HAL
open science

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

Veasna Duong, Arnaud Tarantola, Sivuth Ong, Channa Mey, Rithy Choeung, Sowath Ly, Hervé Bourhy, Philippe Dussart, Philippe Buchy

To cite this version:

Veasna Duong, Arnaud Tarantola, Sivuth Ong, Channa Mey, Rithy Choeung, et al.. Laboratory diagnostics in dog-mediated rabies: an overview of performance and a proposed strategy for various settings.. International Journal of Infectious Diseases, 2016, 46, pp.107-14. ⟨10.1016/j.ijid.2016.03.016⟩. ⟨pasteur-01476330⟩

HAL Id: pasteur-01476330

https://pasteur.hal.science/pasteur-01476330v1

Submitted on 12 May 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

International Journal of Infectious Diseases 46 (2016) 107–114

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Review

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

Veasna Duong [a,1], Arnaud Tarantola [b,1,*], Sivuth Ong [a], Channa Mey [a], Rithy Choeung [a], Sowath Ly [b], Hervé Bourhy [c], Philippe Dussart [a,1], Philippe Buchy [d,1,**]

[a] Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
[b] Epidemiology and Public Health Unit, Institut Pasteur du Cambodge, 5, Bvd Monivong, BP 983, Phnom Penh, Cambodia
[c] Institut Pasteur, Lyssavirus Dynamics and Host Adaptation Unit, National Reference Centre for Rabies, WHO Collaborating Centre for Reference and Research on Rabies, Paris, France
[d] GlaxoSmithKline Vaccines, R&D Asia Pacific, 150 Beach Road, Unit 22-00, 189720 Singapore

ARTICLE INFO

Article history:
Received 12 February 2016
Received in revised form 15 March 2016
Accepted 15 March 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Rabies
Endemic
Virus
Diagnosis
Animal
Human

SUMMARY

The diagnosis of dog-mediated rabies in humans and animals has greatly benefited from technical advances in the laboratory setting. Approaches to diagnosis now include the detection of rabies virus (RABV), RABV RNA, or RABV antigens. These assays are important tools in the current efforts aimed at the global elimination of dog-mediated rabies. The assays available for use in laboratories are reviewed herein, as well as their strengths and weaknesses, 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, the 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, the availability of or access to testing may be severely constrained. Thus, these issues are also discussed along with a proposed 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 suitable for a decreased rabies incidence following the implementation of efficient control measures and when nearing the target of dog-mediated rabies elimination.
© 2016 The Authors. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The development of rabies diagnostic tests began after the routine inoculation of rabbits with rabies virus (RABV)-infected brain and saliva samples in 1880,[1] and the identification of Negri bodies after 1903.[2,3] Several different assays and diagnostic approaches are now available, and these represent important assets in the 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 caseload of dog-mediated rabies? In what sequence? For which expected level of performance?[5] This review comprises an overview of currently available assays and their strengths and weaknesses. Furthermore, a rabies testing strategy based on the authors' field experience is proposed. The assays are examined by type and in terms of the type of sample used and their usefulness in the endemic and non-endemic settings. The aim of this review is to guide virologists on the constraints and priorities of surveillance and detection, as well as animal and human rabies programme managers on the diagnostic methods and their performance.

2. Principles of RABV diagnostic tests

The various available reference diagnostic approaches resulting from international and academic initiatives have been described

* Corresponding author. Tel.: +855 (0) 23 426 009ext.206; fax: +855 (0) 23 725 606.
** Corresponding author. Tel.: +6596173587.
E-mail addresses: atarantola@pasteur-kh.org (A. Tarantola), philippe.x.buchy@gsk.com (P. Buchy).atarantola@pasteur-kh.org
[1] Veasna Duong, Arnaud Tarantola, Philippe Dussart, and Philippe Buchy contributed equally.

http://dx.doi.org/10.1016/j.ijid.2016.03.016
1201-9712/© 2016 The Authors. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

extensively in previous publications.[2,6–8] Their principles, as well as those of non-reference techniques – some recently developed[9–11] – are summarized in Table 1.

