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Use of filter paper (FTA®) technology for sampling, recovery and molecular characterisation of rabies viruses

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Abstract

This study evaluates the feasibility of the use of the FTA® Gene Guard System (a commercial product consisting of filter paper impregnated with patented chemicals supplied by the Whatman company) for the shipment, storage and detection of RNA rabies viruses by a simplified hemi-nested reverse transcriptase polymerase chain reaction. HnRT-PCR of the rabies virus nucleoprotein gene with specific primers showed that viral RNA extracted from crude infected tissues remained stable after fixation on the filter paper under diverse environmental conditions for at least 35 days. The sequence analysis of the products amplified from five out of the seven known genotypes of Lyssaviruses showed the stability of viral RNA viruses after fixation on the filter paper. Furthermore, the sensitivity of the hnRT-PCR following RNA fixation on the filter paper was equivalent to that of standard hnRT-PCR. In conclusion, the stability of viral RNA and the inactivation of infectivity make the FTA® technology useful for the storage, transport, collection and subsequent molecular analysis of viral rabies RNA, facilitating epidemiological investigations in the field.

Keywords: RNA amplification; Rabies virus; RT-PCR; FTA® technology; Epidemiology

1. Introduction

Rabies is a fatal viral zoonosis caused by Lyssaviruses of the Rhabdoviridae family within the mononegavirales order. The genus Lyssavirus includes seven genotypes (Bourhy et al., 1993; Gould et al., 1998): genotype 1 comprises the classical rabies virus which is the archetype of the Lyssavirus genus responsible for most human rabies cases, found in almost every country throughout the world and associated with bats only in the Americas. Rabies, caused by the classical rabies virus, has been maintained for centuries in a mammal reservoir, mainly carnivores (the dog is the main vector and reservoir of rabies) and bats (Rupprecht et al., 2002). Genotypes 2–7 include the rabies-related viruses, as follows: Lagos Bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European Bat Lyssavirus 1 (genotype 5), European Bat Lyssavirus 2 (genotype 6) and Australian Bat Lyssavirus (genotype 7). All seven genotypes, except the Mokola virus, have been isolated from bats. Recently, four additional rabies-related viruses, suggested as new Lyssavirus members, have been isolated from bats in Eurasia: Irkut virus, Aravan virus, Khujand virus and West Caucasian Bat virus (Botvinkin et al., 2003; Kuzmin et al., 2003).

Rabies diagnosis is based currently on three standardised techniques recommended by the World Health Organisation: the fluorescent antibody test, the rabies tissue-culture inoculation test and the mouse inoculation test (Dean et al., 1996; Koprowski, 1996; Webster and Casey, 1996).

For about 10 years, developments in molecular biology have improved knowledge of rabies. Techniques, such as the polymerase chain reaction (PCR), involving the analysis of viral RNA, have become ubiquitous in many rabies research laboratories (Heaton et al., 1997; Kamolvarin et al., 1993; Nadin-Davis, 1998; Picard-Meyer et al., 2004; Sacramento et al., 1991). PCR is an important tool for investigating rabies epidemiology and phylogeny and is used currently to carry out rapid rabies diagnoses, such as the Taq man Real Time PCR (Wakeley et al., 2005) allowing simultaneous Lyssavirus identification when using adequate primers.

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The FTA® is a paper-based system designed to fix and store nucleic acids directly from fresh tissues pressed into the pre-treated paper, allowing the collection and the archiving of nucleic acids. The filter paper matrix of the FTA® Gene Guard system is impregnated with a chaotropic agent that denatures infectious micro-organisms. Proprietary chemicals impregnated into the paper lyse cellular material and fix and preserve DNA and RNA within the fibre matrix (Whatman, 2006a), hence samples are no longer considered infectious. This allows the storage and transport of samples without specific biohazard precautions. The main advantage of the FTA® Gene Guard system is the long-term stability of nucleic acids (>5 years for DNA stored on The filter paper; Whatman, 2006b) at room temperature and the subsequent easy storage of fixed specimens for further molecular testing. Nucleic acids are recovered by punching the impregnated area and eluting with classical reagents. Advantages of FTA technology have been demonstrated for human DNA processing (Dobbs et al., 2002; Whatman, 2006b), for wildlife DNA samples (Smith and Burgoyne, 2004), and recently for the molecular characterisation of DNA and RNA viral pathogens from plant tissues (Ndunguru et al., 2005), but have not been well documented for the use of samples infected with Lyssaviruses.

