Specific-Protocol of Laboratory Techniques in the Diagnosis of Rabies

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Abstract
Many animal-to-human transmissible diseases have been known since ancient times, but the Pasteurian period, which was by far a turning point in the development of microbiology, is the hottest stage of accumulation, substantiation and detection of the causality and pathogenesis of many of these. Peat is an anthropozoosis spread all over the globe, and without being influenced by climate or season, it can have a sporadic, enzootic or epizootic character. The epidemiological aspect correlates with the biology of the species, the main vector. The existence of stray dogs in all regions of a country gives a very scattered character of cases of urban disturbance, with seasonal incidence, favored during the route. Rabies is an acute, sporadic-enzootic encephalomyelitis, found in all homeothermic animal species, transmissible to humans, and characterized by acute evolution with sensory and motor nervous manifestations, expressed by hyperexcitability and aggression, followed by paralysis and death. In all cases of rabies, an immediate diagnosis and urgent action is required, 32 both for animals that are disturbed or suspected of having the disease, and for those that are contaminated or suspected of being infected. As neither the clinical aspects of the macroscopic lesions are pathognomonic, the diagnosis of the disease is based on laboratory exam (virological, biological, histopathological and serological). As it is a major zoonosis, laboratory diagnostic techniques for rabies have been internationally standardized.

Keywords: animal-to-human transmissible diseases, diagnosis of rabies, laboratory techniques.

1. Introduction

Numerous diseases with animal-to-human transmissibility have been known since ancient times, but the Pasteurian period, which was by far the turning point in the development of microbiology, is the hottest stage of accumulation, substantiation and decipherment of the causality and pathogenesis of many of them [1, 2].
Humans can become ill through contact with a variety of viral, bacterial, parasitic and, last but not least, prion pathogens that can be transmitted from animals to humans and vice versa.

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Zoonoses are by no means animal diseases, but, as Bernard Toma, professor of zoonoses at the National Veterinary School in Alfort (Paris) explains, they are "human diseases due to animals" [2].

Every year, the World Health Organization publishes technical reports in which they presented the economic and human losses caused by major zoonotic diseases.

The potential source of infection for humans includes a multitude of animal species, domestic or wild, plus a large number of insect vectors (ticks, mosquitoes, phlebotomists, flies, fleas, lice, etc.) [3].

Humans can be contaminated with the agents of various zoonoses by:

- direct contact with sick or germ-carrying domestic or wild animals;
- Handling dead bodies, meat, blood, hides, or wool from sick animals;
- coming into contact with contaminated objects from sick animals;
- the consumption of food of animal origin from diseased animals incorrectly prepared;
- by stings from vector vectors represented by various insects;
- bites or scratches from sick animals.

Transmission of zoonoses from animals to humans sometimes occurs in a brutal, even traumatic way (by biting or scratching).

Among the major zoonoses, along with brucellosis, tuberculosis, rubella, glanders, leptospirosis, etc., rabies , as it has often been known in the literature, is an infectious disease, of an acute, sporadic-endemic type, common to humans and all warm-blooded animals, characterised by encephalomyelitis-like manifestations and clinically translated by nervous excitability, followed by paresis and paralysis, which invariably lead to death [4-7].

For the diagnosis of a disease caused by a virus in a host, mammal or bird, a systematic approach must be developed, starting first with the initial observation and examination of the host and then the collection and submission of samples to the laboratory. Knowing the time constraints on receiving laboratory confirmation of a virus, (let alone rabies), and interpreting and validating laboratory results is essential in disease control. In the time frame required for laboratory testing, knowledge of the issues related to the spread of virus in susceptible animals and knowledge of palliative treatments is essential [8].

In all cases of rabies, immediate diagnosis and urgent action must be taken, both in relation to rabid or suspect animals and in relation to infected or suspected infected persons. As neither clinical features nor gross lesions are pathognomonic, the diagnosis of the disease is based on laboratory examinations (virological, biological, histopathological and serological) [1, 2].

As a major zoonosis, laboratory diagnostic techniques for rabies have been internationally standardized.

2. Materials and methods

ELISA test for the detection of post-vaccine antibodies

Serological monitoring for qualitative detection of antibodies in serum and plasma produced by rabies vaccination of dogs, cats and foxes. Oral vaccination of wild animals is practised in many countries to eradicate the disease. Assessment seroprevalence in the vaccinated population is one of the methods used to assess the effectiveness of oral vaccination.

The ELIȘA BioPro Rabies kit is used in the control of post-vaccination immunisations in domestic and wild carnivores.

