



PHLN

Public Health Laboratory Network

Australian bat *lyssavirus* infection (Australian bat *lyssavirus*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for Australian bat *lyssavirus*.

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1. Introduction

A rabies-like virus of the genus *Lyssavirus* was isolated from the black flying fox (*Pteropus alecto*) in 1996. The virus was subsequently found in the other mainland Australian megachiropteran species namely the grey-headed flying fox (*Pteropus poliocephalus*), spectacled flying fox (*Pteropus conspicillatus*), and little red flying fox (*Pteropus scapulatus*), as well as an insectivorous microchiropteran species, the yellow-bellied sheathtail bat (*Saccolaimus flaviventris*). It is now assumed that all Australian bats have the potential to carry the virus, which phylogenetic analysis has demonstrated is a new genotype, genotype 7 in the *Lyssavirus* genus, and which is more closely related to serotype 1 classic rabies viruses than to other members of the genus.

The clinical syndrome of Australian Bat *Lyssavirus* (ABL) infection in bats is poorly defined, but anecdotal evidence suggests affected bats may exhibit weakness, paralysis, behavioural changes (docility or aggression, abnormal vocalisation), and dysphagia. Associated histologic lesions have included non-suppurative meningoencephalomyelitis and ganglioneuritis¹. Seropositive animals have also been regularly identified in the absence of clinical disease.

Fatal encephalitis attributable to infection with ABL has been diagnosed in a bat carer who had cared for both insectivorous and fruit bat species and in a second person who sustained an injury following an unprovoked attack from a wild fruit bat.

The natural route of transmission of ABL between bats, and from bats to humans is not yet resolved². Extrapolation from bat rabies where virus has been isolated from saliva and nasal mucosa has led to recommendation that where people are bitten, or even scratched, by bats in Australia and the bat is available for examination then it should be tested for evidence of ABL infection.

Detailed protocols for the management of human patients who have been exposed to bats have been agreed nationally and will not be repeated here³.

2. Laboratory diagnosis

Methods for the diagnosis of Australian bat *lyssavirus* infection are similar whether the sample is of human, bat or other animal origins and are similar to those used for classical rabies. Appropriate laboratories for assessment of samples include the Australian Animal Health Laboratory (AAHL), CSIRO Division of Animal Health, 5 Portarlington Rd, Geelong, 3212 (contact Duty Veterinarian) and Queensland Health Scientific Services, 39 Kessels Road, Cooper Plains, 4108 (contact Greg Smith). These laboratories will provide advice on sample submission and transport procedures.

In the case of domestic animals requiring exclusion of rabies virus infection specimens should be submitted to AAHL.

2.1 Clinical specimens

Animals: Following death from either natural causes or euthanasia, one portion of the longitudinally bisected unfixed brain should be submitted for direct antigen detection, virus isolation by tissue culture or mouse inoculation, and PCR. The remaining portion, fixed in 10% neutral buffered formalin, is suitable for immunocytochemical examination. Flying fox brains may be removed from the cranium at the laboratory conducting initial case assessment. If this is done operators need to minimise opportunity for cross contamination between successive bat specimens which, if it occurs, may lead to particular difficulty in interpretation of any subsequent PCR findings. In the case of specimens relating to potential human exposure, operators may prefer to submit the entire bat to the laboratory conducting specialist testing. In the much smaller microbats considerable architectural damage may occur to brains during removal so it is recommended these species should be submitted whole to the laboratory conducting any specialist testing. Other specimens such as salivary gland may be submitted but do not form part of the standard diagnostic procedure.

All animal specimens submitted for testing should be accompanied by a statement as to whether possible human exposure to ABL is involved. If there is no such statement, clarification should be sought from the person requesting the test. This assists in determining the priority given to testing of the specimen and the specific testing to be carried out.

Humans: In suspect human cases CSF collected during the clinical phase can be tested for virus by isolation and PCR as well as for anti-ABL antibodies. PCR and virus isolation can also be attempted on saliva of suspect human cases. Saliva is usually easy to obtain in significant volumes using non-invasive methods. It is recommended that saliva (1.0-5.0 mL) be centrifuged to pellet cell debris. Once the supernatant has been decanted the pellet can be resuspended in a small volume of sterile water. Both the resuspended pellet and saliva should be examined by PCR. The supernatant should be filtered through a 0.45 µm filter prior to virus isolation attempts. Nuchal and brain biopsy may also be carried out and frozen sections tested by immunofluorescence and PCR, and fixed tissues by immunoperoxidase and also PCR.

Serology for *lyssavirus* antibody determination may assist diagnosis in advanced clinical disease.