In this study, the use of the FTA® Gene Guard system was investigated as a means of storing rabies virus suspensions. By using polymerase chain reaction (PCR) primers that are specific to rabies virus nucleoprotein gene and previously designed to amplify the seven Lyssavirus genotypes, we determined the stability of RNA from five genotype isolates (genotypes 1, 2, 5, 6 and 7) serially diluted and stored between 35 and 48 days on FTA® paper. The study was extended by comparing the PCR product sequences obtained by hemi-nested RT-PCR extracted from undiluted isolates fixed on the filter paper with those of the identical non-fixed undiluted isolates. In addition, cell inoculation tests were undertaken to assess the inactivation of the rabies virus in the FTA Guard System by testing elutes from the filter paper on neuroblastoma cells.

2. Materials and methods

2.1. Materials

Filter paper (FTA® card) was obtained from VWR International (Strasbourg, France). Kits for the viral RNA extraction from the cells fixed on the filter paper (QIAamp spin columns) and for the One Step RT-PCR were obtained from Qiagen (Courtaboeuf, France). Platinium Taq DNA polymerase, 10 × PCR buffer, dNTP and agarose were obtained from Invitrogen (Cergy Pontoise, France). PCR primers previously described by Heaton et al. (1999) to amplify the nucleoprotein gene of the seven genotypes of rabies virus and rabies-related viruses were obtained from Oligo Biotechnologies (Cologne, Germany). The primer sequences for amplifying the rabies virus nucleoprotein gene from nucleotide positions 55–661 as compared with the Pasteur virus strain (Tordo et al., 1986) were JW12 (forward) 5′-ATGTAACACCYCTACAATG, JW6 (reverse) 5′-CARTTVGCRCACATYTTRTG and JW10 (reverse, cocktail of three primers) 5′-GTC ATC AAA GTG TGR TGC TC, 5′-GTC ATC AAT GTG TGR TGT TC, 5′-GTC ATT AGA GTA TGG TGT TC.

Primers from the Ambion Quantum RNA 18S Internal Standards kit (Ambion, UK) were used for amplification of internal control 18S rRNA molecules, corresponding to the amplification of a mouse keeping gene.

2.2. Cells, viruses and propagation of viruses

2.2.1. Cell suspensions

Baby Hamster Kidney cells BHK-21 C13 (ATCC: CCL10) were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, France) supplemented with 10% (v/v) foetal calf serum (Invitrogen, France). Murine neuroblastoma cells (ATCC: CCL131) were grown in DMEM medium supplemented with 5% foetal calf serum.

2.2.2. Viruses

Three genotype 1 rabies virus strains were used: a batch of challenge virus standard CVS-11 (ATCC VR 959), Ariana (Servat et al., 2003) and GS9 (salivary glands isolates from field rabies virus) strains, respectively, of dog and fox origin (Aubert et al., 1991). Four isolates of rabies-related viruses corresponding, respectively, to the genotype 2 (Lagos Bat virus: LBV) (Aubert et al., 2002), genotype 5 (European Bat Lyssavirus type 1: EBLV-1), genotype 6 (European Bat Lyssavirus type 2: EBLV-2) (Brookes et al., 2005) and genotype 7 (Australian Bat Lyssavirus: ABLV) (Fraser et al., 1996) were also included in this study. The characteristics and origins of isolates tested are summarised in Table 1.

2.2.3. Virus propagation

Rabies virus isolates (GS9 and Ariana strains) were propagated serially, respectively, on foxes (Aubert et al., 1991) and dogs (Servat et al., 2003), while CVS-11 was produced in BHK-21 monolayers. Rabies-related viruses isolates were propagated on mice or on BHK-21 cells (Table 1). Suspensions of isolates propagated in animals were clarified by centrifugation at 1500 × *g* for 30 min at 4 °C.

2.3. Preparation of rabies virus dilutions and cells stored on the filter paper

Six 1:10 serial dilutions (10^{-1} to 10^{-6}) of rabies virus isolates corresponding to Lyssavirus genotypes 1, 2, 5, 6 and 7 were prepared in DMEM medium.

A volume of 25 μl for each dilution (pure batch and 10^{-1} to 10^{-6}) was placed onto the centre of the card sampling area. The filter paper was then dried at a temperature of 25 °C for 2 h, and stored in a paper envelope with a packet of dessicant (Silica gel, Sigma, France) at room temperature until use.

2.4. Elution of RNA from FTA® cards

One punch of 6 mm diameter was removed from each region stained with pure and diluted rabies virus suspensions by using a

Table 1
Characteristics of tested rabies and rabies-related viruses used in this study

Genotype	Lyssavirus (genotype)	Identification number	Host species	Country of origin	Biological origin
Genotype 1 (rabies virus)	CVS-11	CVS-11			BHK-21 passaged
	Genotype 1	GS9	*Vulpes vulpes*	France	P4 fox salivary glands
	Genotype 1	Ariana	Dog	Tunisia	P2 dog brain
Genotype 2 (rabies-related virus)	Lagos bat virus (LBV)	119645	*Rousettus aegyptiacus*	Egypt	P2 mouse brain
Genotype 5 (rabies-related virus)	European Bat Lyssavirus type 1b (EBLV-1b)	123008	*Eptesicus serotinus*	France	P4 mouse brain
Genotype 6 (rabies-related virus)	European Bat Lyssavirus type 2 (EBLV-2)	ND	*Myotis daubentoni*	UK	BKH-21 passaged
Genotype 7 (rabies-related virus)	Australian Bat Lyssavirus (ABLV)	ND	*Pteropus alecto*	Australia	P5 mouse brain

Abbreviations used: P: strain passaged serially from the initial field strain origin; ND: not done.

