Quantification of the effectiveness of laboratory diagnostics of rabies using classical and molecular-genetic methods

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ABSTRACT: In comparative experiments the diagnostic effectiveness of four methods of laboratory diagnostics of rabies – the mouse intracerebral inoculation test (MICIT, MIT), the rabies tissue culture infection test (RTCIT), the rapid rabies enzyme immune diagnosis test (RREID) and a molecular-genetic method, the nested reverse transcription polymerase chain reaction (nRT-PCR) – was quantified by the titration of serial dilutions of brain viral suspensions. The threshold value of the tests, i.e., the highest dilution of a specimen, which the method used is able to detect as a positive one, was determined. Further advantages and disadvantages of the tested methods were compared as well. Experimental optimization of procedures for RNA extraction was carried out and the optimum primer for RNA transcription to cDNA was selected. The RREID method was carried out in two variants: detection of the rabies antigen in a clarified (centrifugated) as well as in a non-clarified (noncentrifugated) brain suspension. In the experiments three autochthonous street isolates of rabies virus (in the form of primary isolates) were used; they had been isolated from naturally infected red foxes (*Vulpes vulpes*) and a lynx (*Lynx lynx*). The results of comparative experiments revealed a relative correlation of the diagnostic effectiveness of standard methods (MICIT and RTCIT), with standard MICIT being the more sensitive one, RTCIT however having several other advantages (among others the speed of performance) and thus being preferred. For quantitative comparison of diagnostic effectiveness two other methods (RREID and nRT-PCR) were examined in that street isolates of rabies virus, which revealed the highest titer after titration by MICIT and RTCIT. The sensitivity of the RREID method proved to be rather low. If used with noncentrifugated brain suspensions this method may yield nonspecific reactions. If compared particularly with RREID the nRT-PCR is characterized by a considerably higher diagnostic effectiveness. The sensitivity of nRT-PCR is not affected by preliminary clarification of the brain suspension. The reverse primer N12 seems to be more suitable for transcription of the extracted RNA to cDNA than random hexamers.

Keywords: rabies; rabies virus (RABV); MICIT – mouse intracerebral inoculation test; MIT – mouse inoculation test; RTCIT – rabies tissue culture infection test; RREID – rapid rabies enzyme immune diagnostic test; nRT-PCR – nested reverse transcription polymerase chain reaction

Rabies is an acute lyssavirus disease of warm-blooded animals which may also be transmitted to human (zooanthroponosis). It affects mainly the CNS and virtually in all cases it necessarily takes a fatal course.

The etiological agent of rabies – the rabies virus (genus *Lyssavirus*, family *Rhabdoviridae*) is an RNA virus with a negatively polarized single-stranded RNA. According to the valid taxonomy the genus *Lyssavirus* is classified into seven geno-

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types (Bourhy et al., 1992, 1993; Gould et al., 1998; Svrcek, 2002).

Despite of the long history and the remarkable progress in the knowledge, prevention and control of rabies the disease has maintained its global distribution in animals as well as humans. Each year approximately 60 000 people die of rabies worldwide, mainly in the developing countries (Meslin and Stohr, 1997).

For successful prevention and control of rabies (especially for early postexposition immunization of people) the use of rapid and sensitive diagnostic methods for reliable diagnosis of the disease in animals which exposed human is essential. The diagnosis of rabies is stated on the basis of a complex examination including clinical, epizootiological and laboratory assessments of which the latter are of decisive importance. The laboratory methods of rabies diagnosis are internationally standardized (Meslin et al., 1996; OIE, 2000). In the developed countries only cases confirmed by laboratory tests are officially registered.

