Molecular tools for rabies diagnosis in animals

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Summary

Advances in the study of molecular biology have provided cost-effective technological initiatives that have supported the development of sensitive and specific techniques for rabies diagnosis. In particular, using tests based on the polymerase chain reaction (PCR), it is possible to analyse large numbers of samples in a single day. Developments in reagents and procedures have allowed improvement in the standardisation of techniques, leading to high test sensitivity and specificity. For these reasons, a validated PCR-based test should be considered by the World Organisation for

Animal Health (OIE) as an alternative test for rabies, and included in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Keywords

Diagnostic – Polymerase chain reaction – Rabies – Real-time – TaqMan – Virus.

Introduction

Rabies is caused by all members of the genus *Lyssavirus* (Family *Rhabdoviridae*). Rabies virus (RABV) is the predominant global species, with various host reservoirs, dogs being the most important.

Preliminary clinical diagnosis of rabies in animals is undertaken by veterinarians, based on the signs, available history and epidemiological information associated with each suspect case. However, a clinical diagnosis for rabies in animals is not reliable as there are no clinical signs that are pathognomonic for rabies. In addition, there are no characteristic gross pathological lesions associated with rabies. In most animals, microscopic non-specific lesions with ganglioneuritis may be observed in the nerve centres, as well as histiolymphocytic cuffs and gliosis, but these are suggestive of viral encephalomyelitis and are not exclusive to rabies. The most prominent lesions are usually observed in the cervical spinal cord, pons and hypothalamus. The only specific microscopic changes are intracytoplasmic eosinophilic inclusions (Negri bodies) corresponding to the aggregation of developing rabies virus particles, but their absence does not preclude *Lyssavirus* infection. These pleomorphic neuronal inclusions, measuring from 4 µm to 5 µm, are usually located in the hippocampus. Consequently, diagnosis can only be confirmed by laboratory tests, conducted *post mortem* on central nervous system tissue removed from the cranium. Diagnosis by demonstration of Negri bodies is no longer recommended as a primary test, due to the insensitivity of this method.

The current gold standard World Organisation for Animal Health (OIE) prescribed test is the direct fluorescent antibody test (FAT). For this test, the medulla oblongata, cerebellum and hippocampus are the recommended tissues of choice (9).

Currently recommended confirmatory tests are based on virus isolation, using either the rabies tissue culture infection test (RTCIT) or the mouse inoculation test (MIT). While the FAT can be completed in less than two hours, both the RTCIT and MIT require longer turnaround times (4 days and 28 days, respectively) (Table I). The MIT involves the intracerebral inoculation of mice with a clarified supernatant of a homogenate of brain tissue (medulla oblongata, cerebellum, hippocampus, cortex). Clinical signs of rabies (indicating a positive result) can be observed as early as six to eight days for RABV, and a positive FAT on the brains of the mice confirms the diagnosis. Mice should be observed for a period of at least 28 days for the development of clinical signs. This *in vivo* test is time-consuming, expensive and involves the use of many animals. *In vitro* virus-isolation tests, such as the RTCIT, involve the inoculation of the sample into a susceptible cell line, such as a neuroblastoma cell line. The same fluorescein isothiocyanate conjugate used in the FAT can then be used to confirm the presence of *Lyssavirus* antigens in the infected cell monolayers. The use of virus isolation (RTCIT/MIT) may be recommended from a sample where there is suspicion of infection with RABV, especially to confirm inconclusive FAT results and in cases of human exposure. One advantage of virus-isolation assays is the amplification of the virus isolate for subsequent typing and analysis, enabling the potential detection of viruses other than *lyssaviruses*. Both the RTCIT and MIT are prescribed by the OIE. However, the former is preferable because of the shorter turnaround time to achieve a result and because the test does not involve the use of animals.