Harris 6.0 mm punch and placed into a 1.5 ml Eppendorf tube. A volume of 300 μl of DMEM was added to each Eppendorf tube containing the paper disk and incubated overnight at 4 °C. After the incubation, the disk was removed and viral RNA was purified from 150 μl of eluate. The cell inoculation test was carried out by adding 50 μl of eluate per well containing the neuroblastoma cells.

2.5. Cell inoculation test: control of the inactivation of rabies virus isolates trapped on FTA® cards

The test was performed as described by Webster and Casey (1996), in eight-wells Labtek Tissue Cultures chamber slides (VWR, France) with minor modifications. Briefly, 50 μl of rabies virus eluate from the filter paper was added to one single well containing 4×10^5 neuroblastoma cells. After 48 h in an incubator at 35 °C with 5% CO_2, all supernatant medium was collected from each tested sample and stored at 4 °C until use. The chamber-slides were air dried, fixed in cold acetone, stained with fluorescein isothiocyanate-labeled rabbit anti-rabies nucleocapsid immunoglobulin (BioRad, France) and read under a fluorescent microscope by two trained personnel. In order to confirm the results of the first passage in neuroblastoma cells, 50 μl of the supernatant from the first passage were added to a second well containing the neuroblastoma cells and processed as described above.

2.6. Virus titration

The rabies virus titrations were performed in disposable 96-well microtitre plates just before their storage on the filter paper. A volume of 200 μl of a BHK-21 10^5 cells/ml suspension in DMEM containing 10% of foetal calf serum was added to each well (i.e. 20,000 cells). After a 1 day incubation at 35 °C in a humid chamber containing 5% CO_2, the supernatant was removed from each well and immediately 50 μl of both the undiluted and the serial 10-fold dilutions of non-fixed Lyssavirus isolates (10^0 to 10^{-11}) were added to the wells. After 60 min at 35 °C, 200 μl of fresh growth medium were added to each well. Six replicates were carried out per dilution of virus. Following the incubation of the plates under the same conditions for 72 h, the supernatant medium was removed, cells were washed twice with PBS buffer, fixed for 30 min in cold 80% (v/v) acetone at 4 °C and air dried. Cells were then stained with fluorescein isothiocyanate anti-rabies nucleocapsid conjugate (Biorad, France) for 30 min at 35 °C. The plates were rinsed, then read under a fluorescent microscope by two trained readers. Virus titres were determined according to the graphical method by using Neoprobit paper (Aubert, 1996).

2.7. RNA extraction and RT-PCR

Viral RNA was extracted from 150 μl of rabies virus eluate with Qiagen Viral RNA mini Kit according to the manufacturer's instructions. A control extraction was performed from 25 μl of non-fixed pure isolate according to the technique previously described (Picard-Meyer et al., 2004). The one step RT-PCR amplification was undertaken on 5 μl of extracted RNA (i.e. 250–500 ng) in a final volume of 15 μl containing 1× Qiagen One step RT-PCR buffer, 1 mM each dNTP, 0.6 μl of one step RT-PCR enzyme (0.04%, v/v), 10 pmol each of JW12 and JW6 primers. The cycling conditions were as follows: one cycle of RT at 50 °C for 30 min, a denaturation of 15 min at 95 °C, followed by 35 amplification cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. The amplification was completed by an ultimate elongation of 10 min at 72 °C.

The second amplification was carried out using 1 μl of undiluted one step RT-PCR products in a final volume of 20 μl containing 5 pmol of each JW12 and JW10 primers. This second-round PCR was achieved within 25 cycles.

PCR products were run in a 2% agarose gel electrophoresis in TBE 1× buffer and stained with ethidium bromide (at 1 μg/μl). Gels were then photographed under UV light.

A positive control was performed on each RT-PCR run with 250 ng of viral RNA extracted from a CVS-11 virus and stored at −80 °C until use.

2.8. Evaluation of the sensitivity of the one step RT-PCR

Five 10-fold serial dilutions had been prepared from a CVS-11 batch titrating $10^{4.8}$ $TCID_{50}$ per ml. A volume of 25 μl of pure batch and of each dilution (10^{-1} to 10^{-6}) were deposited onto the centre of FTA® paper. After 2 h at room temperature, the filter paper was punched and the elution was carried out in 150 μl of DMEM. During the same 2 h period, the dilutions were stored in melting ice. Extraction was conducted simultaneously from the 150 μl of elutes and from 25 μl of crude corresponding dilutions.

