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TWO DIFFERENT SYSTEMS FOR THE ISOLATION OF RABIES VIRUS IN CELL CULTURES

M. A. DASCALU[1], A. SERVAT[2], F. DARABAN[1], E. VELESCU[1] & O.-I. TANASE[1]

[1]Department of Public Health, Faculty of Veterinary Medicine, Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine, Iaşi, Romania; [2]Anses, Laboratory for Rabies and Wildlife, WHO Collaborating Centre for Research and Management in Zoonoses Control, OIE Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies, European Union Reference Laboratory for Rabies Serology, Technopôle Agricole et Vétérinaire, Malzéville, France

Summary

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Rabies is one of the fatal zoonotic diseases that affects all warm mammals and humans, caused by a highly neurotropic virus belonging to the *Rhabdoviridae* family, *Lyssavirus* genus. As recommended by the OIE, the RTCIT can be performed on various systems such as: 96-well microtitration plates, 60 or 96 well HLA (human leukocyte antigen) plastic plates, coverslips or multi-chambered glass slides, but with different disadvantages. The aim of our study was to perform the isolation of rabies virus on two different systems, namely: on 96-well microtitration plates (microplate) and on 8 chambered glass slides (LabTek Chamber Slide System) with the purpose to note if the rabies virus multiplies equally well in both plastic and glass systems.

Key words: cell cultures, microplate, multi-chambered glass slides

INTRODUCTION

Rabies is one of the fatal zoonotic diseases that affects all warm mammals and humans, caused by a highly neurotropic virus belonging to the *Rhabdoviridae* family, *Lyssavirus* genus (Tekki *et al.*, 2016). Considering the importance of this disease, the diagnostic tests must be fast and precise, because the results may influence not only the medical decision of a human post-exposure treatment but also the planification of control measures in case of a potential outbreak (Medeiros Caporale *et al.*, 2009). In this regard, three reference techniques are recognised by both WHO and OIE, namely fluorescent antibody test (FAT), Rabies tissue culture infection test (RTCIT) and mouse inoculation test (MIT) (OIE, 2013).

As recommended by the OIE, the RTCIT can be performed on various systems such as 96-well microtitration plates (Webster, 1987; Rudd *et al.*, 1989), 60 or 96 well HLA (human leukocyte antigen) plastic plates (Tollis *et al.*, 1988), coverslips (Chitra *et al.*, 1988) or multi-chambered glass slides (Rudd *et al.*, 1987;

Barrat *et al.*, 1988; Bourhy *et al.*, 1989; Webster *et al.*, 1989), but with different disadvantages. If HLA plate gives inconstant results regarding the samples containing very small amounts of virus, coverslips cultures pose some problems when handling and that because of their fragility, while the multi-chambered glass slides may represent a cost problem when large number of samples are tested (Meslin *et al.*, 1999).

Rabies virus is a neurotropic one and once multiplied in the central nervous system, it may be distributed to the most organs. This has made possible its isolation in a wide range of cells, but with more or less satisfactory results (Webster *et al.*, 1996). Thus, many studies have been demonstrated that the rabies virus is best multiplied in the cells of neural origin and also in cells which possess in the cell membrane structure, muscarinic acetylcholine receptors that are involved in virus attachment in the neuronal cells and synthetic enzyme neurotransmitters, such as murine neuroblastoma cells (N2a) (Chhabra *et al.*, 2007; Madhusudana *et al.*, 2010; Yang *et al.*, 2012; OIE, 2013).

Throughout time, different cell lines of neural origin (e.g. N2a), as well as some non-neural cells (e.g. BSR, BHK-21, CER, HEK-293, McCoy cells) were experimented. The first attempt for the isolation of rabies virus was performed on baby hamster kidney cell line (BHK-21), but the results were not the expected ones, with variable success rates and inferior sensitivity when compared to the MIT (Webster *et al.*, 1996; Meslin *et al.*, 1999; Madhusudana *et al.*, 2010).