2.1. 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 an appropriate level of bio-containment, the RTCT provides results within 24–48 h, 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

Table 1
Summary of RABV laboratory techniques and 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 of isolating 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 fluorescence microscopy facilities; sensitive to toxic and bacterial contamination; amplification of live virus may require adequate biosafety (safety cabinets and BSL-3 laboratory)	12,15
Detecting viral RNA				
Reverse-transcriptase PCR (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 PCR (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 fluorescence 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 a costly fluorescence microscope; less suitable on degraded samples	2,8,12
Antigen capture ELISA: rapid rabies enzyme immunodiagnosis (RREID)	Immunohistochemical technique based on the capture of various rabies antigens by specific antibodies labelled 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 with 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 the 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

RABV, rabies virus; WHO, World Health Organization; BSL, biosafety level; FITC, fluorescein isothiocyanate; OIE, World Organization for Animal Health.

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 of a suitable age in sufficient numbers), as well as animal facilities with a high level of bio-containment (ASL3) to maintain the inoculated animals. Animal ethics regulations also recommend avoiding the use of animals when an efficient cell culture system exists.

As results are usually required urgently and due to animal protection issues, the World Health Organization (WHO),[8,16] as well as the World Organization for Animal Health (OIE),[17] now recommend replacing the MIT with the isolation of RABV in cell culture whenever possible.

2.2. Detecting viral RNA in samples

The international health organizations do not currently consider molecular assays to be reference techniques for the post-mortem diagnosis of RABV in humans and animals. However, they are recommended for intra vitam 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 DFAT for the detection of lyssaviruses.[18]

2.2.1. Reverse-transcriptase PCR (RT-PCR)

RT-PCR is the method most often used to detect RABV RNA for intra vitam 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-h 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 with 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 for variant types of RABV have been developed for precise and geographically limited purposes, especially research. Although time- and resource-intensive, the 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-positives may occur. RT-PCR is highly susceptible to cross-contamination in the operational setting, unless standardization and procedures are stringent for the PCR itself and for the sample extraction[2] and reverse transcription of the RNA.

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

2.2.2. Real-time reverse transcriptase 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] However, as the probes used are highly specific for known sequences, 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]

2.3. Detecting viral antigens

2.3.1. Direct fluorescent antibody testing (DFAT)

DFAT is the main assay used worldwide; it is the WHO and OIE recommended gold standard for the diagnosis of rabies in fresh or frozen brain samples. The latter are important in tropical countries, as preserving fresh samples at 4 °C is often a challenge.[2,8,32,33] This assay is based on attaching fluorescein isothiocyanate (FITC) 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 washing, and can be observed using a fluorescence microscope.[12,33] Results are available within 1–2 h and 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 is best performed on fresh brain samples; the reliability of this assay to diagnose rabies in degraded animal brain samples or corneal smears is low.[12]

2.3.2. Rapid rabies enzyme immunodiagnosis (RREID)

ELISA techniques have been adapted to detect RABV antigens in samples using monoclonal or polyclonal antibodies. These use 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 evaluated qualitatively with the naked eye. However, these techniques 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. These are now implemented in a very limited number of countries, using home-made reagents.

2.3.3. 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 RIDT appears suited to the field and frontline laboratories. It is performant (specificity and sensitivity) for brain samples from any animal and is highly dependent on the antibodies (polyclonal or monoclonal) used. This test has a sensitivity ranging from 91.7% to 96.9% and a specificity ranging from 98.9% to 100% when compared to a fluorescent antibody test.[11,43,44] Furthermore, a result can be obtained within 15–20 min, including the preparation time. This test appears to be very accurate in detecting RABV antigen, but other tests are required if questionable results are obtained. However, the RIDT kit requires further validation before it can be recommended for use by either the OIE or WHO. At this stage, the RIDT should be implemented (1) for research purposes only, or (2) in frontline laboratories to improve the surveillance and control of rabies in remote places from which the shipment of samples to a central laboratory would be difficult or even impossible (or where classical rabies laboratory diagnosis using recommended techniques cannot be established for financial or logistical reasons). Many different products using the same methodology are commercially available and these exhibit highly variable intrinsic properties. Laboratories aiming to

use the RIDT should therefore evaluate the kit carefully and assess the specificity and sensitivity – or should rely on available evaluations performed by international reference laboratories – before routine use.