RT-PCR was then carried out as described above.

2.9. Cloning, sequencing of viral elutes from FTA® and nucleotide sequence analysis

The isolated bands of 589-bp PCR products, obtained with JW12 and JW10 primers from all rabies virus isolates fixed on FTA®, were excised from the gel, purified using the QIA Quick PCR purification kit and cloned into the pDrive Vector (Qiagen PCR cloning, Qiagen, France) according to manufacturer's instructions. The sequencing of cloned products was performed with sense and antisense primers T7 and SP6 by Genome Express (Meylan, France). The sequence comparison was performed, using Vector NTI software (Invitrogen, France) by comparing the amplified products from FTA® with the corresponding pure non-fixed isolates. The sequence comparison was based on the first 580 nucleotides region of the nucleoprotein gene.

3. Controls

Negative and positive controls (CVS-11) were used in each run to assess the reliability of virus titration on BHK-21 cells. The negative control consisted of DMEM medium used for the preparation of all samples.

The usual precautions for PCR were followed strictly in the laboratory (Kwok and Higuchi, 1989) to avoid false-positive results.

4. Results

4.1. Comparison of the sensitivity of RT-PCR with CVS-11 non-fixed or fixed on FTA® paper

The detection limit of the RT-PCR method, using universal primers (JW12-JW6 followed by JW12-JW10) of serial dilutions of CVS-11 and the same dilutions of CVS-11 fixed on the filter paper, was 0.0016 $TCID_{50}$ (corresponding to dilution 10^{-3}), irrespective of fixation or not (Table 2).

4.2. Assessment of virus inactivation on FTA® cards

Fifty microliters of all elutes of the 10-fold serial dilution of CVS-11 deposited onto the filter paper used in part 3.1 above had also been tested by the cell culture inoculation test on neuroblastoma cells. All of them were negative while the cell culture test carried out on the crude dilutions were positive up to the 10^{-4} dilution of the batch, i.e. a theoretical dose of 0.3 $TCID_{50}$ (Table 2).

4.3. Stability and recovering of rabies virus and rabies-related viruses isolates stored on the filter paper

Viral RNA eluted from all rabies virus and rabies-related viruses isolates fixed onto FTA® cards yielded the expected bands of 589-bp in all the seven tested isolates with primers JW12 and JW10, designed to amplify selectively the rabies virus nucleoprotein gene. Representative agarose gels of the amplicons produced by RT-PCR of the seven, tested, fixed isolates, using, respectively, rabies primers JW12 and JW6 followed by JW12 and JW10, are shown in Fig. 1A. Fig. 1B shows the amplification of internal standard 18 S rRNA (324-bp) from the seven, tested, fixed isolates kept for 2 h at room temperature (i.e. 25 °C). Positive results with the rabies amplification products of 589 bp and the amplification of the internal standard corresponding to the mouse keeping gene (324-bp) were observed for the seven

Table 2
Sensitivity of RT-PCR for detecting 25 μl of viral RNA extracted from pure and 10-fold serially diluted CVS-11 virus

Dilution	RT-PCR detection			Cell culture inoculation test		
	Virus dose ($TCID_{50}$)	Non-fixed CVS-11 RT-PCR	CVS-11 from FTA® paper RT-PCR	Virus dose ($TCID_{50}$)	Non-fixed CVS-11 RTCIT	CVS-11 from FTA® paper RTCIT
10^{0}	1.6	+	+	3150	+	−
10^{-1}	0.16	+	+	315	+	−
10^{-2}	0.016	+	+	31.5	+	−
10^{-3}	0.0016	+	+	3.15	+	−
10^{-4}	0.00016	−	−	0.3	+	−
10^{-5}	0.000016	−	−	0.03	−	−
Negative control		−	−		−	−
Positive control		+	+		+	+

Verification of the inactivation of the virus after a 2 h contact with FTA® paper. The viral titre of pure isolate was $10^{4.8}$ TCID50/ml, this virus acting as a positive control throughout the experiments. The positive control (CVS-11) was included in each test run. Abbreviations used: +: positive, −: negative, RTCIT: cell culture inoculation test. Diluted samples, performed with frozen isolate were kept on ice until analysis, while the same dilutions of CVS-11 were fixed on FTA® paper and kept for 2 h at room temperature (i.e. 25 °C). The samples corresponding to the five 1:10 serial dilutions of CVS-11 deposited onto FTA paper were eluted in 150 μl of DMEM. Volumes of 50 and 25 μl of these elutes were, respectively, inoculated on neuroblastoma cells and concomitantly used for determining the sensitivity of the RT-PCR.

