Laboratory diagnostics in dog-mediated rabies – an overview of performance and a proposed strategy for various settings

Veasna Duong[1]*; Arnaud Tarantola[2]*; Sivuth Ong[1]; Channa Mey[1]; Rithy Choeung[1]; Sowath Ly[2]; Hervé Bourhy[3]; Philippe Dussart[1]**; Philippe Buchy[4]**

1. Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
2. Epidemiology & Public Health Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
3. Institut Pasteur, Lyssavirus Dynamics and Host Adaptation Unit, National Reference Centre for Rabies, WHO Collaborating Centre for Reference and Research on Rabies, Paris, France
4. GlaxoSmithKline, Vaccines R&D, 150 Beach Road, Singapore

*, ** these authors contributed equally

Contact authors:

Arnaud Tarantola, MD, Msc
Head, Epidemiology and Public Health Unit
Institut Pasteur du Cambodge
5, Bvd. Monivong
BP 983 - Phnom Penh
Royaume du Cambodge
Mobile: +855 (0) 12 333 650
Tel: +855 (0) 23 426 009 ext. 206
Fax: +855 (0) 23 725 606
Email: atarantola@pasteur-kh.org

Philippe Buchy, MD, PhD
Director Scientific Affairs and Public Health
GlaxoSmithKline Vaccines, R&D Asia Pacific
150 Beach Road, unit 22-00
189720 Singapore
Tel. : +6596173587
Email: philippe.x.buchy@gsk.com

Abstract

Dog-mediated rabies diagnosis in humans and animals has greatly benefited from technical advances in the laboratory setting. Approaches to diagnosis now include detection of rabies virus (RABV), of RABV RNA or of RABV antigens. These assays are an important tool in the current effort for the global elimination of dog-mediated rabies. We review assays available for use in laboratories and their strong or weaker points, which vary with the types of sample analyzed. Depending on the setting, however, the public health objectives and use of RABV diagnosis in the field will also vary. In non-endemic settings, detection of all introduced or emergent animal or human cases justifies exhaustive testing. In dog RABV-endemic settings such as rural areas of developing countries where most cases occur, availability or access to testing may be severely constrained. Therefore, we discuss issues and propose a strategy to prioritize testing while access to rabies testing in the resource-poor, highly endemic setting is improved. As the epidemiological situation of rabies in a country evolves, the strategy should shift from that of an endemic setting to one more suited when rabies incidence decrease due to the implementation of efficient control measures and when nearing the target of dog-mediated rabies elimination.

Keywords

Rabies; endemic; virus; diagnosis; animal; human

Highlights

- Rabies remain a neglected disease in most developing countries
- Significant progress and efforts towards rabies control are recorded in some enzootic countries
- A stepwise approach for global elimination of canine-mediated rabies is now championed by health authorities worldwide and the strategy for rabies diagnosis and surveillance should adapt to the progress
- Several assays are available for diagnosis infection by rabies virus (RABV) in animals or humans.
- The various assays all have strong and weaker points, and their performance varies with the type of sample analyzed, their conservation during shipment, the expertise of the personnel and the environment (equipment, maintenance) of the laboratories.
- The use and the criteria of choice of these assays will vary according to sample analyzed and the objective of the diagnosis, which will also vary depending on whether the setting in endemic or non-endemic.

Introduction

Rabies diagnostic tests were born with routine inoculation of rabies virus (RABV)-infected brain or saliva samples to rabbits in 1880 [1] followed by the identification of Negri bodies reported in 1903 [2,3]. Several different assays and diagnostic approaches are now available, which are important assets in renewed global efforts to eliminate dog-mediated human rabies[4]. But how should these tests be used in the operational setting, especially in countries with a high dog-mediated rabies caseload? In what sequence? For which expected level of performance[5]? We propose an overview of currently available assays, their strengths and weaknesses and propose a rabies testing strategy based on our field experience. We will examine assays, by type and type of sample and their usefulness in endemic and non-endemic settings. Our aim is to guide virologists on the constraints and priorities of surveillance and detection, as well as animal or human rabies program managers on diagnostic methods and their performance.

Principles of RABV diagnostic tests

The various available reference diagnostic approaches have already been extensively described in publications resulting from international or academic initiatives[2,6–8]. Their principle as well as those of non-reference techniques - some recently developed[9–11] - are summarized in Table 1.

Detecting virus: Inoculation tests

Historically and in the research setting, RABV infection is identified by infecting cells and detecting virus. This can be done either through the Mouse Inoculation Test (MIT) or by inoculation of samples onto cultures of murine neuroblastoma or other cells (Rapid Tissue Culture Infection Test - RTCT) [8,12]. Following intracerebral inoculation of mice aged 3-4 weeks, MIT test results are available after an incubation period of up to 28 days. Some strains are associated with a longer incubation period. In laboratories with cell culture facilities and appropriate level of bio-containment, RTCT provides results within 24-48 hours, which is far

quicker than intracerebral inoculation[13,14]. Although it is more sensitive to toxic or bacterial contaminants its sensitivity is comparable to that of the MIT[15]. As RABV does not cause any cytopathic effect, the detection of the virus must therefore be evidenced by direct fluorescent antibody testing (DFAT, see below). In addition, MIT requires animal facilities to produce mice (or a supplier that can quickly provide animals at the suitable age in sufficient number) as well as animal facilities with high level of bio-containment (ASL3) to maintain the inoculated animals. Animal ethics regulations also recommend to avoid using animals when an efficient cell culture system exists.

Because of the usually urgently needed results and for animal protection issues, the World Health Organization (WHO)[8,16] as well as the World Organization for Animal Health (OIE)[17] now recommend replacing MIT by isolation of rabies virus in cell culture whenever possible.

Detecting viral RNA in samples

To date, molecular assays are not considered as reference techniques by international health organizations for *postmortem* diagnosis in humans and animals. However, they are recommended for *intravitam* diagnosis in humans. Although they require laboratory technicians to be trained and validated in molecular techniques, a PCR assay has been shown to be more sensitive than Direct fluorescent antibody testing for the detection of lyssaviruses[18].