Nowadays the detection of cytoplasmic inclusion or Negri bodies (Negri, 1903) is rarely used to diagnose rabies. This method was successfully replaced by rabies antigen detection with the fluorescent antibody test – FAT (Goldwasser et al., 1959). WHO and OIE recommended the FAT as the primary laboratory method for diagnosis of rabies. The mouse inoculation test (MIT), a conventional *in vivo* procedure (Webster and Dawson, 1935) is being gradually substituted by the rabies tissue-culture infection test (RTCIT; Larghi et al., 1975) which is an *in vitro* method. The RREID (rapid rabies enzyme immuno diagnosis test; Perrin et al., 1986) is considered to be an additional method. The indirect immunofluorescence method using antinucleocapsid monoclonal antibodies is also used for the identification of rabies virus (Ondejka et al., 1997). On the other hand, the detection and quantification of rabies antibodies is not usually used for the standart laboratory diagnosis of rabies (Ondrejkova et al., 2002). Nucleic acid hybridization (Bourhy and Sureau, 1990) as well as the RT-PCR (reverse transcription – polymerase chain reaction) method (Tordo et al., 1996) are not standardized either.

The aim of our study was to compare the sensitivity and effectiveness of the MIT, RTCIT, RREID and RT-PCR in the diagnosis of rabies and to state (by titration) the threshold value, i.e., the highest dilution of a specimen, which the method used is able to detect as a positive one. The advantages and disadvantages of tested methods were compared as well. With RT-PCR method two procedures for RNA extraction were optimized and the more suitable of two primers was chosen for RNA transcription to cDNA. In addition, two alternative procedures, rabies antigen detection in a clarified as well as in a nonclarified viral brain suspension were chosen for the RREID test.

MATERIAL AND METHODS

Autochthonous of street isolates of rabies virus

In the experiments three autochthonous street isolates of rabies virus were used, which had been isolated from naturally infected carnivores submitted for laboratory examination. Two of these isolates (No's. 318/18404/01 and 384/24849/01) were isolated from the brain of red foxes (*Vulpes vulpes*) and one (strain No. 403/31139/01) from a lynx (*Lynx lynx*). The isolates (animal brains without previous passages) were kindly provided by the State Veterinary Institute in Zvolen. Detection of the rabies antigen was carried out by the FAT according to Dean et al. (1996).

From the original material a 20% suspension was prepared, the supernatant of which was diluted 1 : 10 and used to cultivate the virus by the MICIT method in new-born inbred mice (Koprowski, 1996; OIE, 2000). From mice which died or were ether-killed in the stage of prostration brains consequently brain membranes were removed. In order to determine the specificity of mortality impression smears of brains were subjected to the FAT test (Dean et al., 1996). Subsequently each brain was divided into two halfs by a longitudinal section. One half of each brain was used to prepare a suspension for virus titration in mice as well as neuroblastoma (N2a) cell cultures. The second half was used for RT-PCR and RREID examination.

Titration of rabies virus isolates (MICIT, RTCIT)

In vivo isolation and titration of the rabies virus (quantitative biological experiment, MIT – mouse inoculation test, MICIT – mouse intracerebral inoculation test) was carried out in white inbred mice weighing 8–10 g. The MICIT (Koprowski, 1996;

OIE, 2000) was used for intracerebral inoculation of mice with 0.03 ml of the brain suspension supernatant. Five different dilutions were used (10^{-3} to 10^{-7}; dilution factor 10). Each dilution was applied in five mice. Observation of the animals continued for 28 days and based on specific death (controlled by FAT detection of the rabies antigen) the titer of the virus was calculated according to Reed and Muench (1938).

In vitro isolation and titration of three rabies virus isolates (RTCIT) was carried out on N2a neuroblastoma cells according to the method recommended by the WHO (Webster and Casey, 1996). Brain suspension dilutions were prepared at rates 1 : 5–1 : 1 935 125 (dilution factor 5). Incubation was performed in an open system for 24–48 hours at 37°C with 5% carbon dioxide (CO_2). Visualization of rabies virus production was carried out by FAT (Dean et al., 1996). The final titer of the virus was calculated accoridng to the formula:

Titer = [average number of fluorescent foci in the last dilutions where fluorescence is still visible] × [dilution] × [1 000/50]