Table 3
Detection of infectious particles and viral RNA by the cell culture inoculation test and RT-PCR from seven isolates of rabies virus and rabies-related viruses after fixation on FTA® paper

Lyssavirus genotype	Virus titer $TCID_{50}$ (ml)	Detection methods	Storage before RNA extraction (days)	Results of cell culture inoculation test and RT-PCR detections				
				Pure and diluted samples fixed on FTA paper				Pure non-fixed samples
				10^0	10^{-1}	10^{-2}	10^{-3}	10^0
Laboratory fixed strains (positive control)								
CVS-11	$10^{4.8}$	Detected (log $TCID_{50}$)	42	3.2	2.2	1.2	0.2	
		RT-PCR (Rabies)		+	+	−	−	+
		RT-PCR (18 s rRNA)		+	−	−	−	+
		RTCIT		−	−	−	−	+
Genotype 1								
GS9	$10^{5.4}$	Detected (log $TCID_{50}$)	44	3.8	2.8	1.8	0.8	
		RT-PCR (Rabies)		+	+	+	+	+
		RT-PCR (18s rRNA)		+	+	+	+	+
		RTCIT		−	−	−	−	+
Ariana	$10^{6.4}$	Detected (log $TCID_{50}$)	42	4.8	3.8	2.8	1.8	
		RT-PCR (Rabies)		+	+	+	-	+
		RT-PCR (18s rRNA)		+	+	+	+	+
		RTCIT		−	−	−	−	+
Genotype 2								
LBV (119645)	10^3	Detected (log $TCID_{50}$)	48	1.4	0.4	/	/	
		RT-PCR (Rabies)		+	−	−	NP	+
		RT-PCR (18s rRNA)		+	+	−	NP	+
		RTCIT		−	−	−	−	+
Genotype 5								
EBLV-1 (123008)	$10^{5.3}$	Detected (log $TCID_{50}$)	35	3.7	2.7	1.7	0.7	
		RT-PCR (Rabies)		+	+	+	−	+
		RT-PCR (18s rRNA)		+	+	−	−	+
		RTCIT		−	−	−	−	+
Genotype 6								
EBLV-2	$10^{4.8}$	Detected (log $TCID_{50}$)	48	3.2	2.2	1.2	0.2	
		RT-PCR (Rabies)		+	+	−	NP	+
		RT-PCR (18s rRNA)		+	+	+	NP	+
		RTCIT		−	−	−	−	+
Genotype 7								
ABLV	$10^{3.8}$	Detected (log $TCID_{50}$)	48	2.2	1.2	0.2	/	
		RT-PCR (Rabies)		+	+	-	NP	+
		RT-PCR (18s rRNA)		+	+	+	NP	+
		RTCIT		−	−	−	−	+

Comparison with results from the cell culture inoculation test and RT-PCR techniques on crude virus suspensions kept at −80 °C for the same period of storage on FTA® paper. The results of the cell culture inoculation tests were obtained after the first passage of cells supernatant on neuroblastoma cells. Abbreviations used: pos: positive, neg: negative, RTCIT: cell culture inoculation test. The detected amount dose (noted log $TCID_{50}$) corresponds to the theoretical quantity of virus contained in 25 μl of the different elutes from FTA® paper. Twenty-five microliters of seven Lyssavirus isolates corresponding to the genotypes 1, 2, 5, 6 and 7 were deposited onto the centre of FTA cards. One punch of 6 mm diameter was taken manually from the centre of the sample section and was used for the RT-PCR; the diameter of the puncher is generally lower than the diameter of the sample section.

isolates stored on the FTA® cards at room temperature for an average of 43 days (range, 35–48 days) before RNA extraction (Table 3). Negative results were observed for all negative controls (negative control of RNA extraction, RT and PCR control negative reaction).

4.4. Comparison of the sensitivity of RT-PCR between RNA extracted from frozen isolates versus fixed isolates

The performance of the RT-PCR method was compared between viral RNA, extracted from pure isolates fixed on the FTA® card, and all corresponding pure non-fixed viruses. Overall, the techniques performed with RNA extracted from pure isolate fixed on the filter paper or non-fixed and non-diluted material gave comparable results for each tested isolate. Whatever the isolate, RT-PCR conducted from the filter paper was positive for theoretical amounts of virus ranging between $10^{0.8}$ and $10^{2.8}$ $TCID_{50}$ (Table 3). The detected-amount dose expressed in log $TCID_{50}$ corresponded theoretically to 25 μl of different isolates placed onto the filter paper. The diameter of the puncher, which is generally smaller than the diameter of the stained area of the paper suggested that the quan-