Principle of the method

The technique is based on a blocking enzyme-linked immunosorbent assay for the detection of rabies virus antibodies. This test can be performed on serum/chest fluid samples from dogs, cats and foxes. The microplate gores are coated with rabies antigen. Diluted samples are incubated in the wells. After washing with wash solution, biotinylated anti-rabies antibodies are added to the wells. If the test sample contains specific anti-rabies antibodies, they block the binding of anti-rabies antibodies to the rabies antigen in the plate. If the test sample does not contain specific anti-rabies antibodies, then the biotinylated antibodies will form an antigen-antibody complex biotinylate. After washing with washing solution, streptavidin peroxidase conjugate is added to the wells. Streptavidin peroxidase will bind to the biotinylated antigen-antibody complex. After a further wash, substrate solution (TMB) is added to the wells forming a blue compound which turns

yellow after the quenching reaction. The colour intensity is read at 450 nm and the decrease in intensity compared to the negative control is proportional to the number of blocking antibodies in the sample analysed.

Materials needed:
The BioPro Rabies ELISA Ab KIT was used for this test (Figure 1).

Figure 1. Components of the ELISA kit

Diagnostic kit
Components of the Bio-Pro Rabies ELISA Ab KIT:
- 2 microtiter plates with 96 wells, 192 reactions;
- 1 bottle washing solution-250 ml;
- 1 vial with negative control-0.5 ml, with 0%
- blocking percentage; blue cap;
- 1 vial with positive control-0.5 ml, with 100% blocking percentage; red cap;
- 1 vial dog positive control serum 1-0.3 ml, with a blocking percentage between 50 and 70%;
- black cover;
- 1 vial dog positive control serum 2-0.3 ml, with a blocking percentage between 30 and 50%;
- white cover;
- 1 vial positive control serum dog 3-0.3 ml, with blocking percentage less than 30%;
- transparent cover;
- 1 vial with diluent for biotinylated antibody-30 ml;
- 1 vial of streptavidin peroxidase conjugate diluent-30 ml;
- 1 bottle with sample diluent-15 ml;
- 1 vial of TMB (tetramethylbenzidine/peroxide) substrate - 30 ml;
- 1 vial anti-rabies biotinylated antibodies-0.3 ml; yellow cap;
- 1 vial streptavidin peroxidase conjugate-0.3 ml; green cap;
- 1 bottle of stop solution (sulphuric acid) - 15 ml;
- 6 adhesive sheets for microplates;
- instructions for use.

Equipment
- Laminar flow hood;
- Spectrophotometer for reading optical densities;
- Washer;
- Computer and software for validation and interpretation of results;
- Centrifuge;
- 1 ml single channel pipette;
- Multichannel pipette 300-1000 µl;
- Refrigerator;
- Stirrer+Thermostat;
- Shaker;
- Vortex;

Other materials
- bidistilled or deionised water;
- Semi-automatic pipettes with variable volume from 10-1000 µl;
- disposable, semi-automatic pipette tips 10-1000 µl;
- micronixers for test sera;
- stand;
- reagent tanks;
- Laboratory glassware: graduated pipettes, Berzelius beakers, graduated cylinders;
- plastics for laboratory use: sterile pipettes, sterilisation boxes;
- gloves;
- filter paper;
- decontamination solutions.

Material under examination
Serum and/or fluid must be expressed and without haemolysis from foxes shot.
Sera to be investigated, with strong haemolysis, contaminated with bacteria or fungi or those collected on anticoagulant (heparin or EDTA) are not tested as they may provide erroneous results. 5-7 ml blood, fluid/sample are required. Test sera should be stored refrigerated at +2°C and +8°C for up to 5 days and frozen at -20°C if longer storage is required.

Working technique
Precautions
Reagents should be stored at +2°C to +8°C;

No expired reagents are used;
Do not mix reagents from different batches;
Before use, all components of the kit should be brought to room temperature (18-25°C);
The bottle should be washed only with distilled or deionised water;
The stop solution can cause serious burns. In case of contact with skin and eyes, flush immediately with water and seek medical attention;
The time between the last wash and the addition of a reagent should not exceed 5 minutes;
During the procedure the plate must not dry out;
Protect the substrate from direct light and antioxidants. Do not use substrate that shows blue colour before spreading in wells;
One pipette tip is used for each sample/step of the method;
The working procedure should not be changed;
The washing of the wells must be followed exactly, watching the filling and emptying of the wells as well as the amount of washing solution. Inadequate flushing can influence the outcome of the reaction.