Because the N2a are highly susceptible to lyssavirus infections and have a superior sensitivity over other cell lines and over the MIT (Smith *et al.*, 1978; Rudd *et al.*, 1980; Umoh *et al.*, 1983; Rudd *et al.*, 1987; Bourhy *et al.*, 1989), they are at the moment the reference cell line recognised by the both WHO and OIE.

The aim of our study was to perform the isolation of rabies virus on two different systems, namely: 96-well microtitration plates (microplate) and 8 chambered glass slides (LabTek Chamber Slide System) with the purpose to note if the rabies virus multiplies equally well in both plastic and glass systems.

MATERIAL AND METHODS

Study area

To carry out this study, a total of 16 brain samples were used. These samples were submitted by the Romanian Sanitary Veterinary and Food Safety Direction (SVFSD) from Bacau,Vaslui, Iasi, Galati, Neamt and by the Regional Center of Veterinary Diagnostic (RCDV) from Republic of Moldavia, being first tested for rabies by Fluorescent Antibody Test and confirmed by the Mouse Inoculation Test (only when FAT results were negative or doubtful) (Fig. 1).

Virus isolates

All 16 brain samples from the North-East of Romania and Republic of Moldavia were isolated between 2012–2016 from 6 cows *(Bos taurus*), 4 dogs *(Canis canis*), 1 goat *(Capra aegragus hircus),* 1 deer *(Capreolus capreolus)* and 4 foxes *(Vulpes vulpes)* (Table 1). The positive samples obtained by SVFSD and RCDV were submitted for researches to the European Union Reference Laboratory for Rabies, from Nancy, France. Prior our tests, all samples were reanalyzed for the detection of rabies antigen using FAT, the reference rabies diagnosis technique, as described in OIE Terrestrial Manual (OIE, 2013).

Table 1. Characteristics of the samples tested from Romania and Republic of Moldavia

No.	Country	Host species	Sample code	Year of isolation	Source
1	Romania	Fox	DR1025	2012	SVFSD* BC
2	Romania	Dog	DR1026	2012	SVFSD BC
3	Romania	Deer	DR1027	2012	SVFSD BC
4	Romania	Cow	DR1028	2013	SVFSD IS
5	Romania	Cow	DR1029	2014	SVFSD IS
6	Romania	Cow	DR1030	2013	SVFSD IS
7	Romania	Cow	DR1031	2015	SVFSD GL
8	Romania	Dog	DR1032	2012	SVFSD NT
9	Romania	Fox	DR1033	2013	SVFSD NT
10	Romania	Fox	DR1034	2013	SVFSD NT
11	Romania	Cow	DR1035	2012	SVFSD NT
12	Romania	Dog	DR1036	2013	SVFSD NT
13	Romania	Fox	DR1187	2014	SVFSD VS
14	Republic of Moldavia	Goat	DR1198	2016	RCDV* RM
15	Republic of Moldavia	Dog	DR1200	2016	RCDV RM
16	Republic of Moldavia	Cow	DR1201	2016	RCDV RM

*SVFSD: Sanitary Veterinary and Food Safety Direction; *RCDV: Regional Center of Veterinary Diagnostic

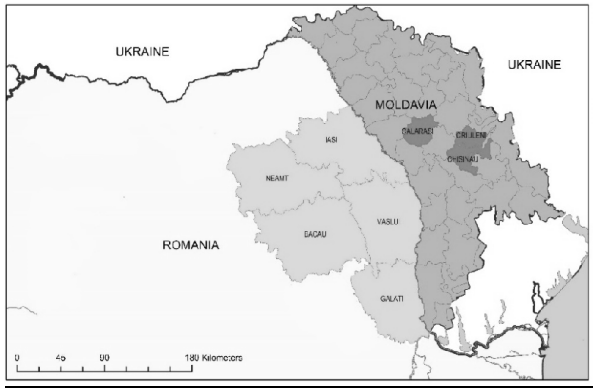


Fig. 1. Map representing the geographical origin of the 16 tested samples from Romania (light gray colour) and Republic of Moldavia (dark gray colour).