2.3.4. Direct rapid immunohistochemical test (dRIT)

This promising method was developed recently by the US Centers for Disease Control and Prevention (CDC). It is based on the detection of rabies N protein in brain smears fixed in formalin, using highly concentrated monoclonal antibodies in the presence of streptavidin peroxidase and a substrate colouring agent. Test results are available within 1 h. This test can be implemented in the field and no fluorescence microscope is required.[9,12,29,45] The estimated sensitivity and specificity approach 100% when compared to a fluorescent antibody test.[9,10] This method can also be used for samples that have been frozen or preserved in glycerol. Cost-effective indirect immunochemistry assays have been developed that can also provide indications on the RABV variant.[46] A major concern, however, is access to uninterrupted supplies of controlled batches of monoclonal antibodies, which are only available through a few laboratories specialized in rabies diagnosis.[12]

2.4. Detecting antibodies

The detection of antibody in serum in the absence of a history of rabies vaccination, or in cerebrospinal fluid (CSF), provides indirect evidence of rabies infection. However, the 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 patients with suspected rabies overwhelmingly die before they can mount an antibody response. The antibody response is only detectable in the blood (or CSF) after 8–10 days,[48] while the majority of human rabies deaths occur around 6 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 for the appropriate immune response in patients receiving post exposure prophylaxis (PEP) as part of research (vaccine evaluation, seroprevalence studies). Considering these constraints and the low sensitivity in the context of rabies diagnosis in humans and animals, the techniques used for serology will not be described here.

3. Why and when should we diagnose rabies?

In non-endemic settings, it is imperative that all suspected human and animal cases be documented. Rabies laboratory diagnosis is essential to detect the importation or emergence of rabies and to guide the public health response or individual case management.

In endemic settings, the early diagnosis of rabies in the head of a suspect animal after euthanasia or natural death (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) offer the possibility of testing the biting animal's head at frontline bite centres to guide timely and adequate PEP for bite victims in rural settings of endemic countries, where most human rabies deaths occur. However, a diagnosis of 'probable rabies' in humans can routinely rely simply on the presence 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 is not available for testing, a positive diagnosis in a patient can help guide PEP in other bite victims if they have remained asymptomatic; these victims should receive PEP even months after the bite.[54] The laboratory diagnosis of rabies in humans is also a priority particularly when PEP with adequate and adequately conserved vaccine has been undertaken and failed, in order to identify the virus.[16,55] Finally, the 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.

4. Using diagnostic techniques in the operational setting

4.1. 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 reference technique 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 a 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 post-mortem human brain samples, DFAT and PCR-based techniques are highly effective for the detection of RABV antigen and genome, respectively. In such samples, RT-PCR and RT-qPCR are useful alternatives to DFAT (Figure 1). These assays can also be used to test saliva or nuchal skin biopsy samples for intra vitam or post-mortem diagnosis in humans.

4.2. 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, however the latter remains more effective 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 their expiry date before being fully used. Method simplicity, the availability of laboratory equipment, and the cost and ease of maintenance of this 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 level 2 biosafety laboratory (BSL-2) is considered adequate containment for diagnosis and other activities, as long as they are performed by duly immunized personnel and are not associated with a high potential for droplet or aerosol production and do not involve large quantities or high concentrations 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 that do not routinely have BSL-3 laboratory capacity.

