RABIES SERO-ANTIBODY MEASUREMENT
WITH SERONEUTRALISATION TESTS

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The reference method for antirabies antibody measurement is seroneutralisation. The
general principle of the method is as follows: a constant amount of virus is mixed with
constant volumes of serum dilutions. After an incubation step, the remaining virus is
detected either by mouse inoculation (clinical signs and death of animals are observed) or
by cell culture. These tests measure the "neutralising power" of the serum. This
neutralisation includes a specific component, due to antibodies and a possible non-specific
component.

When a large number of sera must be tested ELISA techniques may be useful because they
are generally rapid and objective. Different methods may be used: direct estimation of the
amount of specific antibodies, indirect estimation of the antibodies by measuring the
remaining virus after an incubation step or by a competitive test using monoclonal
antibodies as the competitor.

This paper is a summary of remarks and questions which arose during an epidemiological
study performed on dogs.

MOUSE NEUTRALISATION TEST

This technique is well standardised (Atanasiu 1973). The critical points are described
including the dilution of serum to be tested, the challenge strain used, the dose of challenge
virus, the mice to be used and the calculation method.

The comparison of results between laboratories is possible with this method.

CELL CULTURE NEUTRALISATION TEST

In vitro test methods are favoured over in vivo methods because they do not use animals and
also because in vitro tests are generally as sensitive as in vivo ones and give an answer more
rapidly and at a lower cost.

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**First observation**

In 1992 the WHO/OIE collaborative centre was asked by OIE to produce a reference serum of dog origin for rabies. In order to establish the titre of that serum, different freeze dried aliquots were sent to 20 laboratories for titration on cell culture. These laboratories were asked to titrate this dog reference serum against the WHO reference antirabies immunoglobulin.

Up until the present there have been 14 responses. Figure 1 shows the distribution of titres according to the number of laboratories. Two groups of titres are observed: 5 to 10 IU/ml and 12 to 19 IU/ml.

![Graph showing titre distribution](image)

*Figure 1:* Range of titres obtained in 14 different laboratories for different aliquots of the same pool of immunised dogs. Results are expressed as the mean of three different tests.

The conclusion of this observation and of the analysis of the test procedures was that it was not possible to establish a standardised titre without further studies.

**Blind test**

The aim of this test was to control the specificity of the technique, the accuracy and the consistency of dilution factors.

Every participating laboratory received a set of 10 tubes:
- nine unknown dilutions of a pool of sera collected from vaccinated dogs in a pool of sera of naive dogs. Two of these dilutions contained no antibody. Every participating laboratory received identical panels.
- an aliquot of a pool of vaccinated cat sera.
Each panel had to be titrated three times in independent operations.

Up until the present eight out of the ten laboratories that received the panel have responded. Two incomplete answers cannot be used.

All the positive samples were found positive and all the negative ones were found negative. So the specificity of the reaction for this test was 100 percent. The intra-laboratory inter-test variation was generally lower than 15 percent. The repeatability is higher than 15 percent in only one laboratory.

The intra-laboratory consistency is illustrated in Figure 2. It is represented by the regression line between observed titres and the theoretical titres determined from the titre of the more concentrated tube and from the dilution factor. The inter-laboratory variation, that is, the range of titre for the same serum sample, illustrated in Figure 2, is close to the variation illustrated in Figure 1.

![Figure 2: Comparison of results obtained by five participating laboratories, showing their intra- and inter-laboratory variations](image)

One laboratory, different from that described above, has no correlation between titres and dilution factors.

In conclusion, the broad range observed in titres (Figure 1) is seen again during this blind test but the inter-test intra-laboratory repeatability and the intra-laboratory consistency are
generally good. The difference observed in titres established in different laboratories should then come from differences in the test procedures.

**Comparison of testing procedures**

Most of the laboratories follow the principles of the rapid fluorescent focus inhibition test (RFFIT) as described by Smith *et al.* (1973) or Zalan *et al.* (1979). But in fact, the analysis of testing procedures shows few common points, as follows.

**Cell culture support:**
- six laboratories use 96 wells microtitration plates;
- two use Terasaki plates;
- no laboratory uses Lab Tek slides (which is the most frequently described technique!).

