2 ELISA test

Materials and reagents

- ELISA plate reader.
- Microtitre plates.
- 0.1M bicarbonate buffer, pH 9.6.
- TBS buffer, pH 7.5.
- Tween 20.
- NaOH.
- Skimmed milk powder.
- Ovine or caprine immunoglobulin conjugate labelled with alkaline phosphatase.
- ρ-Nitrophenyl phosphate disodium substrate (Sigma Chemical Co, USA) diluted to 1mg/ml in diethanolamine buffer pH 10.2.

Procedure

1. Sensitise the plates overnight at 4°C with 100µl of the optimal antigen dilution (determined at the time of the titration) in bicarbonate buffer.

2. Remove the excess liquid by vigorously shaking the plates.

3. Wash the plates 4 times in demineralised water and dry.

4. Add 150µl of TBS containing 2% skimmed milk to each well to saturate remaining free sites and incubate for 30 minutes at 37°C with shaking.

5. Remove the excess liquid as before.

6. Rinse the plates 4 times with deionised water and wash 3 times for 5 minutes each wash with TBS containing 0.05% Tween 20 (v/v).

7. Add 100µl of the test serum dilutions (in TBS/2% milk) to each well. Add a standard negative serum and a standard positive serum to each set of tests.

8. Incubate for 90 minutes at 37°C with shaking.

9. Remove the excess liquid and wash the plates as in (6).

10. Add 100µl of the conjugate diluted in TBS/2% milk to each well and incubate for 90 minutes at 37°C with shaking.

11. Remove the excess liquid and wash the plates as in (6).

12. Add the substrate and incubate for 90 minutes at 37°C or until an OD of <0.2 is obtained in the negative standard.

13. Stop the reaction by addition of 1N NaOH.

Reading of results

Read the plates at 405nm. A sample is considered to be positive if the OD is >0.4.

Preparation of reagents

- TBS Buffer, pH 7.5

Tris hydroxymethyl aminomethane	2.42 g
NaCl	29.24 g
Demineralised water	1000 ml

Adjust to pH 7.5 with 1M HCl.