Materials

Both biological and chemical materials were used to perform the technique. The biological materials were represented by the 16 brain samples included in the study. Each diagnostic session included 2 positive controls consisting in brains obtained from mice experimentally infected with a fixed rabies virus CVS-27 (Challenge Virus Standard) and from mice experimentally infected with a EBLV-1b lyssavirus isolated in a Serotine bat (European Bat Lyssavirus), respectively.

A negative control, consisting in a brain from healthy mouse, was also included in each session to validate the test.

The chemical materials needed were: murine neuroblastoma cells N2a (ATCC CCL-131), foetal calf serum - FCS (Sigma Life Science, UK), Bio-Rad conjugate (France), DMEM medium (Gibco, UK), trypsin, acetone 80% (Thermo Chemical, UK), sterile distilled water (Sigma Life Science, UK), sterile phosphate buffered saline (Sigma Life Science, UK), and glycerol mounting medium (Light Diagnostic, USA). Other materials like microplate (Falcon, USA), LabTek Chamber Slide System (Thermo Fisher Scientific Inc, USA), Neubauer Chamber (Optik Labor, Germany), UV microscope (Olympus BX41), reversed microscope (Lordil AE 2000), incubater (Incucell, Fisher Bioblock Scientific, MMM Medcenter) and coverslips (Knittel glass) were necessary in order to perform the technique.

RTCIT on LabTek Chamber Slides

Growth medium, cell suspension and inoculum preparation. The DMEM medium was supplemented with 1% mix of antibiotics (penicillin, streptomycin and amphotericin), antimycoplasmic 2.5 µg/mL and 5% FCS, before use. Cell suspension was prepared from murine neuroblastoma cells (N2a) in DMEM growth medium, at a final concentration of 8×10^5 cells/mL. The cells were trypsinised and counted in Neubauer chamber when the cell monolayer was up to 80% confluent. Brain samples were diluted 1/5 in DMEM medium, centrifuged for 30 minutes at 3000 rpm/minute and the supernatant was collected for inoculation.

Inoculation and incubation. Four hundred µL of cell suspension were added in each chamber of the LabTeks and let to rest for 5–10 minutes in a humid chamber containing 5% CO_2, for cell sedimentation. Fifty µL of control brain suspensions, two positive and two negative controls were distributed in the intended wells. All the LabTeks were incubated in a humid chamber with 5% CO_2 at 37 °C for 48 hours. After incubation, medium was discarded from each well using a vacuum pump and then slides were air-dried at room temperature for 5–10 min.

Acetone fixation and slides staining. All slides were filled in ice-cold absolute acetone and fixed at –20 °C for 30 minutes and consecutively air-dried. For slides staining the Bio-Rad conjugate was used, which contain polyclonal antibodies, known for their good specificity, allowing all currently known Lyssaviruses genotypes to be detected. The lyophilised conjugate was reconstituted according to the manufacturer recommendations, in 3 ml of sterile distilled water and centrifuged for 5 minutes at 1500 rpm/minute for clarification. Each well was filled with 50 µL of the supernatant and then incubated at 37°C for 30 minutes in a humid chamber with 5% CO_2.

Slides washing, mounting medium and microscope reading. All slides were washed once in sterile PBS and once in sterile distilled water and air-dried. From each LabTek, both walls and seals were removed and a drop of mounting medium was added on each slide, followed by the coverslips. The reading was performed using a fluorescence microscope with x200 and x400 magnification. Control slides were examined first in order to validate the technique.