**Dilution of serum:**
- threefold dilutions are generally used in six laboratories, other dilution factors used include twofold (1 lab), fourfold (1 lab) and fivefold (2 labs);
- four laboratories (of 9) prepare a tenfold predilution and one uses a fourfold predilution;
- three laboratories make duplicates for each serum.

**Challenge strain:**
- CVS (challenge virus standard) is the challenge strain in eight laboratories;
- SAD (street Alabama Dufferin) is used in one lab;
- SAGI (SAD avirulent Gif-1) is used in one lab.

The challenge virus titre varies greatly from one laboratory to another. The comparison between laboratories is difficult because the calculation method is not standardised: median tissue culture infectious doses (TCID$_{50}$), 50 percent or 80 percent of fluorescence and fluorescent plaque forming units (PFU) are used. Two laboratories express their CVS titre in TCID$_{50}$, one uses 10 000 TCID$_{50}$ per plate and one uses 10 000 000 TCID$_{50}$ per plate. These two laboratories incubate the plates for 24 hours.

The neutralisation step is made at 35 to 37°C and for 60 to 90 minutes.

**Incubation with cells:**
Whatever the laboratory, a cell suspension is added to the tube or plate containing the serum and virus after the incubation step. The cell culture medium is MEM or DMEM with 5, 8 or 10 percent foetal calf serum. Two cell lines are used: BHK21 (7/9 laboratories) and BSR cells (2/9). The density of the cell suspension varies from 3 x 10$^5$ to 1.6 x 10$^6$. The incubation time of the plate varies from 20 to 96 hours at 35 to 37°C.
Observation method:
The observation of the non-neutralised virus is made after fluorescent antibody staining of the fixed cell layer. Two reading techniques may be used:

i. Estimation of the general fluorescence of the cell layer.
Fluorescence is estimated by the proportion of fluorescent cells in the layer. This estimation may vary from one person to another. If a ratio is done when using a reference serum, the "subjective" part of the method disappears as shown in Figure 3.

![Figure 3: Results obtained independently by 2 persons when testing 70 dog serum samples in the same laboratory. The correlation factor is $r = 0.99$.](image)

ii. "Numeration" methods
The method may be strictly qualitative to detect fluorescence in the complete cell layer of the well. The quantitative aspect is given by the number of replicates for every serum dilution. The calculation of the titre is then made with the Spearman-Kärber method or with a neoprobit graphic chart.

Another way is to observe 5 to 10 microscope fields in a well and to determine the proportion of positive fields (i.e. with fluorescence). In this method, the observed fields cannot be considered independent because they are all a small part of a complete layer that has grown in a single well. The observation mean must be reproducible both for magnification (objective, eyepiece and magnifying factors of the additives inserted in the optical path) and for the size of the field really observed. The diameter of the microscope visual field (in mm) is calculated as follows:

\[
\text{eyepiece visual field number} \times \text{objective magnification.}
\]
Therefore, for a constant magnification rate of the optical system, according to the specifications of the eyepiece, this observed diameter may vary from 1 to 3 and the observed surface varies from 1 to 9!

iii. Titre of the challenge virus

If method (i) above is used for calculating and controlling the dose of virus, the titre is estimated from an absolute observation. The remark of point (i) shows that the dose of virus is "observer" related. Great variations are observed in challenge virus dose and in units. In order to have a rapid answer people use high doses of virus, the assumption being also that replication of virus in cells will not be disturbed by a poor quality serum (presence of cytotoxic products or bacteria that are not eliminated by antibiotics).

Calculation of the titre of the serum:

In order for the neutralising power of the serum to be expressed in IU/ml, a reference serum is added and the titre is the result of the following ratio:

\[
\text{dilution of the serum with 50% inhibition of fluorescence} \div \text{dilution of the reference serum with a 50% inhibition of fluorescence.}
\]

Under such conditions, the difference of estimation of fluorescence between persons (the "subjective" part of the reaction) disappears. The effective dilution of the serum is the one with a 50 percent inhibition of fluorescence. The calculation method is either a neoprobit graphic one or uses the Spearman-Kärber method.

Conclusions

Seroneutralisation detects both specific and non specific neutralising activity of a serum, which is important when estimating the immune status of animals, for instance, for cross border controls or as an alternative to quarantine measures. Great variations for in vitro neutralisation tests procedures are used between laboratories and therefore standardisation of techniques is important.

References