Table I
Tests prescribed by the World Organisation for Animal Health

Test	Category/description	Minimum performance time	Turnaround time [a]
FAT (antigen detection)	Primary	2 h	1 day
RTCIT (virus isolation)	Confirmatory	4 days (interim positive results possible at 24, 48, 72 h)	4 days
MIT (virus isolation)	Confirmatory	Up to 28 days	Up to 28 days
RT-PCR (RNA detection)	Confirmatory	24 h first round RT-PCR 30 h hemi-nested PCR	2 days
Real-time RT-qPCR (RNA detection)	Confirmatory	8 h for detection	1 day

a) Turnaround times are the times by which a result would normally be available
FAT: Fluorescent antibody test
MIT: Mouse inoculation test
PCR: polymerase chain reaction
RTCIT: rabies tissue culture infection test
RT-qPCR: reverse-transcription quantitative polymerase chain reaction

The application of molecular biology has aided in the development of tests that result in a more rapid and sensitive diagnosis of infection with *lyssaviruses* (in particular, RABV). These molecular tests could be used for *ante mortem* diagnosis of humans and degraded or putrefied tissue, and enable genetic characterisation. As a result, rapid, differential, flexible molecular assays are becoming more widely accepted (3). For generic approaches intended to detect all *lyssaviruses,* cocktails of primers facilitate either hemi-nested or fully nested amplifications. Alternatively, strain-specific reverse-transcription polymerase chain reactions (RT-PCRs) have been developed to distinguish between various RABV variants in a particular geographical region. By employing fluorogenic probes, the detection of sequence-specific templates can be achieved in real time. In such assays, specificity is ensured by an inherent hybridisation reaction, and cross-contamination is avoided, due to the closed-tube nature of the test. Subsequently, for RABV and other lyssaviruses, various real-time quantitative PCR assays using TaqMan© technology have been reported.

The challenges for rabies diagnostic test developers, beyond a specific and sensitive test, are twofold: firstly, to achieve internationally accepted validation of a test that will lead to its acceptance by organisations globally; and secondly, by ensuring that tests are affordable and require minimal expertise and facilities as these tests are primarily needed mainly in developing regions where financial and logistical barriers may hinder their implementation (3). These barriers are not insurmountable and it is our expectation that, if such tests are accepted and implemented where they are most needed, they will provide substantial improvements for rabies diagnosis and surveillance of this neglected disease.

Materials and methods

Ribonucleic acid extraction

The target of rabies molecular testing is the detection of the viral RNA genome. Viral RNA is extracted from a specimen prior to molecular testing. The viral RNA is non-infectious and can be removed from high-containment laboratories for analysis. The choice of extraction technique is dependent upon the specimen (liquid or tissue) and volume of testing (manual extraction *versus* automated robotics). Tissue lysis and nucleic acid extraction may be chemical, using a TRIzol™ reagent (containing phenol and guanidine isothiocyanate) and chloroform, or enzymatic, with the addition of magnetic beads, filter

tubes, etc., for elution of nucleic acids. Some tissue may require pre-treatment with proteinase K and detergents such as sodium dodecyl sulphate to remove excess protein, whilst some specimens (with low cellular content) may benefit from the addition of carrier RNA prior to extraction (18).

Real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

For RABV and other lyssaviruses, various RT-PCR assays using a quantitative real-time platform have been described (Table II). These sensitive and specific assays are undertaken in a single step in a closed-tube system, thereby substantially decreasing the risk of contamination and potentially allowing for the characterisation of unknown isolates. Such real-time assays can be applied quantitatively and the use of an internal control (e.g. beta-actin RNA or 18S ribosomal RNA) enables the quality of the total RNA template to be assessed, thereby minimising false negatives associated with poor sample quality. All real-time RT-qPCR assays are based on a primer pair, which amplifies a small (usually <200 base pair) amplicon, which is detected in 'real time' either by a specific probe (TaqMan©) or a DNA-binding cyanine dye, such as SYBR Green, as the assay is in progress. It is preferable that the primer pair is designed to be pan-*Lyssavirus* in specificity, i.e. validated against a large panel of representative lyssaviruses covering all known *Lyssavirus* species, with probes specifically designed to discriminate between classical RABV and the other members of the *Lyssavirus* genus (18). However, the genetic diversity among lyssaviruses, even within conserved genomic regions, has precluded the use of a single probe-based assay to reliably detect all lyssaviruses.