RTCIT on microplate

The procedure was performed using two different cell suspension concentration: 5×10^5 cells/mL and 2.5×10^5 cells/mL. For each concentration, 200 µL of cell sus-

pension were added in each well, followed by 50 µL of brain suspension. The plates were incubated for 72 hours in humid chamber with 5% CO_2. Forward, the medium was discarded and the plates washed once in sterile PBS. A first wash with 80% of acetone was performed, followed by a second one and then incubated for 30 minutes at room temperature. Once fixed, another wash with PBS was carried out and the plates were air-dried. Fifty µL of the conjugate were added in each well and then incubated in humid chamber with 5% CO_2 for 30 minutes. The plates were last washed in PBS and read at the fluorescence microscope at ×200 and ×400 magnification.

RESULTS

RTCIT on LabTek Chamber Slides

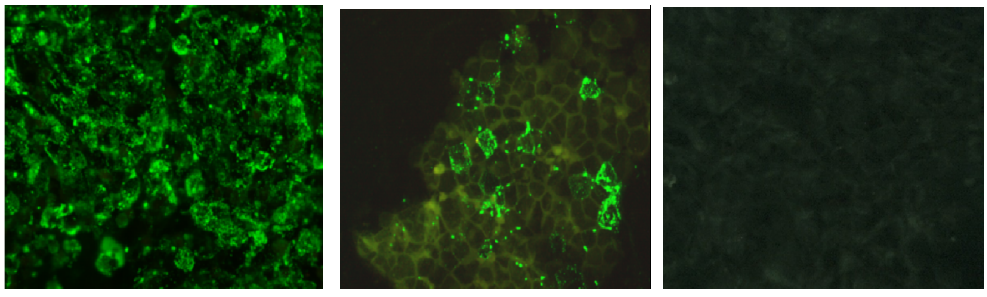
RTCIT on LabTek Chamber slides were conducted using a single cell concentration of $8x10^5$ cells/mL. As expected, CVS-27 and EBLV-1b positive controls were found positive (Fig. 2A, B) and the negative control: negative (Fig. 2C). After an incubation period of 48 hours, out of the 16 tested samples, all of them (100%) were found positive for the isolation of rabies virus in N2a cell cultures.

RTCIT on microplate

RTCIT on microplates were carried out using 2 cell concentrations, namely: 5×10^5 and 2.5×10^5 cells/mL, with an incubation period of 72 hours, each sample being inoculated in duplicate, all the controls being validated (e.g. Fig. 3A).

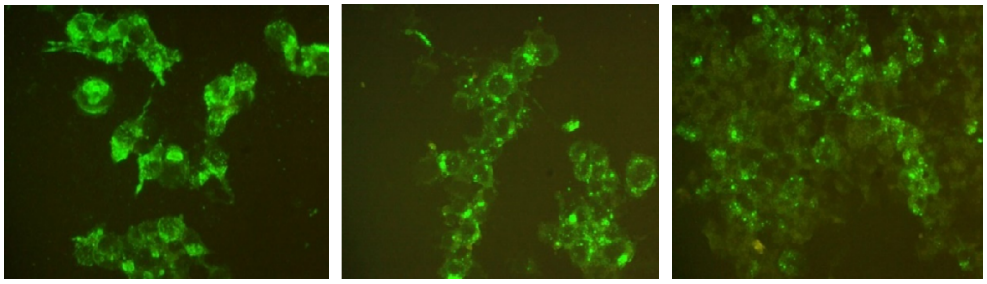
For the first cell concentration of 5×10^5, out of 16 samples, 6 (37.5%) were negative and 10 (62,5%) were positive for the isolation of rabies virus, the same number of negatives being found also for the second cell concentrations of 2.5×10^5, where 6 (37.5%) out of 16 tested samples were found negative and 10 (62.5%) were positive (Table 2).

Five samples (DR1025, DR1027, DR1028, DR1030 and DR1032) were found negative for both cell concentrations of 5×10^5 and 2.5×10^5. Nevertheless, for 2 samples, the results were different for the two cell concentrations used. Thereby, DR1029 was found positive at the first cell concentration, but negative for the second one. Inversely, sample DR1031 was recorded negative at the first cell concentration and positive at the second one.