Preparation of serial dilutions of the viral suspension for RT-PCR and RREID

The second half of mice brains of with the highest titer of the street isolate (specimen No. 318/18404/01 chosen on the basis of previous *in vivo* and *in vitro* titration) was divided into three parts. One part was used for direct Trisol extraction of viral RNA from the brain, the second and third ones were used to prepare a suspension in 900 µl of Dulbeco minimal essential medium. One half of the suspension was centrifugated for five minutes at 2 000 g and the supernatant was used to prepare 10-fold serial dilutions (10^{-1}–10^{-6}) whereas the second half was used to prepare the dilutions without centrifugation.

The individual dilutions of the viral/tissue suspension were consequently used for virus detection (titration) by RREID and RT-PCR.

RREID (rapid rabies enzyme immuno diagnosis)

RREID was performed according to the procedure standardized and recommended by the WHO (Bourhy and Perrin, 1996), however, in addition to standard rabies antigen detection in a clarified brain viral suspension detection was also carried out in a non-clarified sample.

Plastic microtitration plates (strips with eight wells) sensitized with antirabies rabbit antinucleocapsid IgG were used. After wasching with PBS + Tween solution 200 µl of the individual dilutions of the centrifugated suspension or identical amounts of the diluted nonclarified brain suspension were added to each well. In addition to the experimental specimen controls were used as well: 200 µl PBS + Tween solution as a blank control, a positive nondiluted control (200 µl of the supernatant from the brain of a laboratory mouse infected with a CVS strain) and a negative one (200 µl of the supernatant from the negative brain of an intact mouse). The plates were sealed with a plastic adhesive film and incubated in a thermostat for 1 hour at 37°C. The wells were then washed with PBS + Tween solution and 200 µl of polyclonal antirabies mouse antinucleocapsid antibodies were added to each well. Then the plates were incubated again for 1 hour at 37°C. After incubation and washing, 200 µl of goat antimouse antibodies conjugated with horseradish peroxidase were added, the plates were sealed with adhesive plastic film and incubated for 1 hour at 37°C. After thorough washing 200 µl of the substrate with chromogen OPD (o-phenylenediamine) were added and the plates were incubated in the dark for 3–6 minutes at room temperature. Incubation time was chosen in dependence on the intensity and speed of yellow colouring of the positive control. If the latter was evenly yellow, incubation was stopped. Subsequently the reaction was stopped with 50 µl of 4N H_2SO_4 and measurement of optical density (OD) by a spectrophotometer at a wavelength of 492 nm was performed immediately. Absorbancy was calculated by subtracting the OD of the blank from that of the controls and the samples. The test was valid only if absorbancy of the negative and positive controls was below 0.1 and above 1.5 units, respectively. Samples with an absorbancy surpassing that of the negative control by more than 0.08 were considered positive (Bourhy and Perrin, 1996).

nRT-PCR (nested reverse transcription polymerase chain reaction)

For RT-PCR, the brain, the supernatant as well as the diluted non-centrifuged suspension obtained from sample No. 318/18404/01 were used at dilu-

tions identical with those used for RREID. In addition the virus was also detected by RT-PCR directly in undiluted brain tissue. The RT-PCR was performed according to the procedure recommended by the WHO (Tordo et al., 1996; Bourhy et al., 1999) partially modified in our laboratory.

Total RNA was extracted from 200 µl of specimens with Trisol reagent (Invitrogen) according to the manufacturer's instructions. After precipitation with isopropanol (Merck) RNA was washed with 75% ethanol. The pellets were dried at laboratory temperature in a laminar flow box, resuspended in 50 µl nuclease-free water and stored at –20°C for a few days.