Reverse Transcriptase-PCR (RT-PCR)

Reverse-transcriptase polymerase chain reaction (RT-PCR) is the method most often used to detect RABV RNA for *intravitam* diagnosis of rabies in humans. Saliva samples or skin biopsies taken at the nape of the neck (being careful to include hair follicles) [19] can be tested using this technique. The test reaches 100% sensitivity when at least three successive saliva samples are collected at 3 to 6-hour intervals (due to irregular viral shedding) and tested [19]. Positive results can be obtained as soon as the patient is admitted. Human and animal brain samples collected by

various methods can also be tested by this technique, even when they have been kept at relatively high ambient temperatures or when they have become degraded[20–24]. Several sets of more or less consensual primers for RABV have been developed. In some cases, primers specific of variant types of RABV have been developed for precise and geographically limited purposes, especially research. Although time- and resource-intensive, sequencing of PCR amplicons can improve the specificity of the technique. Specificity is very high when the technique is implemented rigorously, but false negatives and false positive may occur. RT-PCR is highly susceptible to cross-contamination in the operational setting, unless standardization and procedures are stringent, both for the PCR itself as well as the sample extraction [2] and reverse transcription of the RNA.

Other promising virus detection techniques such as loop-mediated isothermal amplification (LAMP)[25,26] or nucleic acid sequence-based amplification (NASBA)[27,28] detection have been developed. They are suited to developing settings as they require less sophisticated equipment and are less costly.

Real-time PCR (RT-qPCR)

These assays are based on the transcription of viral RNA to cDNA before amplification (RT). This phase is followed by PCR which uses specific primers and probes or a dye to provide real-time quantification of DNA. These assays reduce the risk of cross-contamination thanks to closed tubes and show an improved sensitivity compared to conventional RT-PCR protocols [2,12,29,30]. As the probes used are highly specific of known sequences, however, sequence mismatch between the primer/probe sequences and the target viral sequence may adversely affect the sensitivity of the test, leading to false negative results. Because the PCR fragments generated are usually very short, the sequencing of the amplicons may provide diagnostic confirmation but cannot be used for in-depth molecular phylogenetic analyses[31].

Detecting viral antigens

Direct fluorescent antibody testing (DFAT)

DFAT is the main assay used worldwide as it is recommended by WHO and OIE as gold standard for the diagnosis of rabies in fresh or frozen brain samples. The latter is important in tropical countries as preserving fresh samples at 4°C is often a challenge [2,8,32,33]. It is based on attaching fluorescein isothiocyanate to polyclonal antibodies targeting the RABV ribonucleocapsid or monoclonal antibodies targeting the RABV nucleoprotein (N). If the targeted RABV antigen is present in the sample fixed on a slide, antibodies attach to it, remain attached despite lavage and can be observed using a fluorescence microscope [12,33]. Results are available within 1-2 hours and results are expressed as positive or negative. The sensitivity and specificity of DFAT nears 99% in an experienced laboratory but is extremely observer-dependent[14,34]. At least two observers must spend enough time on each slide once the quality of the sample has been ensured. This test performs ideally on fresh brain samples: The reliability of this assay to diagnose rabies on degraded animal brain samples or corneal smears is low [12].

Rapid Rabies Enzyme Immunodiagnosis (RREID)

ELISA techniques have been adapted to detect RABV antigens in samples using monoclonal or polyclonal antibodies. These are microplates coated with purified polyclonal or monoclonal anti-RABV IgG targeting the nucleocapsid. Several versions of these assays have been developed (RREID, WELYSSA, etc) and some have been commercially available for some time[35–42]. They have been shown to be sensitive and specific and can be applied even to partially degraded brain samples [12]. Test results can be qualitatively evaluated by the naked eye. These techniques, however, are less sensitive than DFAT (96% agreement between DFAT and RREID test results) [8,12]. For this reason, they should not replace DFAT in laboratories where DFAT is already

performed. They are now implemented in a very limited number of countries, using home-made reagents.

Rapid Immunodiagnostic Test (RIDT)

The Antigen Rapid Rabies Ag Test ® Kit (BioNote, Inc., Gyeongi-do, Republic of Korea) is a lateral flow device based on a qualitative chromatographic immunoassay developed for the detection of RABV antigen in fresh animal brain tissue (e.g., canine, bovine, raccoon dog). This type of new rapid immunodiagnostic test (RIDT) seems suited for the field and for frontline laboratories. It is performant (specificity and sensibility) in brain samples from any animal and is highly dependent of the antibodies (polyclonal or monoclonal) used. This test has a sensitivity ranging from 91.7 to 96.9% and a specificity varying from 98.9 to 100% when compared to a fluorescent antibody test[11,43,44]. Furthermore, a result can be obtained within 15-20 minutes including preparation time. This test appears to be very accurate in detecting RABV antigen but other tests are required if questionable results are obtained. The RIDT kit, however, requires further validation before it can be recommended for use by either OIE or WHO. At this stage, RIDT should be implemented 1/ for research purposes only or 2/ in frontline laboratories to improve surveillance and control of rabies in remote places from which shipment of samples to a central laboratory is difficult or even impossible (or where classical rabies laboratory diagnosis using recommended techniques cannot be established for financial or logistic reasons). Many different products using the same methodology are commercially available and exhibit highly variable intrinsic properties. Laboratories aiming at using RIDT should therefore carefully evaluate the kit to assess the specificity and sensitivity - or should rely on available evaluations performed by international reference laboratories - before routine use.

Direct Rapid Immunohistochemical Test (dRIT)

This promising method was developed recently by the US-CDC. It is based on the detection of rabies N protein in brain smears fixed in formalin, using highly concentrated monoclonal

antibodies in presence of streptavidin peroxidase and a substrate coloring agent. Test results are available within 1 hour, can be implemented in the field and no fluorescent microscope is required [9,12,29,45]. The estimated sensitivity and specificity nears 100% when compared to a fluorescent antibody test [9,10]. This method can also be used for samples frozen or preserved in glycerol. Cost-effective, indirect immunochemistry assays have been developed which can also provide indications on the RABV variant [46]. A major concern, however, is the access to uninterrupted supplies of controlled batches of monoclonal antibodies which are only available through few laboratories specialized in rabies diagnosis [12].