A **B** **C**

Fig. 2. A. CVS-27 positive control, LabTek system, with a cell concentration of 8×10^5 cells/mL, 48 h incubation (x400); B. EBLV-1b positive control, LabTek system, with a cell concentration of 8×10^5 cells/mL, 48 h incubation (x400); C. Negative control, LabTek system, with a cell concentration of 8×10^5 cells/mL, 48 h incubation (×400).



A **B** **C**

Fig. 3. A. CVS-27 positive control, microplate system, with a cell concentration of 2×10^5 cells/mL, 72 h incubation (x400); B. Positive RTCIT on microplate (DR1033) with a cell concentration of 2×10^5 cells/mL, 72 h incubation (x400); C. Positive RTCIT on microplate (DR1033) with a cell concentration of 1.5×10^5 cells/mL, 72 h incubation (×400).

With the purpose to establish which factors had leed to these inconstant results on microplates, another 4 cell concentrations on 5 different samples (DR1033, DR1187, DR1198, DR1200 and DR1201) were used. Thereby, samples which were very positive but also low positive on FAT were chosen in order to verify if there is any corelation between the concentration of rabies virus in a sample and the system used. The cell concentrations used were: 2×10^5, 1.5×10^5, 1×10^5 and 0.5×10^5 cells/mL, with a period of incubation of 72 hours, each sample being inoculated in duplicate.

Three of the samples (DR1198, DR1200 and DR1201) were positive for all 4 cell concentrations, while DR1033 (Fig. 3B, C) and DR1187 were positive for 2×10^5, 1.5×10^5 and negative at lower cell concentrations (Table 2). Under the concentration of 2×10^5, the multiplication and isolation of rabies virus in the cells is inconstant and that because of a small number of cells, low cell concentrations being not indicated to be used.

In an attempt to establish the amount of rabies virus from the samples used in the study, the virus content was determined by titration on N2a cell cultures, with a cells suspension of 2×10^5 cells/mL, each sample being inoculated on microplate in 4 replicates and 6 dilutions, with a period of incubation of 72 hours. The calculation of titres was performed using the Sperman-Karber formula. As a landmark, 2 positive controls (CVS-27 and EBLV-1b) were used. As expected, high amounts of rabies virus were obtained for the CVS-27 positive control, with a titre of $10^{5.50}$ $TCID_{50}$/50 µL. Most samples (DR1025-DR1027, DR1031, DR1033-DR1036, DR1198, DR1200 and DR1201) had a titre ranging between 10^2 and $10^{2.75}$ $TCID_{50}$/50 µL.

For two samples DR1029 and DR1187, titres were quite low, $10^{1.25}$ $TCID_{50}$/50 µL and $10^{0.50}$ $TCID_{50}$/50 µL, respectively. For samples DR1028, DR1030 and DR1032, the titration was not performed, due to insufficient amount of the samples to test.

These results demonstrate a lower amount of rabies virus in the samples when compared to a fixed laboratory strain virus (CVS-27). It must be emphasised that the titration was realised after several freezing and defrosting cycles of the samples.

Table 2. RTCIT results obtained for LabTek and microplate systems

No	Sample code	RTCIT results for Labtek	RTCIT results for microplate (cell concentration)					
		8×10^5 cell/mL	5×10^5 cells/mL	2.5×10^5 cells/mL	2×10^5 cells/mL	1.5×10^5 cells/mL	1×10^5 cells/mL	0.5×10^5 cells/mL
1	DR1025	Pos*	Neg	Neg	NT	NT	NT	NT
2	DR1026	Pos	Pos	Pos	NT	NT	NT	NT
3	DR1027	Pos	Neg	Neg	NT	NT	NT	NT
4	DR1028	Pos	Neg	Neg	NT	NT	NT	NT
5	DR1029	Pos	Pos	Neg	NT	NT	NT	NT
6	DR1030	Pos	Neg	Neg	NT	NT	NT	NT
7	DR1031	Pos	Neg	Pos	NT	NT	NT	NT
8	DR1032	Pos	Neg	Neg	NT	NT	NT	NT
9	DR1033	Pos	Pos	Pos	Pos	Pos	Neg	Neg
10	DR1034	Pos	Pos	Pos	NT	NT	NT	NT
11	DR1035	Pos	Pos	Pos	NT	NT	NT	NT
12	DR1036	Pos	Pos	Pos	NT	NT	NT	NT
13	DR1187	Pos	Pos	Pos	Pos	Pos	Neg	Neg
14	DR1198	Pos	Pos	Pos	Pos	Pos	Pos	Pos
15	DR1200	Pos	Pos	Pos	Pos	Pos	Pos	Pos
16	DR1201	Pos	Pos	Pos	Pos	Pos	Pos	Pos