Reverse transcription of RNA to cDNA was performed in two ways. The first method used the reverse PCR primer N12 (5'-GTA-ACA-CCT-CTA-CAA-TGG-A-3', positions 57 to 75; all the positions of the primers given are based on the PV strain sequence; Tordo et al., 1986a). Considering that the N gene is the most conserved in the lyssa viruses (except some domains of the L protein gene) and that the sequence data concerning this gene are the most exhaustive, we used primers selected from the N gene that were shown to allow amplification of a wide range of genetically diverse lyssa viruses (Bourhy et al., 1993; Kissi et al., 1995). The second method used N6 random hexamers (Vilcek et al., 1994). In the first step the extracted RNA (2 µl) was incubated in the presence of 2 µl primer N12 (10 pmol/µl) or random hexamers (Pharmacia) and 6 µl of sterile water at 72°C for 3 minutes. The reaction solution was cooled on ice and 20 µl of the transcription mixture containing 6 µl of 5 × reaction buffer, 5 µl (2 mM) dNTP, 0.5 µl (20U) RNAsin, 1 µl (200 U) Moloney murine reverse transcriptase and 7.5 µl nuclease free water were added to each sample. Reverse transcription was performed in a total volume of 30 µl at 42°C for 50 minutes. Besides a positive control also negative controls were used in the reaction: the control for negative transcription (all reaction ingredients used for transcription without extracted RNA) and a negative specimen (RNA extracted from negative noninfected brain).

The cDNA was used for *in vitro* amplification by the nested PCR. The external and internal PCR primers designed for the detection of rabies virus flanked a 1 529 bp and 579 bp fragments of the rabies virus genome.

The reaction mixture (50 µl) for the first PCR with external primers contained: 4 µl of cDNA, 5 µl of 10 × reaction buffer, 5 µl (2 mM) dNTP, 2.5 µl (50 mM) $MgCl_2$, 1.5 µl N8 primer (5'-AGT-TTC-TTC-AGC-CAT-CTC-3', positions 1 585 to 1 568; Amengual et al., 1997; concentration 10 pmol/µl) and 1.5 µl N12 primer (5'-GTA-ACA-CCT-CTA-CAA-TGG-A-3', concentration 10 pmol/µl), 0.2 µl (1 U) Taq DNA polymerase (Promega) and 30 µl nuclease free water. The aqueous phase was covered by one drop of mineral oil (Sigma). *In vitro* amplification was carried out in the GeneAmp® PCR System 9700 thermocycler (Biometra) with the following programme: 1 cycle of denaturation at 94°C/5 min, 35 cycles of denaturation at 94°C/40 s, annealing at 56°C/40 s, elongation at 72°C/1 min, followed by 1 cycle 72°C/7 min.

The reaction mixture for the nested PCR comprised 2 µl of amplification product from the first PCR, 5 µl of 10 × reaction buffer, 5 µl (2 mM) dNTP, 2.5 µl (50 mM) $MgCl_2$, 1.5 µl N42 primer (5'-CAC-ATT-TTG-TGA-GTT-GTC-A-3', positions 633 to 651 of the PV sequence, concentration 10 pmol/µl), 1.5 µl N53 primer (5'-GGA-TGC-CGA-CAA-GAT-TGT-AT-3', positions 73 to 92 of the PV sequence, Tordo et al., 1986b; concentration 10 pmol/µl), 0.2 µl (1 U) Taq DNA polymerase (Promega) and 33 µl sterile nuclease free water. After covering the aqueous phase with mineral oil amplification was performed in the following heat regime: 1 cycle denaturation at 94°C/3 min, 35 cycles of denaturation

Table 1. Quantification of diagnostic effectiveness – results of titration of three street isolates of rabies virus *in vivo* on mice (MIT) and *in vitro* on cell cultures (RTCIT)

Isolate of rabies virus – genotyp 1		Result of titration (titer – dilution)	
Number	origin	*in vivo*	*in vitro*
318/18404/01	red fox	$10^{4.32}$	$10^{3.18}$
348/24849/01	red fox	$10^{2.84}$	$10^{1.34}$
403/31139/01	*Lynx lynx*	$10^{3.53}$	$10^{2.68}$

at 94°C/30 s, annealing at 60°C/30 s, elongation at 72°C/40 s, followed by 1 cycle of ultimate elongation at 72°C/7 min. After termination of the amplification reaction the physical lenght of the PCR products was determined by electrophoresis on 2% agarose gel. The gel was stained with ethidium bromide (1 μl/ml) and photographed after visualization on a UV transluminator.