Detecting antibodies

The detection of antibody in the serum in the absence of a history of rabies vaccination or in cerebrospinal fluid (CSF) provides indirect evidence of rabies infection. However, interpretation of test results may be difficult since the host immune response may vary among individuals: the sensitivity and negative predictive value of antibody detection methods in rabies patients is very poor [12,47] as suspect rabies deaths overwhelmingly occur before patients can mount an antibody response. Antibody response is only detectable in the blood (or CSF) after 8-10 days[48], while the majority of human rabies deaths occur around six days after the onset of clinical signs[49]. The poor yield of this technique has been shown in a series of human rabies cases[50,51]. These tests are thus better suited to assess the protection of laboratory or veterinary workers or of pets before transboundary travel, or to check appropriate immune response in patients receiving post exposure prophylaxis (PEP) as part of research (vaccine evaluation, seroprevalence studies). Considering these constraints and low sensitivity in the context of rabies diagnosis in humans and animals, the techniques used for serology will not be described here.

Why and when should we diagnose rabies?

In **non-endemic** settings, all suspect human and animal cases must imperatively be documented. Rabies laboratory diagnosis is essential to detect importation or emergence of rabies and to guide public health response or individual case management.

In **endemic** settings, early diagnosis of rabies in a suspect animals' head after euthanasia or natural death of the biting animal is essential as it can help inform PEP and the consequent need for rabies immunoglobulin. Assays such as RIDT and dRIT (or ELISA assays, such as RREID, if available) open the possibility of testing of biting animals' head at frontline bite centers to guide timely and adequate PEP for bite victims in rural settings of endemic countries, where most human rabies deaths occur. A diagnosis of "probable rabies" in humans, however, can routinely rely simply on the notion of a bite from a potentially rabid dog or other mammal and clinical signs in the patient, especially those compatible with furious rabies[49]. Laboratory diagnosis remains essential in the endemic setting to guide public health surveillance rather than individual patient management for an incurable disease. Testing is also helpful in human cases of "paralytic" rabies, as encephalitis may be caused by RABV or several other pathogens co-circulating in many rabies-endemic settings[52,53]. Finally, when the biting dog's head was not available for testing, a positive diagnosis in a patient can help guide PEP in other bite victims if they have remained asymptomatic, which should receive PEP even months after the bite[54]. Laboratory diagnosis of rabies in humans is also a priority especially when PEP with adequate and adequately-conserved vaccine has been undertaken and failed, in order to identify the virus[16,55]. Finally, laboratory diagnosis of rabies in endemic settings provides reliable, laboratory-confirmed data to complete the few existing estimations of rabies burden documented in humans as part of research efforts[4,56]. Findings from such research projects are crucial to increase awareness about the rabies burden among policy-makers and to prioritize resources towards its control.

Using diagnostic techniques in the operational setting

Performance issues

Assays used will vary, depending on the time elapsed since infection or the objective sought (Figure 1). In practice, direct diagnosis by RABV protein detection using DFAT is the technique of reference that should be implemented in national reference laboratories whenever possible. Direct diagnosis by detection of genome fragments using PCR-based assays must be used only by experienced teams, bearing in mind that this technique is based on validated in-house tests and not standardized, commercially available kits. Cell culture isolation of the virus is usually reserved for reference laboratories and research. Isolation of the virus as well as PCR-based methods may be used as second-line test to confirm negative results of other tests such as DFAT in animals or in the case of isolation to amplify live virus from original samples.

In *postmortem* human brain samples, DFAT and PCR-based techniques are highly performant to detect RABV antigen and genome, respectively. In such samples, RT-PCR or real time RT-PCR are useful alternatives to DFAT (Figure 1). These assays can also be used to test saliva or nuchal skin biopsy samples for *intravitam* or *postmortem* diagnosis in humans.

Operational issues

The choice of assay may vary depending on the nature of the sample to be tested and also its state of preservation [29,57] (Figure 1). DFAT, for example, remains less expensive than RT-PCR and can be used to test samples locally but the latter remains more performant when testing degraded samples[21]. In many endemic settings, the absence of a comprehensive network to transport samples to the laboratory makes it difficult to maintain staff competence in DFAT and fluorescent conjugates may reach expiration date before being fully used. Method simplicity, laboratory equipment availability and cost and ease of maintenance of these equipment are important factors to consider in the choice of laboratory assays before their implementation [29].

Rabies is classified as a Hazard Group 3 pathogen[58,59] but a BSL-2 is considered adequate containment for diagnosis and other activities as long they are performed by duly immunized personnel and are not associated with a high potential for droplet or aerosol production or involving large quantities or high concentration of infectious material[16,60]. Diagnosis and routine activities are undertaken by trained and duly immunized personnel in BSL-2 laboratories in most rabies-endemic developing settings which do not routinely access BSL-3 laboratory capacity.

Practical and logistical issues of rabies diagnosis in animals

Testing live animals for ongoing suspected rabies is unrecommended [16] (Figure 1). Animals should be quarantined for 10-14 days (depending on local regulations) or, in case of suspected rabies, be humanely euthanized with no damage to the head. Brain samples can then be analyzed in the laboratory setting[8,61]. One set of tools should be used for each specimen. The brain must be extracted from the animal's cranium by trained and immunized personnel in a safe and dedicated lab with a clean autopsy table and sterilized equipment. These precautions will prevent false positive results due to sample contamination. After extracting the brain attached to the cerebellum and medulla oblongata, tissue samples are taken from more than one site among Ammon's horns (hippocampus), the medulla oblongata (in the brainstem) and additional samples (cerebral tissue for OIE[62] , cerebral and/or cerebellar tissue for WHO[8,16] , cerebellar tissue for US recommendations[63], while testing all four sites is recommended in the Russian Federation and other countries of the former Soviet Union[64]). Aside from organ transplant, rabies is almost never transmitted by blood or body fluids[65,66] ; standard precautions nevertheless apply to protect personnel safety, even after it has been verified that they are properly immunized [2,67]. A pipette or a straw can also be used to rapidly collect brain samples during field studies in wild or domestic animals or when necropsy in all animal heads is impossible due to too many samples coming to the lab for testing [61,68].