Table II
Real-time quantitative reverse-transcription polymerase chain reaction assays

PCR	Primer/probe name	Role	Specificity	Sequence 5'-3'	Position	Reference
SYBR Green	JW12 primer	F	Pan-*Lyssavirus*	ATGTAACACCYCTACAATG	55-73	5
	N165 -146	R		GCAGGGTAYTTRTACTCATA	165-146	
TaqMan	23	F	RABV	GATCCTGATGAYGTATGTTCCTA	266-288	6
	19	R		RGATTCCGTAGCTRGTCCA	353-335	
	RabGT1-B-FAM	P		CAGCAATGCAGTTYTTTGAGGGGAC	297-321	
	LacZ-Cy5	P		AGT CGG GAA ACC TGT CGT GCC A	56-80	
TaqMan	550B	F	RABV, LBV	CACMGSNAAYTAYAARACNAA	541-561	1
	541lys	R	MOKV & DUVV	GTRCTCCARTTAGCRCACAT	647-666	
	620lyssa	P		CATCACACCTTGATGACAACTCACAA	620-645	
TaqMan	RABVD1 For	F	RABV	ATGTAACACCYCTACAATG	55-73	12
	RABVD1 Rev	R		GCMGGRTAYTTRTAYTCATA	165-146	
	RABVD1 Probe	P		CCGAYAAGATTGTATTYAARGTCAAKAATCAGGT	78-111	
	RABVD2 For	F		TRATGACAACYCACAARATGT	630-650	
	RABVD2 Rev	R		TGARCAGTCYTCRTARGC	764-781	
	RABVD2 probe	P		TAYGACATGTTTTTCTCYCGGATTGARCATC	698-728	
	RABVD3 For	F		AYTTCTTCCAYAARAACTTYGA	846-867	
	RABVD3 Rev	R		CATCCRACAAAGTGRATGAG	1001-1020	
	RABVD3 probe	P		TGYCCYGGCTCRAACATYCTYCTTAT	900-875	

Table II (cont.)

PCR	Primer/probe name	Role	Specificity	Sequence 5'-3'	Position	Reference
TaqMan	1129F	F	RABV	CTGGCAGACGACGGAACC	1129	17
	1218R	R		CATGATTCGAGTATAGACAGCC	1218	
	RB probe	P		TCAATTCTGATGACGAGGATTACTTCTCCGG		
TaqMan	gt1L	F	RABV	TACAATGGATGCCGACAAGA		14
	gt1P	R		CAAATC TTTGATGGCAGGGTA		
	AWgt1	P		TCAGGTGGTCTCTTTGAAGCCTGAGA		
SybrGreen	O1	F	RABV	CTACAATGGATGCCGAC	66-82	13
	R6	R		CCTAGAGTTATACAGGGCT	210-183	
TaqMan	LYSF-YB	F	ABLV	GAACGCCGCGAAGTTGG	191–207	4
	LYSR-YB	R		AGATCCCCTCAAATAACTCCATAGC	240–264	
	LYSF-YB-FAM	P		CGGACGATGTTTGCTCCTACCTAGCTGC	211–238	
	LYSF-FF	F		TCGGGAATGAATGCTGCAA	183–201	
	LYSR-FF	R		GGCAGAYCCCCTCAAATAACTC	267–247	
	LYSF-FF-FAM	P		ACCCCGATGATGTATGTTCTTACTTAGCTGCAG	208–239	
TaqMan	JW12	F	RABV	ATGTAACACCYCTACAATG	55–73	18
	N165–146	R		GCAGGGTAYTTRTACTCATA	165–146	
	LysGT1	P		ACAAGATTGTATTCAAAGTCAATAATCAG	81–109	
TaqMan	23F	F	RABV	CAATATGAGTACAAGTACCCGGC		16
	20R	R		AGCTTGGCTGCATTCATGCC		
	Probe	P		AAGCCCAGTATAACCTTAGGAAA	112-134	
TaqMan	AZ-EF	F	RABV	GAATCCTGATAGCACGGAGGG	278–298	20
		R		CTTCCACATCGGTGCGTTTT	333–352	
		P		CAAGATCACCCCAAATTCTCTTGTGGACA	303–331	
	AZ-SK	F		GTCGGCTGCTATATGGGTCAG	943–963	
		R		ATCTCATGCGGAGCACAGG	995–1013	
		P		TGAGGTCCTTGAATGCAACGGTAATAGCC	965–993	
	CASK	F		TCATGATGAATGGAGGTCGACTC	1226–1247	
		R		TTGATGATTGGAACTGACTGAGACA	1296–1272	
		P		AGAGATCGCATATACGGAGAT	1249–1270	
	NCSK	F		GGTGAAACCAGAAGTCCGGAA	1189–1209	
		R		CCGTATATGCGATCTCTTTAGTCGA	1266–1242	
		P		CTGTCTATACTCGAATCATGA	1211–1227	
	RAC	F		TGGTGAAACCAGGAGTCCAGA	1188–1208	
		R		ATCTTTTGAGTCGGCCCCC	1255–1235	
		P		CGGTCTATACTCGGATCAT	1211–1227	
	SCSK	F		ATGATGAAGACTATTTCTCCGGTGAG	1169–1191	
		R		GTCGGCCTCCATTCATCATG	1246–1226	
		P		CGGAGGCAGTCTATAC	1202–1219	