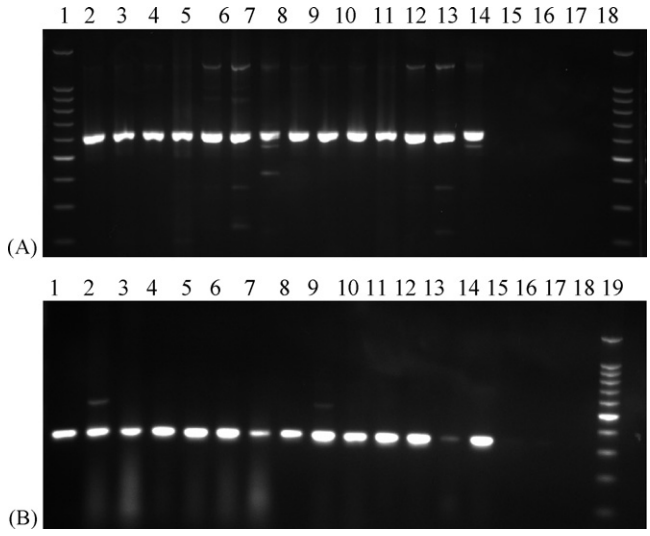

Fig. 1. Comparison of hemi-nested RT-PCR results obtained with RNA extracted from seven pure isolates fixed on FTA® paper for 2 h at room temperature versus RNA extracted from the same seven crude isolates. (A) RT-PCR amplification of rabies virus nucleoprotein gene with rabies specific primers. Lanes 1 and 20: 100 pB DNA ladder; lanes 2–8: amplification of cDNA from pure non-fixed isolates; lanes 9–15: amplification of cDNA from pure isolates fixed on FTA paper; lanes 2 and 9: CVS-11; lanes 3 and 10: Ariana virus; lanes 4 and 11: GS9 isolate; lanes 5 and 12: genotype 2 isolate; lanes 6 and 13: genotype 5 isolate; lanes 7 and 14: genotype 6 isolate; lanes 8 and 15: genotype 7 isolate. Lanes 16 and 17: total RNA extraction negative control, lane 18: RT negative control, lane 19: PCR negative control. (B) RT-PCR amplification of internal control 18S rRNA. Lanes 1–7: amplification of cDNA from pure non-fixed isolates; lanes 8–14: amplification of cDNA from pure isolates fixed on FTA® paper. Lane 19: 100 pB DNA ladder (Promega, France).

tity of virus detected should be higher than the observed results.

4.5. Sequence comparison between fixed and frozen isolates

No significant nucleotide sequence variation was observed between the FTA® and frozen sequences. The comparison of the 584-nt long sequences of the seven isolates showed more than 99% identity. The comparison of the first 580-nt sequences of EBLV-1 gave 99.8% similarity with a single T → C modification in nucleotide position 612 (Fig. 2). The comparison between fixed and frozen isolates for four additional isolates showed 99.5% and 99.8% similarities for LBV and EBLV-2 viruses, for Ariana and GS9 isolates, respectively.

5. Discussion

In this study, it was demonstrated the interest of using the FTA® paper to preserve rabies virus RNA for five out of the seven genotypes.

The hnRT-PCR technique amplifying the 589-bp fragment of the nucleoprotein gene showed no significant differences in the 589-bp amplicons produced from RNA for seven undiluted isolates stored on the FTA® card or kept deep frozen as reference.

The efficacy of the hnRT-PCR to amplify CVS-11 fixed on the filter paper has been shown to be equivalent to that used to amplify the corresponding deep-frozen CVS-11.

Additionally, we have demonstrated that no significant nucleotide sequence variation was observed when the 589-bp fragment of the nucleoprotein gene amplified from fixed sample was compared to the corresponding original deep-frozen sample. This result indicates that viral RNA extracted from infective samples fixed on the card retains fidelity to its nucleotide sequences throughout the sampling, storage and recovery processes. Negative results of the cell culture inoculation test performed on 50 μl of elutes from the paper confirm the inactivation of the five tested genotypes, placed onto the paper and treated after drying for 2 h at room temperature.

The study described in this paper demonstrates that the FTA® technology is a simple and sensitive tool, especially appropriate for molecular studies performed as a complement to referenced rabies diagnosis. When confirmation and further viral characterisation are necessary, the viral RNA can be easily isolated from infected samples fixed on the paper allowing additional molecular studies. However, this technology limits the possibility of further studies of isolated virus, such as its virulence and susceptibility in different species.

National and international regulations on biological transportation currently impose precautionary measures on shipments of specimens for rabies diagnosis or typing in order to avoid exposure to biohazards. The chemical reagents impregnated in the paper are designed to inactivate most pathogens and nucleases during drying. In this study, we demonstrate the ability of the paper to inactivate the infectivity of rabies virus when stored on the paper for 2 h at room temperature. The result of this rabies virus inactivation is that the sample deposited on the filter paper is no longer infectious and subsequently does not induce any biohazard. This method is therefore ideal for shipping specimens to a reference laboratory for molecular study and for further characterisation of rabies strains.