Practical issues of rabies diagnosis in humans

As in animals, *postmortem* diagnosis in humans can easily be performed on brain samples obtained in a pathology laboratory, as above. Necropsies, however, are increasingly refused by next of kin for cultural reasons or when funereal rites must be performed without delay[69]. If *postmortem* necropsy cannot be performed in humans, minimally invasive methods may be used to sample tissue for rabies diagnosis while respecting the corpse's integrity (Figure 2).

Early studies of *intravitam* diagnosis found RABV antigens in hair follicle nerves of human rabies cases[70]. This paved the way for testing of skin biopsies sampled with hair follicles at the nape of the neck, obtained by excision or punch biopsy[19]. Nuchal skin biopsies can be performed *postmortem* but also *intravitam* in sedated patients. An optimal diagnostic strategy will associate nuchal biopsy with testing of at least three consecutive saliva samples (ideally at 3 to 6 hours of interval), which is highly specific and sensitive, irrespective of the time of collection (i.e., one day after the onset of symptoms until just after death)[19,71]. Two other low-invasive methods for *postmortem* brain sampling without craniotomy in humans have also been described: by sub-occipital cisternal puncture or through the retro-orbital route using a catheter (Figure 2).[32,72] A transnasal route has also been described [73].

Shipment issues

Nuchal samples are best shipped frozen but can be shipped simply with ice packs. Saliva samples should be shipped frozen. Brain samples can be frozen or preserved in 50% glycerol-saline solution if freezing is not readily available [10,68,74]. Samples should never be preserved in formalin. The reference laboratory must be contacted before shipment of samples with suspected RABV. They should be sent in a double-enclosed waterproof container, with cooling material and absorbent material. All these are placed in a leak-proof outer container, in observance with national and International Air Transport Association (IATA) guidelines as "Category B"

necessitating UN 3373 / 650 packaging (only rabies virus cultures are considered “Category A”)[75,76]. Sampling and shipping procedures must be well-established and well circulated to lab staff before they have to be put in use. Samples may be sent on filter paper at ambient temperature for easier shipment[77,78]. Filter paper such as FTA also has the advantage of inactivating RABV, reducing hazard and facilitating shipment while preserving nucleic acids.

Conclusion

Laboratory diagnosis of rabies is imperative to guide public health measures in non-endemic settings, document protection (*intravitam* serology on vaccinees’ blood samples) or to conduct research (seroprevalence studies, death despite timely PEP).

Although laboratory testing is desirable, it is extremely limited in resource-poor settings and/or rural areas of endemic countries, where most cases occur worldwide. Public health surveillance needs in the endemic setting with high caseloads may therefore be satisfied by resorting to syndromic case definitions and past history of dog bite[49,79,80]. Diagnosis based on molecular biology tests and virus isolation, however, may help guide PEP, is especially useful in “paralytic” rabies cases, may inform on phylogeny documenting introduction/emergence of RABV in a geographic area[31]. As the epidemiological situation evolves, the strategy should shift from that of an endemic setting to one adapted to a non-endemic setting. Many assays available to date are sensitive and specific but must be implemented by an experienced team with uninterrupted supplies. Rapid tests (dRIT and RIDT) have recently been developed, opening the possibility for first-line rabies laboratory diagnosis in less-equipped laboratories or in more remote areas. Should further external validation of RIDT confirm initial performances and usability in both saliva and brain samples[13–15], this assay may constitute a good alternative in the field, for example in provincial hospitals or vaccination centers where reference laboratory methods of

diagnosis are not readily available. The same is true for dRIT when reagents will become standardized and available on a regular basis with adequate quality control.

Table 1: Summarized RABV laboratory techniques, their advantages and limitations.

Test	Principle	Advantages	Limitations	Ref.
Detecting viral replication				
Mouse inoculation test (MIT)	Intracerebral inoculation into young mice for virus amplification.	Sensitive; Amplifies virus for identification; Easily performed; Possibility to isolate infectious virus.	Delayed results (up to 28 days); More expensive than RTCT; Not recommended by WHO; Requires animal facilities and adequate containment; Potential animal ethics issues as alternative methods exist.	12
Rapid Tissue Culture Infection Test (RTCT)	Inoculation of sample onto cell cultures (e.g. neuroblastoma cells).	Faster and cheaper than mouse inoculation test; Sensitivity comparable to MIT; No mice sacrificed.	Requires training and manpower as well as cell culture systems and fluorescent microscopy facilities; sensitive to toxic and bacterial contamination; Amplification of live virus may require adequate biosafety (safety cabinets and BSL-3 lab);	12,15
Detecting viral RNA				
Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)	Transcribes viral RNA to cDNA and amplifies it using specific primers with further detection of PCR products in agarose gel.	Applicable to any sample; Highly sensitive and specific; PCR products can be used for further nucleotide sequencing.	Time- and resource-intensive; Cross-contamination and false positives are a risk; No commercial diagnostic kits currently available.	2,12,29
Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR)	Transcribes viral RNA to cDNA and amplifies it using specific primers and probes with detection of PCR products in real time.	Less cross-contamination; Applicable to any sample; Sometimes more sensitive than conventional RT-PCR; Highly specific probes.	Single sequence mismatch between the primer or probe sequence and the target viral sequence may alter the sensitivity of the test and even cause false negative results ; PCR products are too short and unsuitable for nucleotide sequencing; No commercial diagnostic kits currently available	12,29,30
Detecting viral antigens/proteins				
Direct Fluorescent Antibody Test (DFAT)	Use of polyclonal or monoclonal FITC-conjugated antibodies for detection of rabies virus antigens by means of fluorescent microscopy.	Gold standard for fresh or fixed brain samples; High sensitivity and specificity, even on fixed specimen; Results obtained quickly; Commercial diagnostic kits are available.	Interpretation requires well-trained personnel (results highly observer-dependent) and costly fluorescent microscope; Less suitable on degraded samples.	2,8,12
Antigen capture ELISA: **Rapid Rabies Enzyme Immunodiagnosis (RREID)**	Immunohistochemical technique based on capture of various rabies antigens by specific antibodies labeled with enzyme.	Highly specific but less sensitive than DFAT (96% agreement between DFAT and RREID test results); Usable even on partly degraded brain samples; Qualitatively readable by the naked eye; A large number of samples can be tested at the same time (screening)	Can be used on brain tissues only; Requires great care to preserve specificity; No commercial diagnostic kits available.	12,29,35–41
Rapid Immunodiagnostic Test (RIDT)	Immunochromatographic assay based on monoclonal antibodies to capture rabies antigens.	Highly sensitive and specific but usually less so than DFAT; Usable on brain and saliva samples from animals; Results obtained rapidly	Dedicated for research purposes only; Need for further validation before either OIE or WHO can recommend its use.	11,43,44
Direct Rapid Immunohistochemical Test (dRIT)	Biotinylated monoclonal antibodies for detection of rabies virus antigens by means of normal light microscopy	High sensitivity and specificity; No need for fluorescence; Results obtained quickly.	Reagents difficult to obtain: need to identify an uninterrupted supply chain of quality-controlled monoclonal antibodies for sustainability.	12,29,45

Figure 1: Proposed rabies testing algorithm, based on objectives and methods to be used in a specialized laboratory.