Preparation of control samples and sera:
Serum samples that were tested were diluted 1:2 in sample diluent (e.g. 60µl PC+60 µl diluent) in a dilution plate or microtube. Positive control serum, negative control serum and control sera were diluted in the same way as serum samples according to the instructions.
Note: Any plate with low protein fixation can be used as a dilution plate.
It is also possible to dilute control sera and samples directly in the plate. 50 µl of sample diluent is put in each well and then place 50 µl positive control serum, negative control serum and control sera in the wells appropriate. Then place 50 µl of the serum samples in the remaining wells.
Note:
1. Insufficient sample volume: (less than 50 µl, dilute the respective serum sample with PBS to obtain a sample volume of 50 µl (e.g. if you have 30 µl of serum, add 20 µl TFS). The sample is marked.
2. Obtain a sample from a fresh blood clot: cut the clot from the heart or large blood vessels and transfer to a 10-12 ml tube; close the tube and leave at room temperature or in the incubator for 2-3 hours; transfer refrigerate for 12 hours overnight; centrifuge the liquid part of the blood clot at 5-10000rpm for 15±10 minutes, then test the liquid obtained.
3. Obtaining a sample from a dried blood clot: cut the dried blood clot, place it in a 10-12 ml tube, and add PBS with antibiotic (penicillin G 100000U/I, streptomycin 100 mg/l, neomycin 50 mg/l) maximum 1:1 ratio, add mark the diluted sample and test.

Reagent preparation was carried out as follows:
Washing solution: is concentrated 10 times. Must be allowed to reach room temperature before use. May form crystals at 2-8ºC, which will disappear at room temperature. If they do not disappear, it should be left at room temperature longer, or it can be heated to 37±2ºC and stirred gently until all crystals dissolve. To prepare the wash solution, mix 1 volume concentrated wash solution with 9 volumes of distilled or deionised water (e.g. 50 ml concentrated wash solution to 450 ml distilled water).
Biotinylated anti-rabies antibody: dilute the biotinylated anti-rabies antibody with the biotinylated antibody diluent in a ratio of 1:100 (e.g. for one plate 110 µl of concentrated botinylated anti-rabies antibody and 11 ml of biotinylated antibody diluent). Before preparing the working solution, the biotinylated anti-rabies antibody must be turbidised. The prepared solution should be used within 8 hours.
Streptavidin peroxidase conjugate: Dilute Streptavidin peroxidase conjugate concentrate with Streptavidin peroxidase conjugate diluent at a ratio of 1:100 (e.g. for one plate 110 µl Streptavidin peroxidase conjugate biotinylate concentrate and 11 ml Streptavidin peroxidase conjugate diluent). Before preparing the working solution Streptavidin peroxidase conjugate must be turbidised. The solution thus prepared should be used within 8 hours.

Work steps
- before work, all reagents (except conjugate) were brought to temperature room.
- open and unused tapes can be stored in plastic bags sealed with desiccant at 2-8ºC, in the dark, but not more than one month.
- order the serum samples and record them on the Worksheet (F-01-PSV-06).

Incubation of samples and control sera
All control sera were homogenized before distribution.
- 100 µl of diluted positive control serum is placed in wells A1 and B1;
- 100 µl of diluted negative control serum is placed in wells A2 and B2;
- 100 µl of diluted control serum 1 is placed in well C1;
- 100 µl of diluted control serum 2 is placed in well D1;
- 100 µl of diluted control serum 3 is placed in well E1;
- 100 µl of the diluted serum samples are placed in the remaining wells;
Cover the plate with foil and incubate overnight (18-24 hours) at 2-8ºC with gentle shaking on an orbital shaker.

Incubation with biotinylated anti-rabies antibody
Remove the foil, empty the contents of the plate and wash 6 times with the washing solution. Press the plate firmly against the filter paper after the last wash. Place 100 µl biotinylated anti rabies antibody in each well.
Cover the plate and incubate for 30±1 minutes at 37±2ºC with gentle shaking on an orbital shaker.

Incubation with Streptavidin peroxidase conjugate
Remove the foil, empty the contents of the plate and wash 6 times with the washing solution. Press the plate firmly against the filter paper after the last wash.
Place 100 µl Streptavidin peroxidase conjugate in each well.
Cover the plate and incubate for 30±1 minutes at 37±2ºC with gentle shaking on an orbital shaker.

Incubation with TMB substrate
- Remove the foil, empty the contents of the plate and wash 6 times with the washing solution. Press the plate firmly against the filter paper after the last wash.
- Put 100 ready-to-use TMB substrate in each well
- Cover the plate and incubate for 15-30 minutes at room temperature (18-25ºC) with gentle shaking on an orbital shaker, away from direct sunlight.