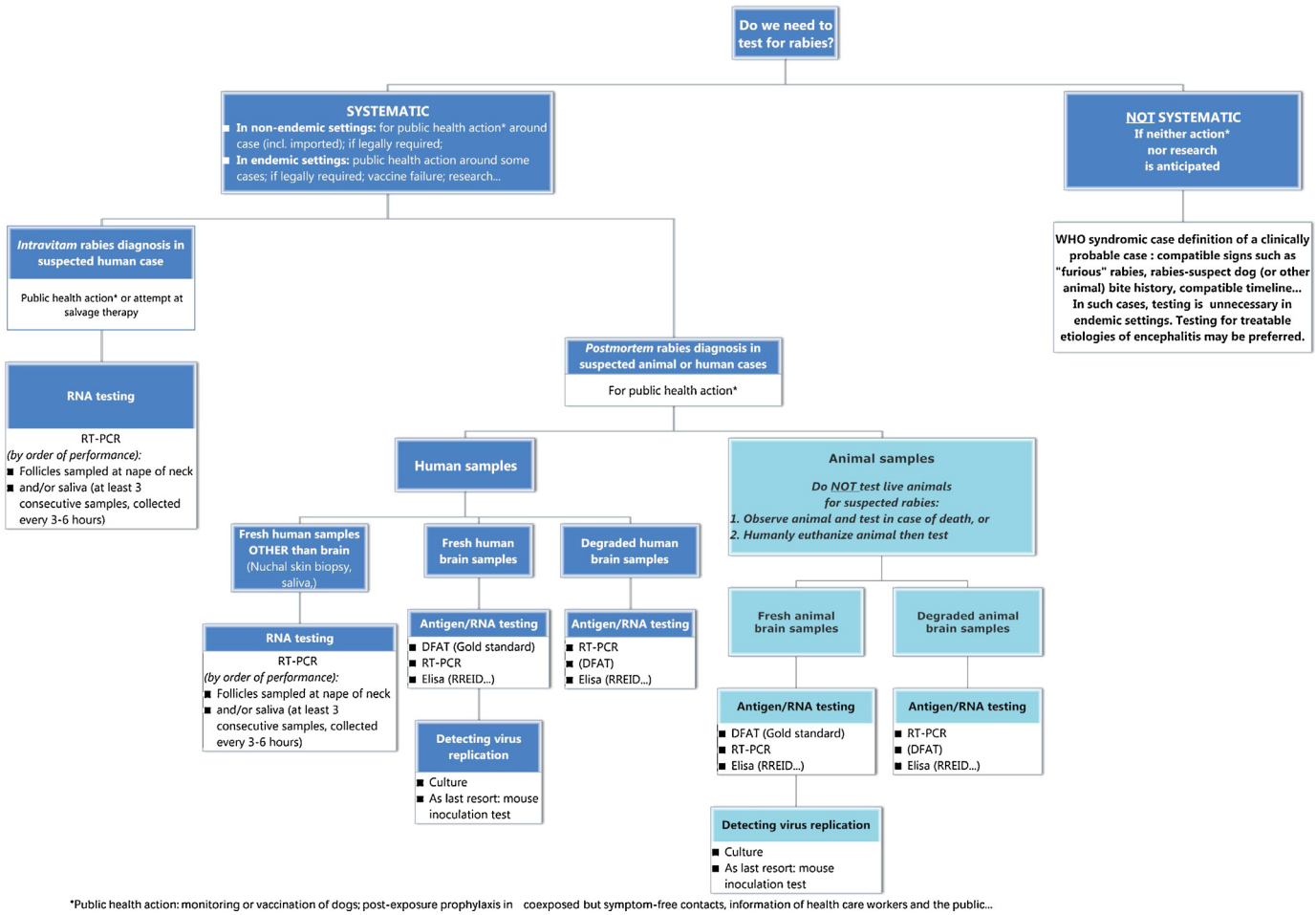

Figure 1. Proposed rabies testing algorithm based on the objectives of testing and the methods to be used in a specialized laboratory.