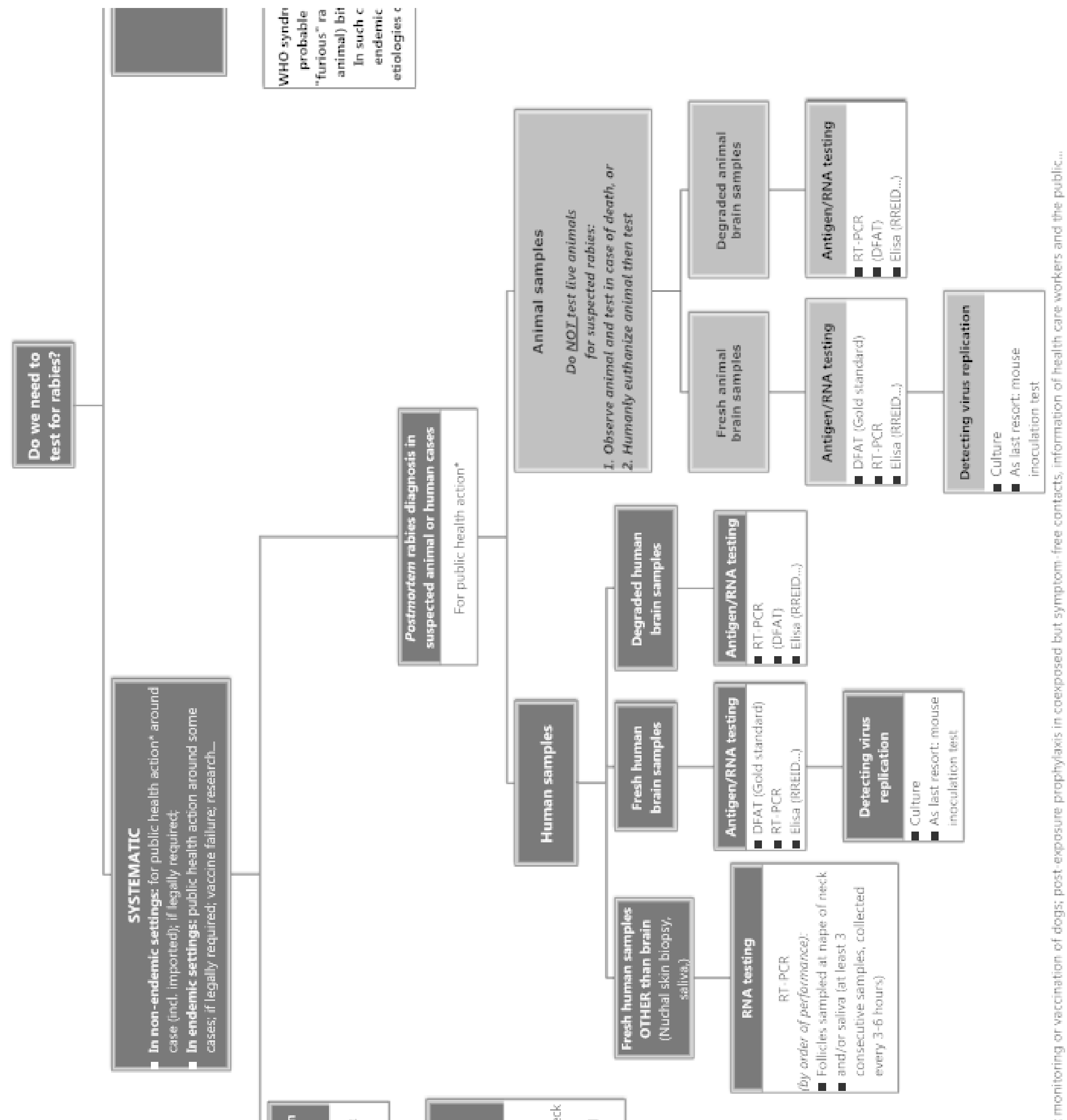
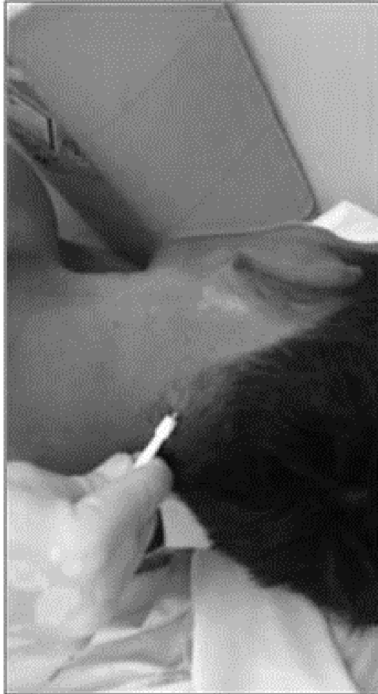
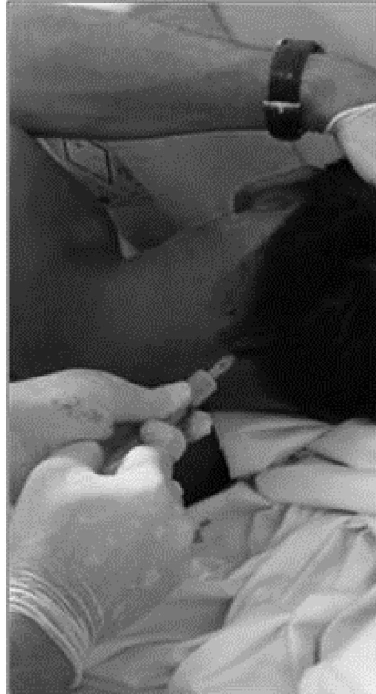
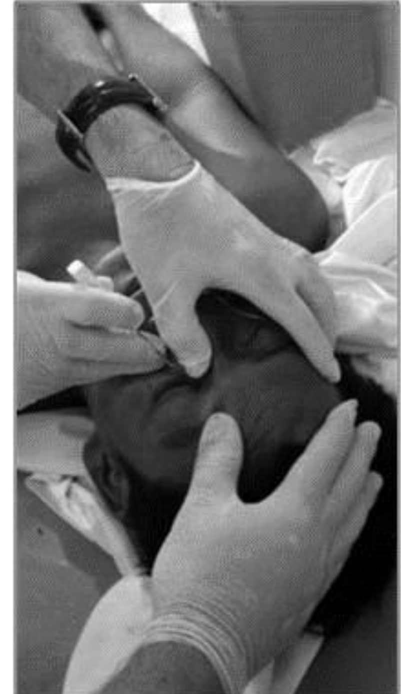


