Progress in the development of a direct rapid immunohistochemical test for diagnosing rabies

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The findings and conclusions in this report are those of the authors only, and do not necessarily reflect the views of their institutions or those of the OIE.

Summary: At present, the direct fluorescent antibody test (FAT) is an OIE-prescribed and WHO-recommended test and is considered the ‘gold standard’ for sensitive and specific rabies diagnosis. The FAT provides consistent results on fresh brain specimens in more than 95% to 99% of cases. As plans for the global elimination of canine rabies advance, the need for additional relevant diagnostic testing is crucial to improve laboratory-based surveillance and monitor programme success, especially in developing countries. A direct rapid immunohistochemical test (DRIT) for rabies diagnosis has been developed as one alternative to meet this need. Similarly to the FAT, the DRIT involves the examination of brain impressions but, rather than immunofluorescence, employs light microscopy and biotin-labelled antibodies. Using the DRIT, tens of thousands of suspect animal brains have been examined since 2008 by diagnosticians and researchers in the Americas, Africa, Asia and Europe. Despite widely varying conditions by locale, species tested, sample quality, viral type, and background conditions, test sensitivity and specificity have approached 100%. All currently recognised lyssavirus species have been identified in naturally or experimentally infected mammals using the DRIT. In the United States, it is applied to laboratory confirmatory testing of equivocal FAT results and to enhance surveillance under field conditions. For example, in support of oral rabies vaccination programmes, more than 60,000 specimens have been tested within North America and, as with the FAT, the DRIT is included in routine proficiency testing. Moreover, preliminary inter-laboratory comparisons, using different monoclonal and polyclonal conjugates, with antibodies tested against a wide variety of viral variants, support the basic premise of the DRIT as a relevant test for future validation and OIE consideration as a prescribed test for rabies diagnosis.

Introduction
During the major historical achievement of the eradication of rinderpest, three interrelated attributes were identified as crucial for long-term veterinary programme success: applicable diagnostics, efficacious vaccines, and the epidemiological knowledge to harness both effectively for practical global prevention and control [1]. All of these facets come into operation when extending a similar programme to another high-impact, rapidly moving, transboundary disease: the acute progressive encephalitis due to rabies. As with rinderpest, pure, potent, safe and efficacious biologics for rabies prophylaxis became available over the past century for domestic animals, as well as for humans and wildlife [2, 3, 4]. Moreover, the case for substantive intervention is well supported by a modern understanding of lyssavirus epidemiology from a theoretical as well as an applied perspective [2, 3, 5, 6]. Lastly, laboratory diagnostics form the cornerstone to any modern rabies management plan [2, 3, 7]. Obviously, without a method to measure success in the initiation of herd immunity, efforts at canine vaccination become exercises in futility. Nevertheless, as with many infectious agents in the developing world, ineficent laboratory-based surveillance systems for rabies are plagued by a debilitating cycle of neglect. Few, or no, reported human or canine cases each year do not capture substantive public attention. A lack of epidemiological focus fails to garner the required resources to manage rabies at its source in reservoir species. Minimal support leads to eroded and ineffective surveillance efforts. Often, with no comprehensive detection mechanisms, political will, national support or local champions, laboratory systems falter. Similar cycles continue for most neglected zoonotic diseases, demanding renewed advocacy [8].

Historical perspectives in rabies diagnosis
Despite the current tenuous diagnostic situation, there has been a significant development of technical acumen related to this viral zoonosis for over a century. Historically, most suspicions about rabies were based upon clinical grounds alone. Gradually, during the late 1800s and early 1900s, the usefulness of laboratory diagnostic methods was demonstrated, indicating a non-specific viral encephalitis, based upon histological examination of the brain. Shortly after, the 1903 description of the Negri bodies, as intra-cytoplasmic inclusions within infected neurons, revolutionised rabies virus diagnosis [9]. By the 1950s, the superiority of other laboratory techniques became clear, such as the direct fluorescent antibody test (FAT) [10]. Over the next several decades, the FAT became the ‘gold standard’ for reliable detection of viral antigens within the brains of rabid animals [2, 3]. Gradually, within this framework, the impetus arose during the 1990s for other diagnostic options from the FAT. Suitable rapid alternatives were needed, equivalent to the FAT, to maintain high standards of sensitivity and specificity and to preserve the underlying concept of an anatomic-pathological basis for detecting intra-neuronal inclusions within the brain, while allowing for greater adaptability without the need for an expensive fluorescence microscope (or even, necessarily, electricity).