RESULTS

According to the results of the *in vivo* (MIT) and *in vitro* (RTCIT) methods presented in Table 1 there was a relative correlation of the diagnostic effectiveness of these methods. At the same time the titers of the autochthonous street isolates of rabies virus proved to be different. Both procedures yielded the highest virus titres in the brains of mice infected with isolate No. 318/18404/01 (originally isolated from the red fox). This isolate was selected for further experiments (quantification of the diagnostic effectiveness of RREID and RT-PCR).

Based on the above results the diagnostic effectiveness of MIT and RTCIT may be stated to be different. MIT appeared to be a very sensitive and effective diagnostic test whereas RTCIT proved to be less sensitive. The highest dilutions in which the virus could be detected were $10^{-2.4}$ and $10^{-3.56}$ for RTCIT and MIT, respectively. RTCIT seems to have several advantages; it provides results in a markedly shorter time than MIT (24–48 hours as compared to 28 days).

Diagnostic nRT-PCR and RREID detection of the nucleocapsid antigen were performed in brain suspensions of mice infected with the virus isolate No. 318/18404/01.

For transcription of extracted RNA to cDNA in the diagnostic nRT-PCR two different primers were used for the same brain sample, a random primer and the N12 reverse PCR primer. Our experiments revealed that the reverse primer allowed to obtain a greater amount of the amplification product. For this reason the N12 reverse primer was used in further experiments to estimate the diagnostic sensitivity of nRT-PCR.

From our experiments summarized in Table 2 it follows that the nRT-PCR method is a markedly more sensitive diagnostic procedure when compared with RREID since its threshold sensitivity approximately reaches dilution 10^{-4} (see also Figure 1) whereas that of RREID is dilution 10^{-2}. If using a nonclarified suspension the highest dilution giving positive diagnostic results with RREID was 10^{-3}. According to our results the different ways of sample preparation did not influence the diagnostic effectiveness or the threshold value of the nRT-PCR method.

DISCUSSION

Diagnosis is an inevitable part of rabies prevention and control with laboratory diagnosis being of utmost importance. According to the recommendation of the WHO in the developed countries only those cases of rabies are officially registered, which had been confirmed by laboratory examination. Rapid and early diagnosis is of extraordinary value in the indication of postexposition immunization of people exposed to suspect animals. In the course of the 20th century the methods of rabies diagnosis were continually improved and they were internationally standardized by the OIE (OIE, 2000) and WHO (Meslin et al., 1996).

Table 2. Quantification of diagnostic effectiveness of the RREID and PCR methods

Rabies virus isolate – genotype 1	Virus titer MICID – LD_{50}	Diagnostic method		Nondiluted sample	Results of detection after dilution of samples					
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Red fox		S	RREID	0	+	+	+	neg.	neg.	neg.
318/18404/01 s	$10^{4.32}$/0.03 ml		PCR	+	+	+	+	+	neg.	neg.
Red fox		CS	RREID	0	+	+	neg.	neg.	neg.	neg.
318/18404/01 ks	$10^{4.32}$/0.03 ml		PCR	+	+	+	+	+	neg.	neg.