Figure 2: Minimally invasive tissue sampling techniques for rabies diagnosis in humans.

Sampling hair follicles at the nape of the neck (*intravitam* or *postmortem*)[19,32]	Sampling CSF or brain tissue by suboccipital cisternal puncture (*postmortem*)	Retro-orbital route to sample brain tissue (*postmortem*) [32,61,81]
		

Note: Clinical staff are asked to routinely wear short sleeves to promote hand hygiene and prevent nosocomial infections in the health care setting. They must don gloves as recommended when performing invasive procedures, to avoid contact with blood and body fluids [82]. Staff may don long-sleeved isolation gowns when a patient has uncontained secretions or excretions, but these are often unavailable even in hospitals of many rabies-endemic countries. Wearing long sleeved isolation gowns may help prevent contact with blood or body fluids, but is not generally indicated to perform biopsies and is not a necessary and specific measure against infection by the rabies virus, which is not a blood-borne pathogen [65,83].

Bibliography

1 Roux EPP. Des nouvelles acquisitions sur la rage. 1883; published online July 30. http://www2.biusante.parisdescartes.fr/livanc/?cote=TPAR1883x398&do=pdf.

2 Hanlon CA, Nadin-Davis SA. Chapter 11 - Laboratory Diagnosis of Rabies. In: Jackson AC, ed. Rabies (Third Edition). Boston: Academic Press, 2013: 409–59.

3 Babes V. Traité de la rage. Paris: Ballière, 1912 http://gallica.bnf.fr/ark:/12148/bpt6k5462676f.

4 WHO | WHO hosts milestone international conference to target global elimination of dog-mediated human rabies. WHO. http://www.webcitation.org/6efTbkujx (accessed Jan 20, 2016).

5 Fardy JM, Barrett BJ. Evaluation of diagnostic tests. *Methods Mol Biol Clifton NJ* 2015; **1281**: 289–300.

6 Rupprecht C, Nagarajan T, editors. Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention. Academic Press, 2015 http://www.sciencedirect.com/science/book/9780128000144 (accessed Feb 3, 2016).

7 Barrat J, Picard-Meyer E, Cliquet F. Rabies diagnosis. *Dev Biol* 2006; **125**: 71–7.

8 Meslin F-X, Kaplan MM, Koprowski H, World Health Organization, editors. Laboratory techniques in rabies, 4th ed. Geneva: World Health Organization, 1996.

9 Madhusudana SN, Subha S, Thankappan U, Ashwin YB. Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans. *Virol Sin* 2012; **27**: 299–302.

10 Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg Infect Dis* 2006; **12**: 310–3.

11 Voehl KM, Saturday GA. Evaluation of a rapid immunodiagnostic rabies field surveillance test on samples collected from military operations in Africa, Europe, and the Middle East. *US Army Med Dep J* 2014; : 27–32.

12 Mani RS, Madhusudana SN. Laboratory Diagnosis of Human Rabies: Recent Advances. *Sci World J* 2013; **2013**: e569712.

13 Dacheux L, Bourhy H. Chapter Three - Virus Isolation in Cell Culture: The Rabies Tissue Culture Infection Test. In: Rupprecht C, Nagarajan T, eds. Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention. Academic Press, 2015: 25–31.

14 Bourhy H, Rollin PE, Vincent J, Sureau P. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. *J Clin Microbiol* 1989; **27**: 519–23.

15 Barrat J, Barrat MJ, Picard M, Aubert MF. [Diagnosis of rabies by cell culture]. *Comp Immunol Microbiol Infect Dis* 1988; **11**: 207–14.

16 World Health Organization. WHO expert consultation on rabies (Second report). Geneva, Switzerland, 2013.

17 OIE - World Organisation for Animal Health, editor. Manual of diagnostic tests and vaccines for terrestrial animals, 7. ed. Paris: OIE, 2012.

18 Picard-Meyer E, Bruyère V, Barrat J, Tissot E, Barrat MJ, Cliquet F. Development of a hemi-nested RT-PCR method for the specific determination of European Bat Lyssavirus 1. Comparison with other rabies diagnostic methods. *Vaccine* 2004; **22**: 1921–9.

19 Dacheux L, Reynes J-M, Buchy P, *et al.* A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2008; **47**: 1410–7.

20 Rojas Anaya E, Loza-Rubio E, Banda Ruiz VM, Hernández Baumgarten E. Use of reverse transcription-polymerase chain reaction to determine the stability of rabies virus genome in brains kept at room temperature. *J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc* 2006; **18**: 98–101.

21 Beltran FJ, Dohmen FG, Del Pietro H, Cisterna DM. Diagnosis and molecular typing of rabies virus in samples stored in inadequate conditions. *J Infect Dev Ctries* 2014; **8**: 1016–21.

22 Araújo DB, Langoni H, Almeida MF, Megid J. Heminested reverse-transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC Res Notes* 2008; **1**: 17.

23 Albas A, Ferrari CI, da Silva LH, Bernardi F, Ito FH. Influence of canine brain decomposition on laboratory diagnosis of rabies. *Rev Soc Bras Med Trop* 1999; **32**: 19–22.