Conception of an alternative diagnostic test for rabies
The preliminary work for an alternative method was undertaken during 1999, initially as an indirect immunohistochemical test, using an experimental cocktail equivalent to five anti-rabies virus monoclonal antibodies purified from mouse ascites fluid. Within a few years, the number of antibodies was reduced to two and both were directly linked to biotin to simplify the test. Conceptually based on the antibody-antigen detection of viral inclusions in the mammalian central nervous system (CNS), as in the FAT, the direct rapid immunohistochemical test (DRIT) was developed by 2002, but with several distinct differences (Table I). In
contrast to the FAT, one prominent feature of the DRIT included a brief fixation of brain impression slides in 10% buffered formalin, which not only provides excellent morphological preservation and ready access for antibody diffusion into tissue, but also inactivates lyssavirus infectivity, as an important biosafety improvement over acetone. Also, instead of the fluorochrome-linked conjugates used in the FAT, the DRIT employs antibodies linked covalently to biotin (either hyperimmune serum, such as goat anti-rabies immunoglobulin G or murine monoclonal antibodies), and light microscopy, using a colorimetric streptavidin-biotin, sensitivity-boosting detection system, with resistance to organic solvents, denaturants, detergents, proteolytic enzymes, and extremes of temperature and pH, with post-impression results obtained in approximately less than one hour (Table II).

Hence, the DRIT was developed upon the same immunological premise for focal post-mortem diagnosis as the FAT. Moreover, it was built upon the

### Table I
Comparison of direct fluorescent antibody test and direct rapid immunohistochemical test procedures for diagnosing rabies

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Fluorescent antibody test (FAT)</th>
<th>Direct rapid immunohistochemical test (DRIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Brain</td>
<td>Brain</td>
</tr>
<tr>
<td>Material</td>
<td>Impressions on microscope slides</td>
<td>Impressions on microscope slides</td>
</tr>
<tr>
<td>Reagent</td>
<td>Anti-rabies monoclonal or polyclonal antibody</td>
<td>Anti-rabies monoclonal or polyclonal antibody</td>
</tr>
<tr>
<td>Principle</td>
<td>Antibody-antigen recognition</td>
<td>Antibody-antigen recognition</td>
</tr>
<tr>
<td>Observation</td>
<td>Intra-neuronal inclusions</td>
<td>Intra-neuronal inclusions</td>
</tr>
<tr>
<td>Usefulness</td>
<td>High sensitivity and specificity</td>
<td>High sensitivity and specificity</td>
</tr>
<tr>
<td>Equipment</td>
<td>Fluorescence microscope</td>
<td>Light microscope</td>
</tr>
<tr>
<td>Conjugate</td>
<td>Fluorescein isothiocyanate</td>
<td>Biotin</td>
</tr>
<tr>
<td>Fixative</td>
<td>Acetone</td>
<td>Formalin</td>
</tr>
<tr>
<td>Time</td>
<td>–1.5–4 hours (or longer)</td>
<td>&lt;1 hour</td>
</tr>
</tbody>
</table>