The genome of lyssaviruses is approximately 11,000 base pairs in length. For RABV, the nucleoprotein gene is located at position 71–1423 (GenBank accession number M13215)
ABLV: Australian bat *Lyssavirus*
DUVV: Duvenhage virus
LBV: Lagos bat virus
MOKV: Mokola virus
PCR: polymerase chain reaction
RABV: rabies virus
F: Forward primer
R: Reverse primer
P: Probe

The validation of probe-based assays relies heavily on the availability of representative viruses or nucleic acid. However, for some *Lyssavirus* species, only a single virus isolate or sequence is

available for primer/probe design, and may not be representative of all currently circulating or emerging variants. In addition, a single mutation in the region of the primers or the probe may alter the sensitivity of the assay, thus demanding continued vigilance and validation. This is particularly relevant when testing in animals because all of the most recently detected lyssaviruses since the report of Australian bat *Lyssavirus* in 1998 have been in animals not humans. Therefore, to reduce the possibility of failing to detect a novel or variant *Lyssavirus,* the use of universal pan-*Lyssavirus* primers in conjunction with SYBR Green, rather than specific probes, is perhaps the optimum confirmatory assay for the rapid real-time detection of all the known *Lyssavirus* species (5). As the application of real-time assays has become more widespread and routine, the availability of commercial master mixes is increasing, whereby operators simply add their local primers, probes and RNA. The use of such one-tube reagents reduces set-up times, increases reproducibility and may assist efforts to standardise and harmonise these assays. There is still no consensus on the particular region of the *Lyssavirus* genome to target for PCR-based assays. The majority of laboratories however, have chosen to target the nucleoprotein gene (N-gene). This is due to the large number of N-gene sequences available publicly, the relative sequence conservation across the N-gene and the abundance of N-gene messenger RNA (Table II).