Stopping the reaction
Place 50 µl of stop solution in each well.

Reading
Read the optical density at 450 nm, 5-15 minutes after stopping in a spectrophotometer.90

Validation of results
Test results are validated if the following conditions are met:
- The negative control serum obtuse density must be greater than 1.0;
- The difference between the mean optical density of the negative control serum and the positive control serum must be equal to or greater than 0.8.
If the validation criteria are not met, the results of that plate are invalid and the samples must be retested.
For the control the blocking percentages of the control sera can be calculated and the results should be as follows: control 1 between 45% and 70%, control 2 between 25% and 45%, and control 3, less than 30%.

3. Results and discussion

The blocking percentage (BP) was calculated for each sample:
PB%=OD cn-DO proba/DO cn-DO cp * 100
- Samples with serum PB<40% are considered negative for anti-rabies antibodies;
- Samples with serum PB≥40% are considered positive for anti-rabies antibodies;
- Samples with serum PB≥70% are considered serum samples with an anti-rabies antibody level equal to or greater than 0.5 IU/ml based on the FAVN test. It is recommended to use the first positive cut-off (PB equal to or greater than 40%) for the purpose of assessing oral immunization. It is not recommended to use the second positive cut-off (PB equal to or greater than 70%) for interpretation of fox serum results due to the fact that most fox serum samples are actually body fluids with unknown dilution factor, which will make it almost impossible to measure the exact level of protection.
The study analyzed 1170 thoraco-abdominal fluids from foxes shot between 2011 and 2018, of which 382 were positive and 788 negative.
The breakdown of the samples analyzed by year is shown in the tables 1, 2 and figures 3, 4.

Table 1. Elisa tests in shot foxes

Year	Number of shot foxes	Elisa test (thoraco-abdominal fluid)	
		Positive result	Negative result
2011	147	15	132
2012	31	4	27
2013	96	27	69
2014	89	30	59
2015	214	54	160
2016	206	51	155
2017	234	116	118
2018	153	85	68

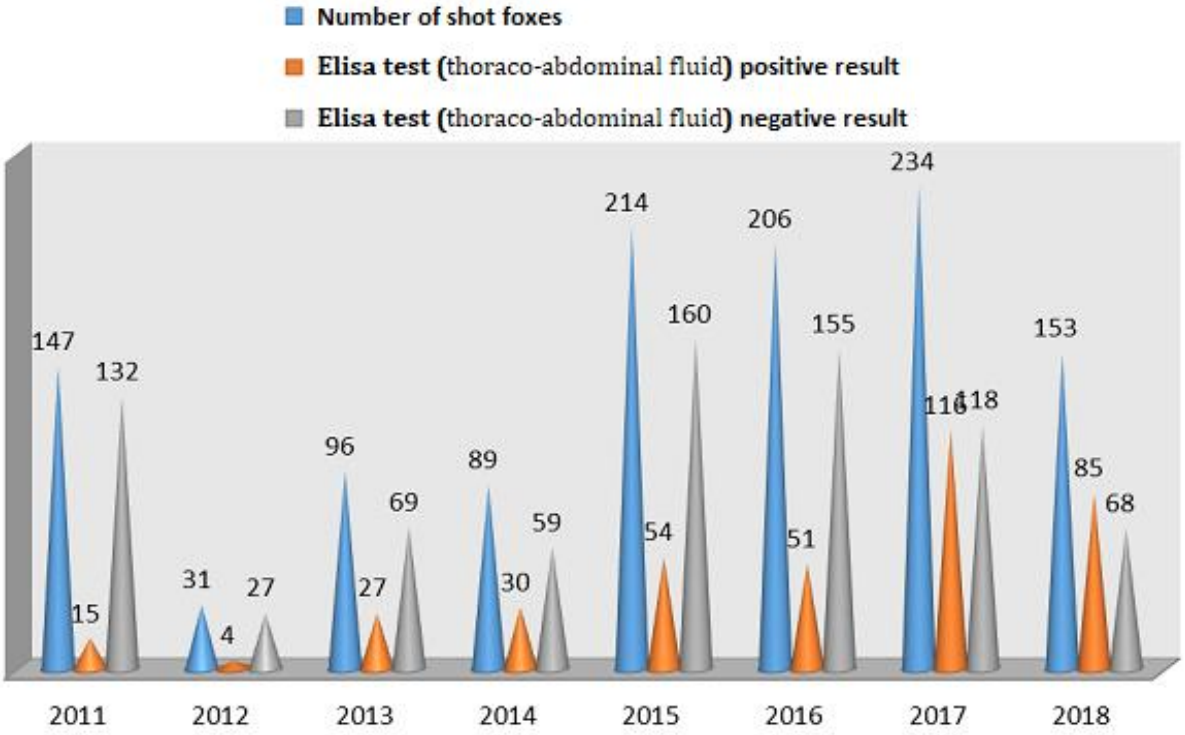

Figure 2. Elisa test

Table 2. Elisa tests in shot foxes (percentages)