24 David D, Yakobson B, Rotenberg D, Dveres N, Davidson I, Stram Y. Rabies virus detection by RT-PCR in decomposed naturally infected brains. *Vet Microbiol* 2002; **87**: 111–8.

25 Muleya W, Namangala B, Mweene A, *et al.* Molecular epidemiology and a loop-mediated isothermal amplification method for diagnosis of infection with rabies virus in Zambia. *Virus Res* 2012; **163**: 160–8.

26 Boldbaatar B, Inoue S, Sugiura N, *et al.* Rapid detection of rabies virus by reverse transcription loop-mediated isothermal amplification. *Jpn J Infect Dis* 2009; **62**: 187–91.

27 Sugiyama M, Ito N, Minamoto N. Isothermal amplification of rabies virus gene. *J Vet Med Sci Jpn Soc Vet Sci* 2003; **65**: 1063–8.

28 Wacharapluesadee S, Phumesin P, Supavonwong P, Khawplod P, Intarut N, Hemachudha T. Comparative detection of rabies RNA by NASBA, real-time PCR and conventional PCR. *J Virol Methods* 2011; **175**: 278–82.

29 Fooks AR, Johnson N, Freuling CM, *et al.* Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. *PLoS Negl Trop Dis* 2009; **3**: e530.

30 Hayman DTS, Banyard AC, Wakeley PR, *et al.* A universal real-time assay for the detection of Lyssaviruses. *J Virol Methods* 2011; **177**: 87–93.

31 Mey C, Metlin A, Duong V, *et al.* Evidence of two distinct phylogenetic lineages of dog rabies virus circulating in Cambodia. *Infect Genet Evol* 2016; **38**: 55–61.

32 Bourhy H, Sureau P. Méthodes de laboratoire pour le diagnostic de la rage. Paris: Institut Pasteur, 1991.

33 CDC - Diagnosis: Direct Fluorescent Antibody Test - Rabies. http://www.webcitation.org/6YLMcxOSW (accessed May 7, 2015).

34 Robardet E, Andrieu S, Rasmussen TB, *et al.* Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates. *J Virol Methods* 2013; **191**: 88–94.

35 Perrin P, Sureau P. A collaborative study of an experimental kit for rapid rabies enzyme immunodiagnosis (RREID). *Bull World Health Organ* 1987; **65**: 489–93.

36 Perrin P, Gontier C, Lecocq E, Bourhy H. A modified rapid enzyme immunoassay for the detection of rabies and rabies-related viruses: RREID-lyssa. *Biol J Int Assoc Biol Stand* 1992; **20**: 51–8.

37 Perrin P, Rollin PE, Sureau P. A rapid rabies enzyme immuno-diagnosis (RREID): a useful and simple technique for the routine diagnosis of rabies. *J Biol Stand* 1986; **14**: 217–22.

38 Xu G, Weber P, Hu Q, *et al.* A simple sandwich ELISA (WELYSSA) for the detection of lyssavirus nucleocapsid in rabies suspected specimens using mouse monoclonal antibodies. *Biol J Int Assoc Biol Stand* 2007; **35**: 297–302.

39 Xu G, Weber P, Hu Q, *et al.* WELYSSA: a simple tool using mouse monoclonal antibodies for the detection of lyssavirus nucleocapsid in rabies suspected specimens. *Dev Biol* 2008; **131**: 555–61.

40 Oelofsen MJ, Smith MS. Rabies and bats in a rabies-endemic area of southern Africa: application of two commercial test kits for antigen and antibody detection. *Onderstepoort J Vet Res* 1993; **60**: 257–60.

41 Miranda NL, Robles CG. A comparative evaluation of a new immunoenzymatic test (RREID) with currently used diagnostic tests (DME and FAT) for dog rabies. *Southeast Asian J Trop Med Public Health* 1991; **22**: 46–50.

42 Saxena SN, Madhusudana SN, Tripathi KK, Gupta P, Ahuja S. Evaluation of the new rapid rabies immunodiagnosis technique. *Indian J Med Res* 1989; **89**: 445–8.

43 Kang B, Oh J, Lee C, *et al.* Evaluation of a rapid immunodiagnostic test kit for rabies virus. *J Virol Methods* 2007; **145**: 30–6.

44 Yang D-K, Shin E-K, Oh Y-I, *et al.* Comparison of four diagnostic methods for detecting rabies viruses circulating in Korea. *J Vet Sci* 2012; **13**: 43–8.

45 Dürr S, Naïssengar S, Mindekem R, *et al.* Rabies diagnosis for developing countries. *PLoS Negl Trop Dis* 2008; **2**: e206.

46 Dyer JL, Niezgoda M, Orciari LA, Yager PA, Ellison JA, Rupprecht CE. Evaluation of an indirect rapid immunohistochemistry test for the differentiation of rabies virus variants. *J Virol Methods* 2013; **190**: 29–33.

47 Wasniewski M, Labbe A, Tribout L, *et al.* Evaluation of a rabies ELISA as an alternative method to seroneutralisation tests in the context of international trade of domestic carnivores. *J Virol Methods* 2014; **195**: 211–20.

48 Johnson N, Cunningham AF, Fooks AR. The immune response to rabies virus infection and vaccination. *Vaccine* 2010; **28**: 3896–901.

49 WHO recommended surveillance standards, Second edition: Rabies. Geneva: World Health Organization.

50 Noah DL, Drenzek CL, Smith JS, *et al.* Epidemiology of human rabies in the United States, 1980 to 1996. *Ann Intern Med* 1998; **128**: 922–30.

51 Kasempimolporn S, Hemachudha T, Khawplod P, Manatsathit S. Human immune response to rabies nucleocapsid and glycoprotein antigens. *Clin Exp Immunol* 1991; **84**: 195–9.

52 Mallewa M, Fooks AR, Banda D, *et al.* Rabies encephalitis in malaria-endemic area, Malawi, Africa. *Emerg Infect Dis* 2007; **13**: 136–9.

53 Tarantola A, Goutard F, Newton P, *et al.* Estimating the burden of Japanese encephalitis virus and other encephalitides in countries of the mekong region. *PLoS Negl Trop Dis* 2014; **8**: e2533.