S – nonclarified suspension

CS –suspension clarified by centrifugation nondiluted sample = brain tissue

0 – brain tissue without dilution cannot be used for RREID

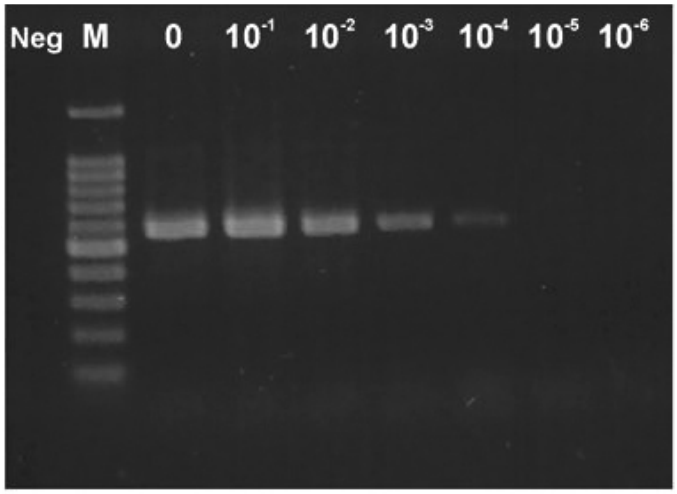

Figure 1. Demonstration of sensitivity of nested RT-PCR. Positive brain suspension diluted 10^{-1}–10^{-6} (factor of dilution 10)

Neg. – negative control (RNA extracted from negative noninfected mouse brain)
M – 100 bp molecular size marker
0 – undiluted brain tissue
10^{-1}–10^{-6} – dilutions of brain tissue

The aim of our study was to the compare sensitivity and diagnostic effectiveness of two standard methods (MIT, RTCIT), one recommended procedure (RREID) and one perspective but not yet internationally standardized method (RT-PCR) in rabies diagnosis and to state the threshold value, i.e. the highest dilution of the samples for these methods which were able to be recognized as positive. As a standard for our experiments the titer of street virus, which has been determined by MICIT on white laboratory mice was used.

The fluorescent antibody test (FAT) is the standard and most frequently primary used method of rabies diagnosis (Goldwasser et al., 1959; Dean and Abelseth, 1973; Dean et al., 1996). It is rapid, cheap and reliable. On the basis of our experiments as well as the data reported by other authors (Bourhy and Sureau, 1990; Meslin et al., 1996) FAT can be considered to have almost 100% specificity and sensitivity. One disadvantage of this method is that in case of formalin fixation the sample has to be treated with enzymes – most frequently with trypsine (Umoh and Blenden, 1981; Barnard and Voges, 1982). In addition, FAT detection of the rabies antigen in older decomposed brain samples may sometimes yield false negative results (David et al., 2002). Heaton et al. (1997) performed the FAT in a positive brain sample and obtained negative results after 72 hours of incubation at 37°C whereas using nRT-PCR in samples from the same brain they detected viral RNA even after 360 hours of incubation at 37°C.

The mouse inoculation test (MIT, MICIT) developed by Webster and Dawson (1935) is time consuming (incubation period 9–20 days for adult mice or 5–7 days for suckling pink mice), expensive and nowadays it is considered to be unethical. For this reason it is being replaced by the rabies tissue culture infection test on cell cultures (RTCIT). This test was initially developed by Larghi et al. (1975). It yields results after 24–48 hours (Bourhy and Sureau, 1990), it is cheaper and more ethical than the MIT, however, it may be performed only in well equipped laboratories with skilled staff. RTCIT and MIT are both directed at the detection of live virus and thus they are generally used mainly as supplementary methods in cases when FAT yields negative results in suspect animals that exposed people or domestic animals. Like FAT, both RTCIT and MIT may yield false negative results in older decomposed samples (David et al., 2002).

In our experiments MICIT showed to be a highly sensitive and effective diagnostic test: while the highest dilution in which RTCIT detected a virus reached an average of $10^{-2.4}$, the highest dilution still positive with MICIT had an average value of $10^{-3.56}$. In spite of some disadvantages RTCIT is more suitable for rabies diagnosis than MICIT since it allows to invesigate greater numbers of samples in a very short time (18–48 hours). However, in our opinion this method should only be used as a supplementary method to FAT and RREID since it does not allow to detect the virus in an inactive state. With prolonged sample storage the amounts of live virus in positive specimens decrease, consequently the diagnostic effectiveness of RTCIT also decreases.