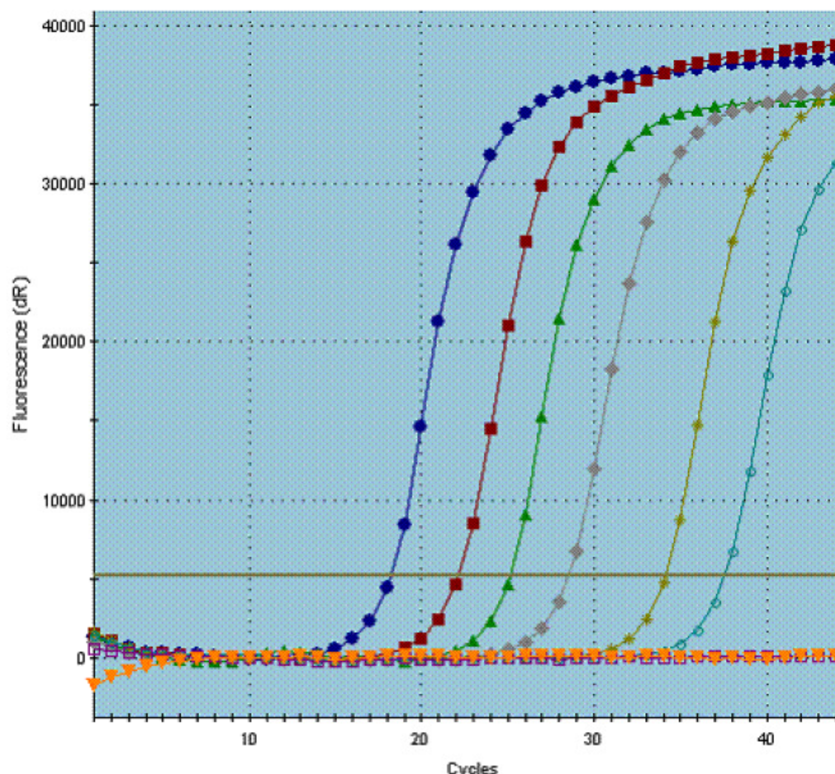
Results

The real-time assay amplification curve illustrates a ten-fold dilution series of RNA extracted from RABV (CVS-11), using pan-*Lyssavirus* primers JW12 and N165-146 in a real-time SYBR Green RT-qPCR assay (Fig. 1) (Table II). Figure 1 demonstrates that total RNA with a concentration of 1 µg/µl is detected before cycle 20 (Ct[#] < 20), and this assay is able to detect viral RNA at least up to a dilution of 0.01 ng/µl (0.00001 µg/µl) before cycle 40.

Fig. 1
RT-PCR amplification curve for serially diluted RABV (CVS-11 strain) RNA*

* The 1:10 serial dilution starting at 1 µg/µl and finishing at 10 pg/µl. Two negative controls were included, showing no detectable fluorescence

[#] The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).



The high sensitivity of this assay has enabled it to be successfully employed in the United Kingdom (UK) for the rapid diagnosis and *Lyssavirus* species confirmation of multiple European bat lyssavirus (EBLV)-2 infected bats, the *intra vitam* detection and genetic typing of a human rabies case within five hours of sample receipt, and the detection of a rabid puppy in UK quarantine during 2008 (data not shown).

Discussion

Irrespective of the outcome of any clinical diagnosis, the conventional OIE diagnostic tests are entirely dependent on the nature and quality of the sample supplied. Validated OIE diagnostic tests that confirm the presence of RABV or other lyssaviruses have been the foundation of rabies control strategies in many countries (19). Prescribed tests are required by the *OIE Terrestrial Animal Health Code (Terrestrial Code)* for the international movement of animals and animal products, and are considered optimal for determining the disease status of animals. Alternative tests are those that are suitable for the diagnosis of disease within a local setting and can also be used in the import/export of animals after a bilateral agreement.

Diagnosis of rabies is undertaken using OIE-prescribed tests, usually by FAT in the first instance, with a confirmatory diagnosis based routinely on virus isolation, if necessary. The FAT test detects *Lyssavirus* antigens (nucleoprotein) with fluorescently labelled antibodies in samples of brain tissue taken from a suspect animal. The FAT is regarded as the gold standard OIE test for rabies diagnosis and gives reliable results on fresh specimens within a few hours in 95% to 99% of cases. It is the only OIE-approved direct validated method that allows the identification of *Lyssavirus*-specific antigens in a short time and at a reduced cost, irrespective of geographical origin and status of the host. It should be regarded as the first step in diagnostic procedures for all rabies laboratories. Autolysed samples can, however, reduce the sensitivity and specificity of the FAT and other diagnostic tests. When the FAT is inconclusive, it is imperative that rabies diagnosis is obtained using separate OIE-prescribed diagnostic assays (Table I). A second test is required because, on rare occasions, a single test that appears to be negative, even when the sample is taken late in the disease, has at times proven to be unreliable, resulting in a false-negative test result. These OIE-prescribed methods have provided accurate and timely information on animal rabies cases, thereby supporting surveillance for rabies and providing a greater understanding of the epidemiology of this disease. For numerous laboratories in rabies-endemic regions in the developing world, cost and simplicity are vital factors in the delivery of disease diagnosis and cannot be neglected, even when the principal consideration is for rapid diagnosis. Therefore, cost and simplicity need to be considered if new technologies are to be adopted in the regions of the world where they are most needed (3).