4.3. Practical and logistical issues of rabies diagnosis in animals

Testing live animals for ongoing suspected rabies is not recommended[16] (Figure 1). Animals should be quarantined for 10–14 days (depending on local regulations) or, in the 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 laboratory 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 should be taken from more than one site among Ammon's horns (hippocampus) and the medulla oblongata (in the brainstem). Additional recommended samples include the following: cerebral tissue for OIE,[62] cerebral and/or cerebellar tissue for WHO,[8,16] and cerebellar tissue for the US CDC.[63] The testing of all four sites is recommended in the Russian Federation and other countries of the former Soviet Union.[64] Aside from organ transplantation, rabies is almost never transmitted by blood or body fluids.[65,66] Standard precautions nevertheless apply to protect the safety of personnel, even after verification that they have been 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 of all animal heads is impossible due to too many samples coming to the laboratory for testing.[17,61]

4.4. Practical issues of rabies diagnosis in humans

As in animals, post-mortem diagnosis in humans can easily be performed on brain samples obtained in a pathology laboratory, as above. Autopsies, however, are increasingly refused by next of kin for cultural reasons, or when funeral rites must be performed without delay.[68] If a post-mortem autopsy cannot be performed for a human subject, minimally invasive methods may be used to sample tissue for rabies diagnosis while respecting the corpse's integrity (Figure 2).

Early studies of intra vitam diagnosis found RABV antigens in hair follicle nerves of human rabies cases.[69] 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 post-mortem but also intra vitam in sedated patients. An optimal diagnostic strategy will associate nuchal biopsy with testing of at least three consecutive saliva samples (ideally at 3–6 h intervals), which is highly specific and sensitive, irrespective of the time of collection (i.e., 1 day after the onset of symptoms until just after death).[19,70] Two other minimally invasive methods for post-mortem brain sampling without craniotomy in humans have also been described: sub-occipital cisternal puncture and the retro-orbital route using a catheter (Figure 2).[32,71] A trans-nasal route has also been described.[72]

4.5. Shipment issues

Nuchal samples are best shipped frozen, but can be shipped simply with ice packs. Saliva samples should be shipped frozen.

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

Brain samples can be frozen or preserved in 50% glycerol–saline solution if freezing is not readily available.[10,17,73] Samples should never be preserved in formalin. The reference laboratory must be contacted before the shipment of samples with suspected RABV. They should be sent in a double-enclosed waterproof container, with cooling material and absorbent material. All of these should be 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 RABV cultures are considered 'category A').[75,75] Sampling and shipping procedures must be well-established and communicated effectively to laboratory staff before they have to be put into use. Samples may be sent on filter paper at ambient temperature for easier shipment.[76,77] Filter paper such as FTA also has the advantage of inactivating RABV, reducing the hazard and facilitating shipment while preserving nucleic acids.

5. Conclusions

The laboratory diagnosis of rabies is imperative to guide public health measures in non-endemic settings, document protection (intra vitam serology on vaccinees' blood samples), and 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 a dog bite.[49,78,79] Diagnosis based on molecular biology tests and virus isolation, however, may help guide PEP, is especially useful in 'paralytic' rabies cases, and may provide information on phylogeny for documenting the introduction/emergence of RABV in a geographic area.[31] As the epidemiological situation evolves, the strategy should shift from that suited for 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, offering the possibility for first-line rabies laboratory diagnosis in less-equipped laboratories or in more remote areas. Should further external validation of the RIDT confirm its initial performance and usability in both saliva and brain samples,[13–15] this assay may become a good alternative in the field, for example in provincial hospitals or vaccination centres where reference laboratory methods of diagnosis are not readily available. The same is true for dRIT when reagents become standardized and available on a regular basis, with adequate quality control.

Conflict of interest: The authors have no conflict of interest. Philippe Buchy is an employee of GSK Vaccines.