### Table II
Synopsis of the direct rapid immunohistochemical test procedure for rabies diagnosis

1. Make brain touch impressions on microscope slides.
2. Allow slides to air-dry.
3. Fix brain impression slides in 10% buffered formalin for 10 minutes.
4. Treat slides with hydrogen peroxide for 10 minutes.
5. Incubate slides with primary biotinylated antibodies for 10 minutes.
6. React slides with streptavidin-peroxidase for 10 minutes.
7. Add peroxidase substrate to slides for 10 minutes
   (3-amino-9-ethylcarbazole, AEC).
8. Counterstain (with Gill’s haematoxylin) for 2 minutes.
9. Mount the cover slip with water-soluble mounting media.
10. Read the slides using light microscopy to detect stained viral inclusions.
technical evolution in the rabies diagnostic field, from the advent of immunohistochemistry in pathology, in addition to hybridoma production of monoclonal antibodies directed against linear or conformational determinants of viral antigens [11, 12, 13, 14, 15]. The initial selection of monoclonal antibodies was dependent upon prior years of use in the antigenic typing of viral variants and the broad recognition of some antibodies, versus the discriminatory use of other monoclonal antibodies, to differentiate lyssavirus serotypes [16, 17]. Additionally, not all monoclonal antibodies were equally useful in the DRIT after formalin fixation, without the need for prior digestion, so selection was dependent upon a number of properties. For example, monoclonal antibodies WI 502 (developed at the WHO Collaborating Centre, Wistar Institute, Philadelphia, Pennsylvania, USA, during 1978) [15, 16] and FLI 239.17 (developed at the WHO Collaborating Centre, Tubingen, Germany, during 1982), directed against the rabies virus nucleoprotein, not only recognised epitopes on all known lyssaviruses (and became antibodies used in commercial FATs), but also maintained ideal binding in the DRIT, after the brief formalin fixation step. Depending upon the type of antibody, the choice of colour development system and the counterstains selected for detection via simple light microscopy, viral inclusions appear as discrete magenta bodies to finer, reddish ‘antigenic dust’, distributed against a bluish-purple background of neuronal tissue (Figs 1 & 2).

Application of the DRIT in enhanced surveillance of canine and wildlife rabies

The preliminary experimental work in the evolution of the DRIT between 1999 and 2002 was focused upon the selection, purification and biotinylation of the most suitable, broadly cross-reactive monoclonal antibodies, simplification of the protocol steps, and the determination of reliable common sources for the other reagents. The advantages of the DRIT included the ability to perform the test at ambient temperatures in the laboratory, without the need for 4°C or –20°C refrigeration during fixation, or incubation at 35°C to 37°C during the antibody-antigen reactions. Thereafter, in collaboration with state health departments throughout the United States (USA), phase I testing began to evaluate operator ability to perform the test upon local species and variants. All New World rabies virus variants were recognised by the DRIT, as were all other lyssaviruses that had been identified by the start of the new millennium (Table III). Between 2005 and 2012, the DRIT was being used experimentally in phase II comparisons for limited, decentralised, enhanced rabies surveillance attempts in the Americas, Africa and Eurasia, primarily for diagnosis in domestic animals, but later in wildlife as well [18, 19, 20, 21, 22, 23, 24, 25]. As shown by prior comparative testing, the brainstem is a readily accessible and pathologically relevant major brain region for selection in the DRIT, rather than focusing upon less critical CNS
Table III

Global experience in the use of the direct rapid immunohistochemical test for rabies diagnosis

<table>
<thead>
<tr>
<th>Location</th>
<th>Primary taxa</th>
<th>Findings (a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada (Quebec, Ontario)</td>
<td>Wildlife (n &gt; 3,500)</td>
<td>Concordant to fluorescent antibody test</td>
<td>Unpublished(b)</td>
</tr>
<tr>
<td>Chad</td>
<td>Dogs (n &gt; 35)</td>
<td>~100% sensitivity</td>
<td>18</td>
</tr>
<tr>
<td>China</td>
<td>Dogs, humans (n &gt; 70)</td>
<td>~100% sensitivity</td>
<td>19, 20</td>
</tr>
<tr>
<td>India</td>
<td>Dogs (n &gt; 400)</td>
<td>~100% sensitivity</td>
<td>21</td>
</tr>
<tr>
<td>Middle East (Afghanistan, Iraq)</td>
<td>Dogs (n &gt; 370)</td>
<td>~100% sensitivity</td>
<td>22</td>
</tr>
<tr>
<td>South Africa</td>
<td>Dogs, wildlife (n &gt; 250)</td>
<td>~100% sensitivity</td>
<td>23</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Dogs, wildlife (&gt;150)</td>
<td>~100% sensitivity</td>
<td>24</td>
</tr>
<tr>
<td>United States</td>
<td>Wildlife (&gt;60,000 samples tested to date)</td>
<td>&gt;95% sensitivity</td>
<td>25</td>
</tr>
</tbody>
</table>

(a) Compared for diagnostic agreement to the gold standard FAT upon brain tissue from suspect animals

areas which are more difficult to access, such as the cerebral hemispheres or hippocampus. The brainstem is also more easily dissected in a few minutes by use of a scalpel, or other simplified sampling methods [26].