2.2 Rapid diagnosis – direct immunofluorescence

A provisional diagnosis of ABL infection may be obtained by detection of lyssaviral antigen by immunofluorescence in brain smears⁴. Adequate sampling is important to maximise the accuracy of this test and so the test is most applicable to post mortem diagnosis. At least four smears should be examined and these should include tissue from the following anatomic regions of the brain: cerebral cortex, cerebellum, hippocampus and medulla (brain stem). Suitability of specimens such as unfixed nuchal biopsies from humans is unknown.

- a. Suitable specimens – unfixed brain
- b. Sensitivity - > 99% for rabies virus relative to mouse inoculation test⁴.
- c. Specificity - will be affected by the commercial fluorescent antibody conjugate used, consult product insert.
- d. Predictive values – unknown
- e. Suitable test acceptance criteria – control slides of mouse brain infected with *lyssavirus* should show positive staining of *lyssavirus* antigen. Negative control smears from uninfected brains should show no evidence for conjugate binding. Sample processing should have produced satisfactory smears of the test material from the four CNS sites.
- f. Suitable internal controls – positive control slides containing *lyssavirus* infected cells should contain intracellular cytoplasmic apple-green fluorescence present as either finely scattered or as smoothly outlined inclusions. Negative control smears from uninfected mouse brains with added conjugate should appear negative with minimal background staining. The smears must be examined by two operators. Control smears may be pre-prepared from material held at – 80°C and slides stored at – 20°C.
- g. Suitable test validation criteria – Auditors should have available evidence of records of accession date of the diagnostic specimen and subsequent method of storage.
- h. Suitable external QC program – There is currently no external proficiency program for *lyssavirus* antigen detection in Australia. Participation of testing laboratory to internationally recognised standards (eg NATA accreditation) will ensure general quality assessment.
- i. Special considerations – Staff working with live rabies virus or clinical specimens that might reasonably be considered to contain ABL must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

Detection of ABL antigen by immunofluorescence in human tissue should be notified to health authorities in accordance with relevant legislation in the state in which the test was performed.

2.3 Isolation of ABL

Tissue culture infection tests have essentially replaced mouse inoculation tests (MIT) in rabies diagnosis for isolation of field strains as they are relatively easy to perform, less expensive than the MIT and results may be obtained more quickly⁵. Similarly, inoculation of mouse neuroblastoma cells may be performed for isolation of ABL. However, mouse inoculation has been useful for some virus isolations that were otherwise unsuccessful in cell culture.

- a. Suitable specimens – cerebrospinal fluid, brain biopsies (from humans) or unfixed brain collected as soon as possible after death. Isolation from unfixed nuchal biopsies from humans may also be attempted.
- b. Sensitivity – the neuroblastoma cell line should be one recognised as effective for isolation of *lyssavirus* (eg. Biowhittaker MNA line or ATCC Neuroblastoma 2a line). Cultures should be

maintained for 4 to 7 days prior to fixation and staining of cells. Test sensitivity is affected by specimen contamination with fungi or bacteria, preservation and storage conditions, and incubation period.

- c. Specificity – will be affected by the commercial fluorescent antibody conjugate used, consult product insert.
- d. Predictive value – unknown
- e. Suitable test acceptance criteria – Uninoculated control cultures should remain healthy over the course of the isolation period. Inoculated cultures should not show bacterial contamination or significant decline due to toxic effects of the inoculated sample.
- f. Suitable internal controls – Cell cultures set up at the same time as those for the test material, and maintained under the same conditions.
- g. Suitable test validation criteria – auditors should have available evidence of records of inocula and records of time specimen stored in the laboratory before inoculation.
- h. Suitable external QC program - participation in the National Association of Testing Authorities (NATA) quality control programs
- i. Special considerations - improper handling or storage of specimens may lead to leading to loss of infectivity and a *lyssavirus* negative isolation result despite an antigen/PCR positive test result on clinical material. Staff working with live rabies virus or clinical specimens which might reasonably be considered to contain ABL must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

Detection of ABL antigen by virus isolation from human tissue should be notified to health authorities in accordance with relevant legislation in the state in which the test was performed.

2.4 Identification of ABL by immunocytochemistry

ABL antigen may be identified in tissue sections by immunocytochemistry employing an anti-rabies Mab (clone 'HAM'). Immunopositivity has been observed in bat brains as round intracytoplasmic masses in large neurons and smaller round to ovoid masses in neuronal processes, cell bodies and in the neuropil throughout the central nervous system. The most frequent and intense staining is noted in the hippocampus, thalamus and midbrain, and medulla oblongata and pons. Immunopositivity may occur in the absence of detectable inflammatory lesions¹.