References

1. Roux EPP. Des nouvelles acquisitions sur la rage. Paris: A. Parent, Imprimeur de la Faculté de Médecine; 1883, Available at: http://www2.biusante.parisdescartes.fr/livanc/?cote=TPAR1883x398&do=pdf (accessed January 20, 2016).
2. Hanlon CA, Nadin-Davis SA. Laboratory diagnosis of rabies. In: Jackson AC, editor. *Rabies.*. Third ed., Boston: Academic Press; 2013. p. 409–59.
3. Babes V. Traité de la rage. Paris: Ballière; 1912, Available at: http://gallica.bnf.fr/ark:/12148/bpt6k5462676f (accessed January 20, 2016)
4. World Health Organization. WHO hosts milestone international conference to target global elimination of dog-mediated human rabies. Geneva, Switzerland: WHO; 2016 , Available at: http://www.webcitation.org/6efTbkujx (accessed January 20, 2016)
5. Fardy JM, Barrett BJ. Evaluation of diagnostic tests. *Methods Mol Biol* 2015;**1281**:289–300.
6. Rupprecht C, Nagarajan T. Current laboratory techniques in rabies diagnosis, research and prevention. Cambridge, Mass. (USA): Academic Press; 2015 , Available at: http://www.sciencedirect.com/science/book/9780128000144 (accessed February 3, 2016)
7. Barrat J, Picard-Meyer E, Cliquet F. Rabies diagnosis. *Dev Biol* 2006;**125**:71–7.
8. Meslin FX, Kaplan MM, Koprowski H. Laboratory techniques in rabies, 4th ed., Geneva, Switzerland: 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. Virus isolation in cell culture: the rabies tissue culture infection test. In: Rupprecht C, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention*. Academic Press; 2015. p. 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: WHO; 2013.
17. OIE – World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals, 7th 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 JM, Buchy P, Sivuth O, Diop BM, Rousset D, Rathat C, et al. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis* 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 Invest* 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, Zulu L, Fandamu P, Banda D, Kimura T, 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, Noguchi A, Orbina JR, Demetria C, Miranda ME, 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* 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, Wakeley PR, Banyard AC, McElhinney LM, Marston DA, 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 DT, Banyard AC, Wakeley PR, Harkess G, Marston D, Wood JL, Cunningham AA, 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, Ong S, In S, Horwood PF, Reynes JM, 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. US Centers for Disease Control and Prevention CDC – Diagnosis. Direct fluorescent antibody test—rabies. Atlanta, GA: CDC; 2011, Available at: http://www.webcitation.org/6YLMcxOSW
34. Robardet E, Andrieu S, Rasmussen TB, Dobrostana M, Horton DL, Hostnik P, Jaceviciene I, 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. *Biologicals* 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, Xue H, Audry L, Li C, Wu J, 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, Xue H, Audry L, Li C, Wu J, 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, Park BK, Park Y, Hong K, Lee K, et al. Evaluation of a rapid immunodiagnostic test kit for rabies virus. *J Virol Methods* 2007;**145**:30–6.
44. Yang DK, Shin EK, Oh YI, Lee KW, Lee CS, Kim SY, Lee JA, 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, Diguimbye C, Niezgoda M, Kuzmin I, Rupprecht CE, 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, Rieder J, Labadie A, Schereffer JL, Cliquet F. 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. World Health Organization. WHO recommended surveillance standards. *Rabies*. 2nd ed., Geneva, Switzerland: WHO; 1999.
50. Noah DL, Drenzek CL, Smith JS, Krebs JW, Orciari L, Shaddock J, Sanderlin D, 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, Chikungwa P, Mankhambo L, Molyneux E, Molyneux ME, et al. Rabies encephalitis in malaria-endemic area, Malawi, Africa. *Emerg Infect Dis* 2007;**13**:136–9.
53. Tarantola A, Goutard F, Newton P, de Lamballerie X, Lortholary O, Cappelle J, Buchy P. Estimating the burden of Japanese encephalitis virus and other encephalitides in countries of the Mekong region. *PLoS Negl Trop Dis* 2014;**8**:e2533.
54. World Health Organization. Criteria for application withholding of post-exposure treatment. Geneva, Switzerland: WHO. Available at: http://www.who.int/rabies/human/generalconsid/en/ (accessed February 5, 2016).