Rapid rabies enzyme immune diagnosis (RREID) is another supplementary but very important diagnostic procedure recommended by the WHO which allows to examine greater amounts of samples and yields results within 3–4 hours (Bourhy and Sureau, 1990). Perrin et al. (1986) and Perrin and Sureau (1987) were the first who used this method, which

is focused on the detection of rabies nucleoproteins and thus is not influenced by the method of sample fixation. RREID is a suitable supplementary method, which allows to detect inactivated viruses as well. Our experiments showed that with a clarified centifugated suspension of a virus-containing brain sample the threshold value of RREID was 10^{-2} however with a nonclarified suspension the threshold value reached 10^{-3}. The WHO methodic standard for RREID (Bourhy and Perrin, 1996) recommends the use of a clarified suspension. The results of our experiment aimed at simplification of the RREID procedure (ELISA, which consisted in omitting clarification of the samples by centrifugation, revealed this step to be inappropriate. In spite of the lower threshold level of RREID (in comparison to MIT) its great advantage is the possibility to examine a great number of samples within a very short time (3–4 hours) and to evaluate the results with the naked eye without using a spectrophotometer, which predetermines this method to be used in laboratories with a lower level of technical equipment.

Recently the suitability of RT-PCR as a molecular genetic method with high specificity and sensitivity has been discussed. Tordo et al. (1996) stated the RT-PCR to be a highly sensitive method, more sensitive than other ones that are used to diagnose rabies. However, they pointed that time, which is needed to perform this method (18 hours) is essentially longer than with FAT or RREID. Since the speed of obtaining results is the most important criterion in the diagnosis of rabies, Tordo et al. (1996) recommend the RT-PCR only as a supplementary method. According to the results of Heaton et al. (1997) and David et al. (2002) RT-PCR successfully detects rabies virus RNA also in older decomposed samples which gave negative results in FAT, MIT as well as RTCIT.

In agreement with literary data the results of our experiments confirmed the high sensitivity of RT-PCR. Nested RT-PCR proved to be a markedly more sensitive diagnostic procedure than RREID, MIT and RTCIT: its threshold value was dilution 10^{-4} whereas that of RREID only 10^{-2}. Our experience revealed that the use of differently prepared samples, either clarified (centrifugated) or nonclarified (noncentrifugated), had no infulence on the diagnostic effectiveness or on the threshold value detectable by nRT-PCR.

For the transcription of extracted RNA to cDNA in diagnostic PCR two different primers were used for one sample: a random primer and the N12 reverse primer. Due to possibility to use cDNA prepared by random hexamers with any primer pairs specific for different pathogens in PCR Vilcek et al. (1994) recommend to use this approach for the preparation of cDNA, especially for pestiviruses. However, our results revealed that in case of rabies virus synthesis of cDNA using the N12 reverse primer yielded greater amounts of the PCR product. We decided in further experiments to use for the preparation of cDNA the N12 primer.

In spite of the relatively high threshold of sensitivity we suppose RT-PCR to be used only as a method supplementary to FAT, RREID and RTCIT. It may also be used in controversial and exigent cases of *intra vitam* diagnosis of rabies (Crepin et al., 1998). If suitable primers are used, RT-PCR enables to detect a wide spectrum of different genotypes of lyssaviruses as well (Heaton et al., 1997).

According to WHO the RT-PCR method is not recommended for the routine dignosis of rabies and should only be carried out by molecular biology laboratories with the necessary facilities and expertise. The reason of the presently restricted possibilities of using PCR in the daily diagnostic routine can be found in the lacking standardization, high risk of cross-contamination and the extremely demanding character of the method. On the other hand, RT-PCR in combination with the restriction fragment length polymorphism method or sequencing of a part of viral genome is useful to obtain data for epizootiological, epidemiological a phylogenetical studies (Tordo et al., 1996; Amengual et al., 1997; Bourhy et al., 1999; Holmes et al., 2002).

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