- a. Suitable specimens - brain tissue from animals or people. For postmortem samples from animals, at least one half of the longitudinally sectioned brain should be submitted, preferable collected as soon after death as possible and fixed in 10% neutral buffered formalin. The condition of the sample is important as autolysed brain makes the interpretation of the immunoperoxidase test very difficult. Adequate fixation is important and close attention to immediate fixation utilizing small pieces of tissue will help ensure this occurs. Staining in portions of the specimen where inadequate fixation has taken place will be non-specific and should be ignored. Detectable antigen varies in frequency and distribution thus wide examination of brain tissue is required. Brain and nuchal biopsies from clinically affected humans may also be submitted. The accuracy of this test performed on human nuchal and brain biopsies is unknown. Samples from the hippocampus and cerebellum should be included if possible.
- b. Sensitivity – Similar to FAT (close to 100%) where adequate sample size including representative areas of brain is available⁶.

- c. Specificity – The HAM MAb may not bind well to some *lyssaviruses* (eg. Lagos bat, Mokola). Close to 100% for rabies and closely related viruses.
- d. Predictive value – unknown
- e. Suitable test acceptance criteria – Appropriate positive immunostaining, present as intracellular cytoplasmic finely scattered or smoothly outlined inclusions, of a specimen known to contain *lyssavirus* antigen. A specimen, processed identically to the test specimen, that does not contain the antigen in question should be negative.
- f. Suitable Internal controls – Sections containing rabies antigen (positive control) and sections not containing rabies antigen (negative control) are included in each test. Positive control material is provided by brain tissue sections from mice infected with C.V.S. [Challenge virus strain]('fixed') strain of Rabies virus
- g. Suitable test validation criteria for ABL antigen detection by immunocytochemistry – records of mouse inoculation with CVS rabies virus, clinical signs, tissue harvesting and processing , and records of performance of test controls
- h. Suitable External QC Program - participation in the National Association of Testing Authorities (NATA) quality control programs
- i. Special considerations –

Detection of ABL antigen by immunocytochemistry in human tissue should be notified to health authorities in accordance with relevant legislation in the state in which the test was performed.

2.5 Serology – rapid fluorescent focus inhibition test (RFFIT)

The RFFIT is a virus neutralisation test used to detect the presence of rabies antibody⁷. As rabies and ABL are both serotype 1 *lyssaviruses* the RFFIT may also be used to detect anti-ABL antibody. The method utilises observation of foci of rabies infected cells which are observed by fluorescent antibody staining. It is more sensitive than the mouse neutralisation test in detecting antibody in rabies post vaccinal sera in people.

In rabies, seroconversion occurs late in the course of clinical disease. The relationship between clinical disease due to ABL and seroconversion is unclear in both bats and people. It should be remembered that apparently healthy bats may carry antibody to ABL and also that a negative result may not guarantee the animal is free of *lyssavirus*.

- a. Suitable specimen – non hemolysed serum or cerebrospinal fluid
- b. Sensitivity- Selection of cell type and quality, CVS-11 virus and reference positive standard will affect sensitivity.
- c. Specificity – Non specific neutralisation may occur at low serum dilutions so that evidence for neutralisation at levels of less than 0.5 IU/mL should be appropriately commented on in reports.
- d. Predictive value – unknown
- e. Suitable test acceptance criteria – Virus input, and reference positive and negative sera should fall within suitable limits. Test serum should not have disrupted the growth of the cell monolayer. Uninfected cell controls should be free of virus.
- f. Suitable internal controls – The WHO rabies reference standard should titre to expected levels.
- g. Suitable test validation criteria – Maintenance of records of sample handling and acceptable performance in the control charting of positive serum titre.

- h. Suitable external QC program - participation in the National Association of Testing Authorities (NATA) quality control programs
- i. Special considerations - Staff working with live rabies virus or clinical specimens that might reasonably be considered to contain ABL must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

Detection of ABL antibody in human CSF or serum should be notified to health authorities in accordance with relevant legislation in the state in which the test was performed.

Enzyme Linked Immunosorbent Assay ELISA

Sanofi Pasteur market an ELISA kit suitable for testing human serum which expresses antibody levels in international units and produces results comparable to those of the RFFIT. The antigen used in the tests is purified envelope glycoprotein obtained from inactivated virus preparations. The tests involves comparison of OD values obtained for specimens to a standard curve obtained by testing a dilution series of positive control serum of known IU/mL of anti-rabies antibody. ELISA readers must be capable of absorbent measurements at 492 nm. As the test utilizes a protein-A linked conjugate it can also be used for species such as dog, cat and mouse providing that the laboratory performing the test has access to appropriate positive control serum (of known IU/mL as determined by the RFFIT) of the relevant species.