55. Velasco-Villa A, Messenger SL, Orciari LA, Niezgoda M, Blanton JD, Fukagawa C, Rupprecht CE. Identification of new rabies virus variant in Mexican immigrant. *Emerg Infect Dis* 2008;**14**:1906–8.
56. Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, Barrat J, et al. Estimating the global burden of endemic canine rabies. *PLoS Negl Trop Dis* 2015;**9**:e0003709.
57. Dacheux L, Wacharapluesadee S, Hemachudha T, Meslin FX, Buchy P, Reynes JM, Bourhy H. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. *PLoS Negl Trop Dis* 2010;**4**:e765. http://dx.doi.org/10.1371/journal.pntd.0000765
58. Lherm C. Classement des agents biologiques. France: INRS; 1999.
59. Advisory Committee on Dangerous Pathogens. The approved list of biological agents. Bootle, United Kingdom: Health and Safety Executive; 2013, Available at: http://www.hse.gov.uk/pubns/misc208.pdf (accessed)
60. Chosewood LC, Wilson DE. US Centers for Disease Control, National Institutes of Health. Biosafety in microbiological and biomedical laboratories. Washington: US GPO; 2010.
61. Barrat J. Simple technique for the collection and shipment of brain specimens for rabies diagnosis. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. 4th ed., Geneva, Switzerland: WHO; 1996. p. 425–32.
62. OIE – World Organisation for Animal Health. Terrestrial manual. OIE; 2012, Available at: http://www.oie.int/en/international-standard-setting/terrestrial-manual/ (accessed February 4, 2016)
63. US Centers for Disease Control and Prevention. Diagnosis in animals and humans—rabies. Atlanta, GA: CDC; 2011, Available at: Available at: http://www.cdc.gov/rabies/diagnosis/animals-humans.html (accessed February 5, 2016)
64. Interstate Council for Standardization, Metrology and Certification Moscow, Russian Federation. Methods of laboratory diagnostic of rabies. Atlanta, GA: CDC; 2013, Available at: http://files.stroyinf.ru/Data2/1/4293777/4293777838.pdf (accessed January 20, 2016)
65. Aguèmon C, Tarantola A, Zoumènou E, Goyet S, Assouto P, Ly S, Mewanou S, et al. Rabies transmission risks during peripartum—two cases and a review of the literature. *Vaccine* 2016;**34**(15):1752–7. http://dx.doi.org/10.1016/j.vaccine.2016.02.065. Epub 2016 Mar 3.
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, editors. *Current protocols in microbiology*. Hoboken, NJ: John Wiley and Sons, Inc; 2009. p. 1A.1.1–1A.1.14.
68. Tarantola A, Crabol Y, Mahendra BJ, In S, Barennes H, Bourhy H, Peng Y, et al. Caring for rabies patients in developing countries—the neglected importance of palliative care. *Trop Med Int Health* 2016. http://dx.doi.org/10.1111/tmi.12670
69. Bryceson AD, Greenwood BM, Warrell DA, Davidson NM, Pope HM, Lawrie JH, Barnes HJ, et al. Demonstration during life of rabies antigen in humans. *J Infect Dis* 1975;**131**:71–4.
70. 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.
71. Tong TR, Leung KM, Lee KC, Lam AW. Trucut needle biopsy through superior orbital fissure for the diagnosis of rabies. *Lancet* 1999;**354**:2137–8.

72. Sudarsanam TD, Chacko G, David RD. Post-mortem trucut transnasal brain biopsy in the diagnosis of encephalitis. *Trop Doct* 2008;**38**:163–5.
73. Andrulonis JA, Debbie JG. Effect of acetone fixation on rabies immunofluorescence in glycerine-preserved tissues. *Health Lab Sci* 1976;**13**:207–8.
74. International Air Transport Association. UN 3373—biological substance, category B. IATA; 2015. Available at: https://www.iata.org/whatwedo/cargo/dgr/Documents/packing-instruction-650-DGR56-en.pdf (accessed January 20, 2016).
75. World Health Organization. Guidance on regulations for the transport of infectious substances 2009–2010. Geneva, Switzerland: WHO; 2008, Available at: http://www.who.int/csr/resources/publications/biosafety/WHO_HSE_EPR_2008_10/en/ (accessed February 4, 2016)
76. 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* 2015;**77**:461–5.
77. 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.
78. 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* 1998;**3**:436–46.
79. Hampson K, Dobson A, Kaare M, Dushoff J, Magoto M, Sindoya E, Cleaveland S. Rabies exposures, post-exposure prophylaxis and deaths in a region of endemic canine rabies. *PLoS Negl Trop Dis* 2008;**2**:e339.