The most widespread use of the DRIT has occurred in North America, due to a need for improved wildlife rabies surveillance. It is worth noting that a key proof of concept for the oral rabies vaccination (ORV) of wildlife occurred during the 1960s to 1970s [27]. Since that time, ORV has emerged as a viable method for rabies management in multiple species of wild mesocarnivores, including red (Vulpes vulpes) and grey foxes (Urocyon cinereoargenteus), raccoon dogs (Nyctereutes procyonoides), coyotes (Canis latrans) and raccoons (Procyon lotor), at landscape scales. For example, the elimination of rabies in the red fox over broad areas of western Europe and eastern Canada, and the management of raccoon dog rabies in the Baltic region, as well as the elimination of the disease in coyotes in Texas (due to a focus on domestic dog sources in Mexico), and the prevention of any appreciable westward spread of raccoon rabies from the mid-Atlantic region of the USA, highlight the value of intervention with ORV in specific wildlife reservoir species [28, 29, 30, 31, 32, 33]. As with canine rabies prevention, the efficient and effective application of ORV requires a clear understanding of the spatial-temporal distribution of specific viral variants in real time. Independent human and domestic animal exposure-based surveillance alone does not provide the sampling scope and intensity to delineate rabies distribution among wild reservoir species, which is required to make sound ORV decisions. Moreover, due to uncertain economic pressures, many cooperating agricultural and public health laboratories have experienced budget reductions over the past decade, which inhibit their ability to process increasing numbers of suspected rabid wildlife samples by the FAT, beyond those that must be tested as a priority to protect public health. This course of events created a specialised niche for a simple, field-efficient, rabies diagnostic test that could be applied by trained biologists to evaluate an increasing sample burden – one unrelated to human or domestic animal exposures, which are the ostensible responsibility of local public health and agricultural officials.

To this effect, during 2004, limited enhanced rabies surveillance began near ORV zones in the USA, as a complement to routine public health surveillance, to ensure more sensitive decision-making for disease control programmes [34, 35, 36]. In addition, a paradigm shift took place in 2005, when the DRIT became available to United States Department of Agriculture Wildlife Services rabies biologists, who had been trained in basic testing procedures to provide diagnostic results based upon enhanced wildlife surveillance. After selected wildlife
biologists were given thorough training in biosafety, standard operating procedures, etc. the DRIT was implemented in earnest, with all positive samples (and 10% of random negative samples) of brain tissue being sent for confirmation by FAT. By 2008, DRIT proficiency testing was established with the Wisconsin State Laboratory of Hygiene as a standard means of providing quality assurance in comparison to the FAT. Between 2005 and 2011, by comparison to the approximately 100,000 suspect animal samples tested each year by public health laboratories, approximately 49,000 of more than 62,000 enhanced wildlife surveillance samples, collected in or within 80 km of ORV zones, were tested using DRIT, identifying the location of nearly 900 additional rabid animals to refine management strategies [25]. Enhanced wildlife rabies surveillance included the following types of samples obtained from free-ranging meso-carnivores, without a known human or domestic animal exposure history: animals brought to the attention of local field biologists, or collected by cooperators, with unusual behaviours suggestive of rabies, which typically have the highest rabies prevalence (among animals not involved in human exposure events); fresh road kills; animals live-captured in preparation for or during ORV monitoring, with gross lesions or behaviours suggestive of rabies; animals collected in close proximity to recently detected rabies cases that pose a risk to ORV success; and occasional nuisance animals trapped in areas of dense human habitation [32]. Based upon positive outcomes, DRIT remains a central method of rabies evaluation in wild meso-carnivores in the USA, with more than 60,000 samples tested by DRIT from over 77,000 suspected wildlife cases, collected for enhanced rabies surveillance by 2013. The use of DRIT has been similar in Canada, for enhanced ORV surveillance among suspect wildlife in both Ontario and Quebec, focused primarily on carnivores1.