54 WHO | Criteria for application withholding of post-exposure treatment. WHO. http://www.who.int/rabies/human/generalconsid/en/ (accessed Feb 5, 2016).

55 Velasco-Villa A, Messenger SL, Orciari LA, *et al.* Identification of New Rabies Virus Variant in Mexican Immigrant. *Emerg Infect Dis* 2008; **14**: 1906–8.

56 Hampson K, Coudeville L, Lembo T, *et al.* Estimating the Global Burden of Endemic Canine Rabies. *PLoS Negl Trop Dis* 2015; **9**: e0003709.

57 Dacheux L, Wacharapluesadee S, Hemachudha T, *et al.* More Accurate Insight into the Incidence of Human Rabies in Developing Countries through Validated Laboratory Techniques. *PLoS Negl Trop Dis* 2010; **4**. DOI:10.1371/journal.pntd.0000765.

58 Lherm C. Classement des agents biologiques. INRS, 1999.

59 Advisory Committee on Dangerous Pathogens. The Approved List of biological agents. 2013 http://www.hse.gov.uk/pubns/misc208.pdf.

60 Chosewood LC, Wilson DE, Centers for Disease Control (U.S.), National Institutes of Health (U.S.). Biosafety in microbiological and biomedical laboratories. Washington: U.S. G.P.O. : For sale by the Supt. of Docs., U.S. G.P.O., 2010.

61 Barrat J. Simple technique for the collection and shipment of brain specimens for rabies diagnosis. In: Meslin F-X, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies, 4th ed. Geneva: World Health Organization, 1996: 425–32.

62 Terrestrial Manual: OIE - World Organisation for Animal Health. http://www.oie.int/en/international-standard-setting/terrestrial-manual/ (accessed Feb 4, 2016).

63 CDC - Diagnosis: In Animals and Humans - Rabies. http://www.cdc.gov/rabies/diagnosis/animals-humans.html (accessed Feb 5, 2016).

64 Methods of Laboratory Diagnostic of Rabies. 2013. http://files.stroyinf.ru/Data2/1/4293777/4293777838.pdf.

65 Aguèmon C, Tarantola A, Zoumènou E, *et al.* Rabies transmission risks during peripartum - two cases and a review of the literature. *Vaccine*; : pii: S0264–410X(16)00260–7.

66 Tillotson JR, Axelrod D, Lyman DO. Epidemiologic notes and reports: Rabies in laboratory worker - New York. *MMWR Morb Mortal Wkly Rep* 1977; **26**: 183–4.

67 Burnett LC, Lunn G, Coico R. Biosafety: Guidelines for Working with Pathogenic and Infectious Microorganisms. In: Coico R, McBride A, Quarles JM, Stevenson B, Taylor RK, eds. Current Protocols in Microbiology. Hoboken, NJ, USA: John Wiley & Sons, Inc., 2009: 1A.1.1–1A.1.14.

68 Chapter 2.1.13: Rabies. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012, 7th ed. OIE - World Organisation for Animal Health, 2012. http://www.oie.int/en/international-standard-setting/terrestrial-manual/ (accessed Feb 4, 2016).

69 Tarantola A, Crabol Y, Mahendra BJ, *et al.* Caring for rabies patients in developing countries - the neglected importance of palliative care. *Trop Med Int Health* 2016; published online Jan 25. DOI:10.1111/tmi.12670.

70 Bryceson ADM, Greenwood BM, Warrell DA, *et al.* Demonstration during Life of Rabies Antigen in Humans. *J Infect Dis* 1975; **131**: 71–4.

71 Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J Clin Microbiol* 1998; **36**: 1117–21.

72 Tong TR, Leung KM, Lee KC, Lam AW. Trucut needle biopsy through superior orbital fissure for the diagnosis of rabies. *Lancet Lond Engl* 1999; **354**: 2137–8.

73 Sudarsanam TD, Chacko G, David RD. Postmortem trucut transnasal brain biopsy in the diagnosis of encephalitis. *Trop Doct* 2008; **38**: 163–5.

74 Andrulonis JA, Debbie JG. Effect of acetone fixation on rabies immunofluorescence in glycerine-preserved tissues. *Health Lab Sci* 1976; **13**: 207–8.

75 IATA. UN 3373 - Biological substance, Category B. 2015. https://www.iata.org/whatwedo/cargo/dgr/Documents/packing-instruction-650-DGR56-en.pdf.

76 World Health Organization. Guidance on regulations for the Transport of Infectious Substances 2009-2010. 2008. http://www.who.int/csr/resources/publications/biosafety/WHO_HSE_EPR_2008_10/en/ (accessed Feb 4, 2016).

77 Sakai T, Ishii A, Segawa T, Takagi Y, Kobayashi Y, Itou T. Establishing conditions for the storage and elution of rabies virus RNA using FTA(®) cards. *J Vet Med Sci Jpn Soc Vet Sci* 2015; **77**: 461–5.

78 Picard-Meyer E, Barrat J, Cliquet F. Use of filter paper (FTA) technology for sampling, recovery and molecular characterisation of rabies viruses. *J Virol Methods* 2007; **140**: 174–82.

79 Chandramohan D, Maude GH, Rodrigues LC, Hayes RJ. Verbal autopsies for adult deaths: their development and validation in a multicentre study. *Trop Med Int Health TM IH* 1998; **3**: 436–46.

80 Hampson K, Dobson A, Kaare M, *et al.* Rabies exposures, post-exposure prophylaxis and deaths in a region of endemic canine rabies. *PLoS Negl Trop Dis* 2008; **2**: e339.

81 Hirose JA, Bourhy H, Sureau P. Retro-orbital route for brain specimen collection for rabies diagnosis. *Vet Rec* 1991; **129**: 291–2.

82 Siegel JD, Rhinehart E, Jackson M, Chiarello L, Health Care Infection Control Practices Advisory Committee. 2007 Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Health Care Settings. *Am J Infect Control* 2007; **35**: S65–164.

83 Tarantola A, Abiteboul D, Rachline A. Infection risks following accidental exposure to blood or body fluids in health care workers: a review of pathogens transmitted in published cases. *Am J Infect Control* 2006; **34**: 367–75.