Owing to the neurotropic nature of RABV and other lyssaviruses, infection results in enormous viral replication in the central nervous system in the final stage of the disease, which leads to significant antigen and viral genome concentrations. This makes the detection of viral RNA in brain tissue by molecular tests an unambiguous indicator of infection. As for the detection of viral genome, molecular approaches are now available which process multiple specimens from nucleic acid extraction through to genetic typing, with considerably reduced risks of contamination. It is evident that RT-PCR dominates genetic detection of *lyssaviruses* and it seems probable that this technique will continue to dominate rabies diagnosis in the 21st Century (3). 'Real-time' quantitative RT-qPCR techniques allow the visualisation of results as they occur during the amplification and, with some development, enable quantification of genomic RNA in the original sample. However, the highly sensitive nature of RT-qPCR assays to detect very low levels of target molecules also renders such tests extremely sensitive to contamination by extraneous nucleic acids. It is therefore essential that all laboratories employing PCR-based assays have stringent laboratory quality control standards that reduce, as far as is practicably possible, the risk of sample or test contamination. These do not have to involve

expensive infrastructure, but include a 'clean' area containing all equipment necessary for reagent and test preparation and a separate area with dedicated equipment for amplification and analysis. Gloves must be worn and replaced frequently at all stages of the procedure, thereby protecting the specimens and reagents from contamination by ubiquitous RNAses and cross-contamination by nucleic acid. Good laboratory practice must be adhered to throughout the entire process, with appropriate use of positive and negative controls for each stage of the process. Reverse-transcription PCR generates DNA amplicons, with sequences that can be defined. While this adds further cost and another level of technology, it has proven invaluable in assessing the specificity of the test and has contributed to numerous epidemiological studies on RABV. The importance of sequencing PCR products was highlighted for a highly sensitive nested RT-PCR that yielded host genomic amplicons of the same size as the target amplicons, but this result was only confirmed as a false positive following direct sequencing (7). False negatives and inefficiencies in sample extraction can be determined using a housekeeping RT-PCR, e.g. β-actin or 18s ribosomal RNA (rRNA) (3).

A further benefit of RT-PCR has been for the detection and classification of novel members of the *Lyssavirus* genus. The genetic characterisation of new viruses has been realised with the use of molecular tools such as PCR. This has been highlighted through the detection of rabies in an African civet (*Civettictis civetta*) from Tanzania (11). Following the clinical suspicion of rabies in the African civet, infection was confirmed using a *Lyssavirus* antigen detection method. Molecular analysis of brain samples subsequently demonstrated the infection to be caused by a novel and highly divergent *Lyssavirus* (11).

Human rabies diagnosis based on clinical symptoms is also unreliable, and contributes to the gross under-reporting of rabies (10). From a human health perspective, assays have been developed that measure RNA in *ante mortem* nuchal skin biopsies and in brain biopsies taken at autopsy using real-time quantitative PCR-based techniques, although these tests are not recommended by the World Health Organization (WHO) for routine *ante / post mortem* diagnosis of rabies (17). In addition, as for diagnosis in animals, strict quality control standards, including the development of international standards, are required in laboratories employing PCR-based technologies that undertake human rabies diagnosis, to avoid false-positive results.

There are numerous examples of technology transfer between diagnostic rabies laboratories in developed and developing countries that have successfully demonstrated the feasibility of undertaking rabies diagnosis using PCR-based technologies. With strict quality control procedures in place, and demonstrable experience and expertise, these molecular techniques have been successfully applied for confirmatory rabies diagnosis in animals in Africa (Tanzania, Namibia, Ghana, Nigeria) China and Turkey. The OIE Twinning Programme has prompted the OIE reference laboratory in Germany to assist in preparing a laboratory in Turkey as a potential future OIE reference laboratory supporting rabies diagnosis in the Middle East region. Moreover, the OIE Twinning Programme, in collaboration with the OIE Reference Laboratory in the UK, has assisted China to meet the requirements as an OIE reference laboratory for rabies (2). In 2012, the OIE officially approved the Changchun Veterinary Research Institute in China as a recognised OIE Reference Laboratory supporting rabies diagnosis in Asia.