Reference testing and inter-laboratory comparisons

Given the growing laboratory and field experience in the use of this test for domestic animal and wildlife rabies surveillance, additional evaluation of the DRIT took place at several OIE Rabies Reference Centres during 2013. A variety of lyssavirus isolates and animal species were compared in each laboratory. Both monoclonal and polyclonal conjugates were employed in the DRIT evaluation (Fig. 2). In this preliminary analysis, there was 100% concordance between the FAT and the DRIT, if the results from all individual monoclonal antibodies were considered together (Table IV).

Three additional findings were apparent from the comparative testing at the reference laboratories:

a) to avoid confusion, inter-laboratory harmonisation of procedures and standardisation of supplies are essential for any new test evaluation in comparison to a gold standard, as minor variations in biotinylation quality, the choice of reagents, fixation times, etc. may produce subtle differences in interpretation;

b) to prevent over-dilution, antibody conjugates must be titrated carefully to determine a standard working concentration, one which is more than adequate to cover the taxonomic breadth of the viral genus and locally important variants; and

c) to ensure the broadest possible range of sensitivity, a combination of at least two pan-reactive monoclonal antibodies should be considered, recognising different viral epitopes. Although scores of monoclonal antibodies have been generated during the past 35 years to differentiate among lyssavirus antigens, truly pan-reactive antibodies (e.g. WI 502, FLI 239.17, etc.) are uncommon.

Conclusions

As with the general acceptance of Negri body detection in the early 1900s, and its gradual replacement by the FAT during the later 20th Century, other tests for rabies diagnosis are expected to be developed throughout the 21st Century [2, 3, 37]. In addition to the use of other antigen detection or serological assays, molecular methods have increasingly proven their capability in the diagnostic arena, in support of FAT findings. While real-time polymerase chain reaction (RT-PCR) and next-generation sequencing offer high sensitivity and specificity when applied to lyssavirus diagnostics, and are decreasing in cost, such tests may be limited at the present time to centralised reference laboratories [38, 39]. It is unlikely that these applications will be readily available at the local
level in most developing countries, where canine rabies persists, and prevention, control and elimination plans are focused [6, 40, 41]. Point-of-care diagnostics, such as lateral flow assays, might permit rapid suggestions of viral detection in suspect animal fluids, but these are costly, lack adequate sensitivity and specificity for all lyssavirus species, and are totally dependent upon the importation of foreign devices [2, 3, 42].

Alternatives to fluorescence microscopy, such as the DRIT, offer comparable sensitivity and specificity to the current gold standard FAT; allow the observation of viral inclusions of the correct size, shape, distribution, colour and association in mammalian CNS tissues and provide concomitant antigenic typing of positive samples, while allowing the local development of monoclonal or polyclonal conjugates in a rapid, economic, flexible and reliable format [43]. Considering its promising results, the DRIT is an asset for all laboratory-based rabies diagnosis and, as such, will foster improved rabies surveillance in many parts of the world, particularly in developing countries, with support from reference laboratories. After further test validation and consideration by the OIE, the DRIT should, in the future, be approved as an OIE-prescribed and WHO-recommended test for rabies, to provide local diagnostic support for continuing efforts towards the global elimination of canine rabies.

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### Table IV

Comparison of lyssavirus diagnosis by the fluorescent antibody test and the direct rapid immunohistochemical test

<table>
<thead>
<tr>
<th>Lyssavirus</th>
<th>Fluorescent antibody test (FAT)</th>
<th>Direct rapid immunohistochemical test (DRIT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>ABLV(^{(a)})</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>BBLV(^{(b)})</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DUVV(^{(c)})</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>EBLV-1(^{(d)})</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>EBLV-2(^{(e)})</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IKOV(^{(f)})</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LBV(^{(g)})</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MOKV(^{(h)})</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>RABV(^{(i)})</td>
<td>92</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>116</td>
<td>66</td>
</tr>
</tbody>
</table>

\(a\) Australian bat lyssavirus; \(b\) Bokeloh bat lyssavirus; \(c\) Duvenhage virus; \(d\) European bat lyssavirus-1; \(e\) European bat lyssavirus-2; \(f\) Ikoma virus; \(g\) Lagos bat virus; \(h\) Mokola virus; \(i\) Rabies virus (samples included bats, camels, cats, cattle, deer, dogs, ferrets, foxes, hedgehogs, humans, jackals, mongoose, raccoons, rodents, sheep, skunks, wolves).
References