It was demonstrated previously that glycerol preservation protected the rabies virus RNA (genotype 1 isolates) for at least 60 days at room temperature (Aguilar-Setién et al., 2003) allowing the use of molecular tests in addition to the recommended rabies diagnosis techniques. In this study, we have shown that the rabies RNA of seven tested isolates from five genotypes were stable for at least 43 days at room temperature. The stability of DNA on the filter paper has been well studied and documented previously (Dobbs et al., 2002; Smith and Burgoyne, 2004). DNA has been reported by the manufacturer to be stable on the cards for at least 5 years. Unfortunately, only few studies have been performed on the stability of RNA trapped on the technology (Moscoso et al., 2005; Natarajan et al., 2000; Ndunguru et al., 2005). The stability of RNA on the card was previously established in 2003 when Wacharapluesadee et al. (2003) showed that specific RT-PCR followed by a second PCR giving a 260-bp amplicon with specific rabies primers (LISEBL1F and R or LISEBL2F or R) could be obtained for positive samples (all infected with genotype 1 Lyssavirus) stored for 222 days at room temperature on the filter paper. The stability of DNA or RNA is the result of the technology; nucleic acids are instantly captured and stabilised while pathogens and nucleases are inactivated.

The archival storage of samples for future molecular analysis could be highly facilitated by using the technology because

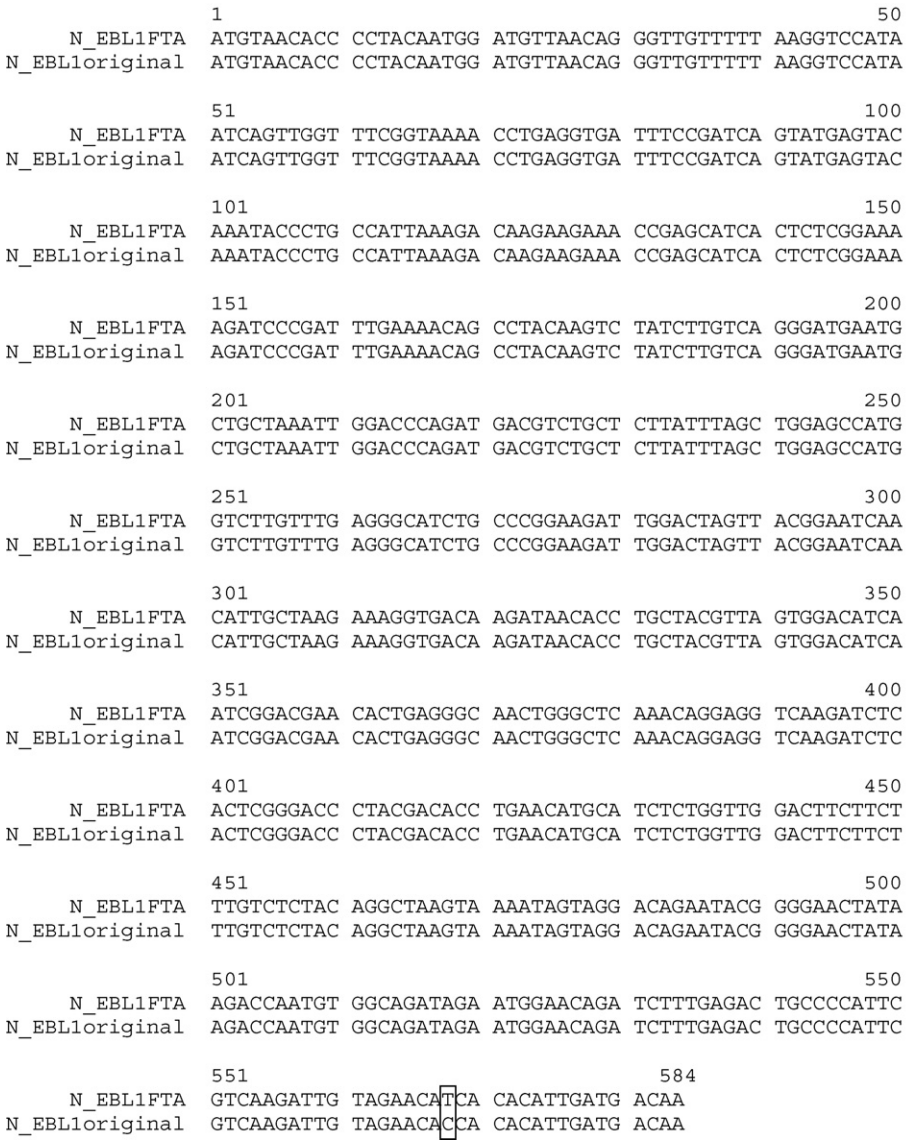

Fig. 2. Schematic alignment of European Bat Lyssavirus type 1 (EBLV-1) sequenced PCR products obtained from original isolate (non-fixed) and isolate fixed on FTA® paper. The comparison of the 580-nt sequences gives 99.8% similarity between the two sequences. Alignment was performed with Vector NTI software (Invitrogen, France).