With the introduction of laboratory accreditation, quality control measures are being implemented in an increasing number of diagnostic facilities worldwide. Such quality controls for diagnostic rabies RT-qPCRs should encompass several measures, such as the inclusion of appropriate positive, negative and inhibition controls in assay runs, monitoring of equipment performance and staff training. For an assay to be accepted as a confirmatory test, both sensitivity and specificity analyses are crucial to avoid false-negative and false-positive test results. The consistency and the inter-assay reproducibility should also be ensured over time by monitoring test performance using blinded proficiency testing. When used with appropriate quality controls, RT-qPCR has shown comparable or

superior concordance of results compared to the prescribed laboratory tests in inter-laboratory trials. In the 2009 European Union (EU) ring trial, RT-qPCR showed 91% concordance between laboratories, compared to 87% for FAT and 70% for RTCIT (15). For these reasons, it is likely that international bodies will accept the use of molecular tests for routine rabies diagnosis in the future. However, the lack of standardisation is a major obstacle to the general use of PCR for rabies diagnosis, especially in developing countries. It is important to highlight the fact that the quality control measures discussed above are equally important, and should already be implemented in any current OIE Reference Laboratory performing FAT. The use of PCR should not be restricted only to a confirmatory diagnostic test for decomposed samples but should also be recognised as a powerful tool for virus typing and molecular epidemiology studies (8). There are a number of diseases for which PCR-based assays have been accepted by the OIE for international trade as either prescribed tests (e.g. bluetongue virus [BTV] and Infectious bovine rhinotracheitis) or alternative tests (e.g. enzootic bovine leukosis virus, malignant catarrhal fever and African horse sickness), for the diagnosis of specific animal diseases (Table III).

For other viral diseases, the presence of virus-specific nucleic acid does not always indicate the presence of infectious virus. For example, non-infectious virus (or defective virions) could also be detected by PCR (e.g. the detection of BTV RNA in the blood long after the virus can be isolated) (5). There is, however, no evidence for this situation in lyssavirus-infected animals, due to the fact that lyssaviruses do not cause viraemia in infected animals, simplifying the interpretation of PCR-based results.

Further simplification and standardisation of molecular diagnostic techniques will allow their increased application in developing countries. It is likely that new developments will focus on generating low volume and yet affordable diagnostic tests for rabies. In the future, these technologies will have a demonstrable impact on people living in developing countries, especially where rabies is still considered a 'neglected' disease.

Table III

Diseases listed by the World Organisation for Animal Health for which polymerase chain reaction-based assays have been accepted as either a prescribed or alternative assay for international trade (20)

Terrestrial Code Chapter No.	Disease name	Prescribed tests	Alternative tests
2.1.3	Bluetongue	RT-qPCR	Not available
2.4.11	Enzootic bovine leukosis	Not available	PCR (pro-viral DNA)
2.4.13	Infectious bovine rhinotracheitis / infectious pustular vulvovaginitis	PCR (BoHV-1 DNA)	Not available
2.4.15	Malignant catarrhal fever	Not available	PCR (AIHV-1 & OvHV-2 DNA)
2.5.1	African horse sickness	Not available	Real time RT-qPCR (AHSV RNA)

AHSV: African horse sickness virus
AIHV: Acelaphine herpesvirus
BoHV: Bovine herpesvirus
OvHV: Ovine herpesvirus
PCR: Polymerase chain reaction
RT-qPCR: Reverse-transcription quantitative polymerase chain reaction

Recommendations

– Rabies should be considered a notifiable disease in all countries.

– The OIE should consider the recommendation of a validated PCR-based test that enables rapid *Lyssavirus* detection and typing with the option of quantification in real time as an alternative test for rabies. This should be included in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.*

– Collaborative projects and technology transfer of rabies molecular testing that link laboratories from developed and developing countries should be encouraged.

– Training in both OIE alternative and newly developed diagnostic tests for rabies should be supported financially, with logistical support, by national governments in partnership with the OIE and the OIE Reference Laboratory network.

– Standardised diagnostic procedures as prescribed by the OIE should always be used to confirm a 'suspected' clinical case of rabies in animal(s).

– All rabies diagnostic laboratories should follow the OIE guidelines for quality assurance in veterinary laboratories.

– All laboratories equipped to undertake rabies diagnosis should participate in inter-laboratory ring trials and proficiency schemes.

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