Year	Percentage of shot foxes	Elisa test (thoraco-abdominal fluid) %	
		Positive result	Negative result
2011	100	10.204	89.796
2012	100	12.903	87.097
2013	100	28.125	71.875
2014	100	33.708	66.292
2015	100	25.234	74.766
2016	100	24.757	75.243
2017	100	49.573	50.427
2018	100	55.556	44.444

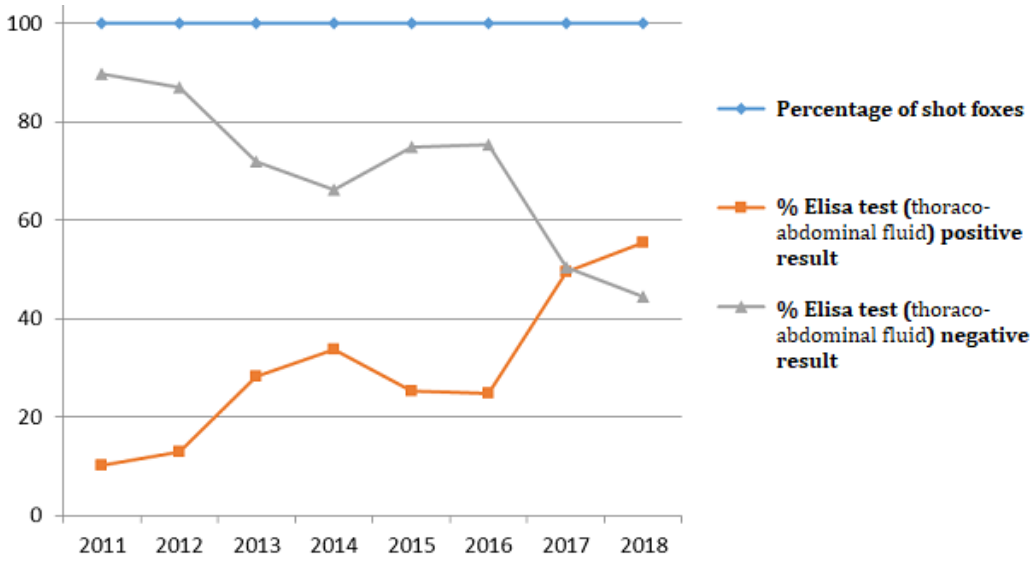

Figure 3. Elisa test (percentages)

From the analysis of the data presented in the above tables and graphs, it appears that, on the same number of samples tested, as in the case of the vaccine marker (1170), the number of serologically positive cases is lower than the number of negative cases (382 positive and 788 negative samples), which would indicate poor immunisation of the vaccinated and tested foxes. The situation, although somewhat paradoxical in relation to the degree of immunisation revealed by the presence of the vaccine marker, could be explained by the possible degradation of protein fractions, components of immunoglobulins (antibodies) in the thoraco-abdominal fluids collected from fox carcasses not always qualitatively suitable for an eloquent serological test.

4. Conclusions

Rabies currently remains a public health problem for both Sibiu County and for the whole country.
Laboratory diagnosis of all diseased animals with manifestations of nervous and that could be attributed to rabies, is the main measure that any doctor veterinary can undertake to confirm or refute the diagnosis. The methods currently used in any veterinary diagnostic laboratory are standardized and capable of establishing the incriminating etiological agent. The 8-year study (2011-2018) highlighted the effectiveness of the measures implemented by Decision 2011/807/EU of 30 November 2011 approving the 10-year, nationwide rabies eradication programme in Romania.
The implementation of the measures laid down in Decision 807/2011/EU by vaccinating foxes, considered the main reservoir of rabies virus, with vaccine baits, followed by strict monitoring through control hunts and diagnostic examinations of all shot foxes has resulted in a reduction to zero of disease cases in both wild and domestic animals.
The absence of contact with sick animals also reduced to zero the cases of illness in human.

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