the cards are small and can be stored for short-term storage at room temperature or at 4 °C for longer term storage. This stability alleviates the need for the long-term storage of all infected samples in a deep-freezer or liquid nitrogen, which is expensive. When specimens are stored at −70 °C, mechanical electrical failure of the deep-freezer is an important risk. Once a frozen sample thaws, severe degradation of DNA and RNA may occur and may result in the inactivation of infectious particles, unless the samples are immediately transferred to another deep-freezer.

In the same way, the transport of fresh or frozen samples from the point of collection involves leakage risks and the possibility of sample degradation due to temperature variation. Extracted RNA or stored tissues may also degrade in the laboratory, resulting in possible problems for re-testing of infected samples.

According to WHO and World Organisation for Animal Health (OIE) recommendations, the use of molecular techniques, such as PCR are not currently recommended for routine diagnoses of rabies and should only be used in laboratories under strict quality control procedures that have experience and expertise with these techniques (WHO, 2005; World Organisation For Animal Health, 2004). Molecular techniques are useful for epidemiological studies in combination with conventional techniques, such as the Fluorescent Antibody Test, the "gold standard" in laboratory diagnosis of rabies, and the cell culture inoculation test for further characterisation of the isolate. To date, many rabies virus isolates from domestic and wild animals (including bats) as well as humans have been studied and compared with monoclonal antibodies or molecular techniques (Amengual et al., 1997; Badrane and Tordo, 2001; Holmes et al., 2002; Kuzmin et al., 2004; Mansfield et al., 2006; Nadin-Davis et al., 2001). These studies permitted a significant improvement in the understanding of epidemiology of rabies, allowing the establishment of geographical links between virus isolates and

showing differences that can be used to identify the principal reservoir. Thus, for example, the artic fox has been identified as the principal rabies host in Artic regions while the rabies virus was apparently associated with foxes (red fox and steppe fox) in Russia and raccoon dogs in the Far East of Russia (Kuzmin et al., 2004).

The filter paper demonstrated previously by Smith and Burgoyne (2004) to be eminently suitable for the collection of biological samples, including blood and saliva, from a wide range of wildlife species, could be easily used in the future for epidemiological surveys of bats and others mammals, by collecting micro-samples from living animals. Little data are known about the "real" geographical distribution of European Bat Lyssaviruses in Europe, because the bat rabies, passive surveillance remains limited to a few European countries: The Netherlands, Denmark, Germany, Poland, UK and France. Between 1977 and 2003, 729 European bats were diagnosed as rabid in Europe, 95% of them being identified as *Eptesicus serotinus*, all infected with the two isoforms a and b of European Bat Lyssavirus type 1. The other 5% of cases which occurred in the pond bats *Myotis dasycneme* and *Myotis daubentonii* were reported infected with infectious European Bat Lyssavirus type 2. Since 2000, other species of bats including *Myotis myotis*, *Myotis naterreri*, *Miniopterus schreibersii* and *Rhinolophus ferrumequinum* were reported infected in Spain with only viral RNA of the European Bat Lyssavirus type 1 virus (Serra-Cobo et al., 2002), suggesting that the sensitivity of the bat species to European Bat Lyssaviruses infection seems to differ among the different European bats.

Active surveillance of bat populations should be initiated to complement passive surveillance in close collaboration between scientists and bat biologists in order to investigate the transmission of European Bat Lyssaviruses within and outside identified bat colonies. Some potential transmission routes have been suggested by several rabies scientists since the 1950's, like the aerosol transmission, or the transmission via the placenta, urine or saliva (Messenger et al., 2003). More recently, Johnson et al. (2006) reported that bat lyssaviruses, particularly the Lyssavirus genotypes 1 and 6, might be transmitted via the airborne route in a dose dependent manner. To confirm any of these hypotheses, it will be necessary to improve bat surveillance, by collecting micro-samples (saliva, blood and urine) taken from live bats captured and released at roosting sites which can be collected easily in the field, using the FTA technology, and then analysed by molecular techniques.

It has been requested by WHO (WHO Expert Consultation on Rabies, 2005) that a constant molecular epidemiological surveillance should be maintained to trace spill-over transmissions from reservoir to non-reservoir animals and humans. It is also a way to detect the emergence of rabies virus variants in new species and geographical areas.

6. Conclusion

This study demonstrates that the FTA methodology is useful for easy sampling and rapid, safe and cheap shipment of samples for typing in national reference laboratories by molecular analysis. This technology might make easier the detection of new outbreaks or the epidemiological analysis of local human cases or translocated human and animal cases.

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