- a. Suitable specimen – non hemolysed serum or plasma
- b. Specificity – Non specific reactivity may occur at low serum dilutions so that evidence for levels of less than 0.5 IU/mL should be interpreted with caution.
- c. Predictive value – unknown
- d. Suitable test acceptance criteria – reference positive and negative sera should fall within suitable limits
- e. Suitable internal controls – The supplied reference standards should titre to expected levels.
- f. Suitable test validation criteria – Maintenance of records of sample handling and acceptable performance in the control charting of positive serum titre.
- g. Suitable external QC program - participation in the National Association of Testing Authorities (NATA) quality control programs
- h. Special considerations - Staff working with live rabies virus or clinical specimens that might reasonably be considered to contain ABL must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

2.6 Molecular biology – Identification of ABLV by RT-PCR and sequence analysis

PCR is a newly developed test to detect the presence of the ABLV from a variety of tissues – either fixed or preserved by formalin fixative. The test has been utilised on autolytic tissue with some success, however this process usually causes degradation of nucleic acid such that it invalidates the test. The agent is inactivated by SDS/Phenol prior to extraction of the nucleic acid which is then precipitated by ethanol. Full conditions are given below⁸ for the preparation of complementary DNA and hemi-nested PCR reactions. Primers used to detect either the pteropid or insectivorous ABLV isolate in primary PCR are NP601-CAAATTTTAGATTTTTGGCTGGAAGT and NP220-GACTTTCCGCTAAGTCCTAG. Differentiation between the pteropid or insectivorous ABLV isolates is done by nested PCR using NP620-TATGACATGTTCTTTTCTCGAGT and NP830C-AGGAACGGCAGTTTCTTGTCCG (for insectivorous isolate) or NP620 with NP220 for the pteropid

isolate of ABLV. PCR conditions are denaturation: 94°C for 1 min.; annealing: 37°C for 2 min and an extension time of 2min at 72°C. Primary PCR is done for a total of 40 cycles with nested PCR done for 25 cycles. Expected product size for the primary product is 290 b.p. and for the nested products are 219 and 263 for insectivorous and pteropid ABLV respectively. Sequence analysis is required for confirmation of product specificity.

- a. Suitable specimens – cerebrospinal fluid, saliva, formalin fixed tissue, fresh tissues ie nuchal biopsy, brain and salivary gland inactivated by homogenisation, phenol-chloroform/SDS extraction.
- b. Sensitivity: Nested PCR usually required to amplify nucleic acid sequences. One tube PCR reactions as well as multiplex PCR reactions have been utilised but lack the sensitivity of single tube PCRs^{8,9}.
- c. Specificity: Specific for the insectivorous and pteropid isolates of ABLV
- d. Predictive value –
- e. Suitable test acceptance criteria – sequence identity with known ABLV isolates
- f. Suitable internal controls: Tissue known to either i) contain ABLV or ii) free of the agent able to generate a PCR fragment that results in appropriate sequence upon DNA sequence analysis
- g. Suitable test validation criteria – Sequence analysis of amplified DNA
- h. Suitable external QC program - participation in the National Association of Testing Authorities (NATA) quality control programs
- i. Special considerations - Staff working with live rabies virus or clinical specimens that might reasonably be considered to contain ABL must have a rabies vaccine induced antibody titre of at least 0.5 IU/ml. This level should be checked at intervals of six months.

Detection of ABL by RT-PCR in human tissue should be notified to health authorities in accordance with relevant legislation in the state in which the test was performed.

3. Summary

Fatal encephalitis attributable to ABL infection has been identified sporadically in the Australian population. In each case there has been history of direct contact with bats. It is advised that when people are bitten or scratched by bats in Australia then, where possible, the bat should be tested for evidence of ABL infection to assist in guiding post-exposure prophylaxis.

In suspected human clinical cases, specimens suitable for direct immunofluorescence, virus isolation, immunocytochemistry, serology and/or RT-PCR should be collected for confirmation of the diagnosis. These should be submitted to the Australian Animal Health Laboratory (AAHL), CSIRO Division of Animal Health, 5 Portarlington Rd, Geelong, 3212 (contact Duty Veterinarian) or Queensland Health Scientific Services, 39 Kessels Road, Cooper Plains, 4108 (contact Greg Smith). These laboratories will provide advice on sample submission and transport procedures.

4. References

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5. PHLN laboratory definition

5.1 Condition

Meningoencephalitis in people and animals caused by Australian bat *lyssavirus*.

5.1.1 Tests

Definitive Criteria

- a. Appropriate result from sequence analysis of PCR product derived from cerebrospinal fluid, nuchal biopsy, brain, salivary gland, saliva, tissue culture supernatant or formalin fixed tissue.

Suggestive Criteria

- a. Positive fluorescent antibody test result for lyssaviral antigen on fresh brain smears.
- b. Isolation of *lyssavirus* from central nervous system tissue, cerebrospinal fluid, saliva, and/or salivary gland.
- c. Specific immunostaining for lyssaviral antigen of formalin fixed paraffin sections of central nervous system tissue.
- d. Presence of antibody to serotype 1 *lyssaviruses* in serum or cerebrospinal fluid.