*Pos: Positive; *Neg: Negative; *NT: not tested.

DISCUSSION

The discordant results between those obtained on microplate and LabTek Chamber Slides may be due to the quality of the samples, considering the fact that some of them were stored for a long time since 2012 in different storage conditions, the repeated freezing and defrosting cycles and year of the samples isolation were factors that could have influenced the results of the tests.

It has been also demonstrated that the poor condition of the samples, those in a state of decomposition, next to the neuroblastoma cells lysis and clumping may be the reason for unsatisfactory results (Rudd *et al.*, 1989).

Regarding the fixative of choice in immunofluorescence microscopy for rabies virus detection, this is represented by the absolute acetone, which is well adapted for multi-chambered glass slides, but not also for the 96-well plates, being prefered the diluted acetone of 80% for a optimal fixation of cells to the plastic plate, as it was demonstrated by Webster (1987) and Rudd *et al.* (1989) and in order to avoid the damage of the plastic device.

Concerning the optimal incubation period, different variants have been suggested, from 18 hours (Bourhy *et al.*, 1989) to 24 hours (Portnoi *et al.*, 1982; Chitra *et al.*, 1988), while others studies recommend to allow the cells to grow for 3 or 5 days before giving the results (Webster, 1987; Rudd *et al.*, 1989). Also, efficient replication of the rabies virus in a cell culture can be influenced by the concentration of the virus in the samples. Thereby, in a sample containing a high

virus concentration, the infection in N2a cells can be visualised as early as after 24 hours, but in one containing smaller amounts of virus, it required up to 3–4 days to produce infection (Umoh *et al.*, 1983; Webster, 1987).

It should be noted that before our researches, most part of the samples used in the study were stored at –20°C for a long period of time. This, next to the many freezing and defrosting cycles performed, illustrates the loss of infectivity, that's why the long-term storage of rabies virus samples should be at considerably lower temperature, such as –80°C, as was shown by Wachendorfer *et al.* (1985).

As a disadvantage for the LabTek system, can be mentioned the possibility of contamination when the walls and the seals have to be removed, thereby this step it has to be done very carefully. This is an issue that it is not found when performing RTCIT on microplate, because the wells do not communicate with each other. The economic side should not be neglected, taking into account that microplates cost much less than LabTeks.

CONCLUSIONS

The best results were obtained on multichambered glass, the cell concentration of 8×10^5 cells/mL being considered optimal for a good multiplication and isolation of the rabies virus on this type of system.

Regarding the other system used, namely the 96-well titration plate, the results were inconstant, being acceptable for the cell concentration of 5×10^5 and 2.5×10^5 cells/mL, but not for those under 2×10^5 cells/mL, the low number of cells did not allow virus multiplication and it is not recommended to be used.

The results obtained on microplate, could have also been influenced by the several freezing and defrosting cycles performed on the samples together with the storage conditions, which resulted in a loss of infectivity.

Changing the growth medium after 24 hours of incubation, changing the strain of neuroblastoma cells for a better fixation on a plastic support, use of a counterstaining to decrease background signal due to the plastic are some of the aspects which can be implemented in order to obtain better results on microplate.

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