Australian Bat Lyssavirus

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SUMMARY

Australian bat lyssavirus (ABL) is a potentially lethal zoonotic virus, which is closely related to rabies virus. Standard methods for the diagnosis of infection with rabies virus have been modified for the diagnosis of ABL infection. Since there are no pathognomonic clinical signs of disease or gross lesions, a definitive diagnosis can be made only in the laboratory. The tissues of choice for the preferred laboratory tests include central nervous system (CNS) tissue from four standard sites within the brain.

Identification of the virus

The fluorescent antibody test (FAT) is the preferred test for exclusion of ABL. A drop of anti-lyssavirus immune serum, previously conjugated with fluorescein isothiocyanate, is added to a fixed smear of brain tissue, preferably made from four sites within the brain including the parietal cortex, hippocampus, cerebellum and brainstem at the level of the caudal cerebellar peduncles. The FAT provides a reliable diagnosis in 98-100% of rabies cases, and is likely to be similar for ABL cases. However, since a single negative FAT on fresh material does not rule out the possibility of infection, further tests may need to be done simultaneously. Results are expressed in International Units relative to a standard antiserum.

Microscopic appearance may reveal aggregates of ABL material (equivalent to Negri bodies in rabies virus infections) in the cytoplasm of neurones. The sensitivity of methods for microscopic examination depends on the degree of autolysis of the specimen: up to 15% false-negative results occur with tests of autolysed tissues submitted for diagnosis of rabies virus.

Virus isolation may be attempted on a monolayer culture of neuroblastoma cells inoculated with a suspension of brain tissue. A FAT on the cells carried out 48-72 h later will show the presence or absence of viral antigen. Alternatively, newborn mice or 3- to 4-week-old mice that are inoculated intracerebrally with a suspension of brain tissue are observed for 28 days. A FAT is carried out on tissues from the brain of all mice that die between 5 and 28 days after inoculation.

ABL can be identified in specialised laboratories by using a polymerase chain reaction (PCR) followed by DNA sequencing of the PCR product.

Serological tests

Virus neutralisation (VN) assays may be conducted in cell cultures. Results are expressed in International Units relative to a standard antiserum.

Status of Australia and New Zealand

ABL was first identified in bats in Australia in 1996, but retrospective studies on stored tissues from bats have demonstrated that the earliest known case was in early 1995. ABL has not been identified in New Zealand. In Australia, viral antigen has been detected in the central nervous system (CNS) of the four major species of fruit bats and in one species of insectivorous bat throughout the combined geographical range of these species. Disease associated with infection

has been reported in bats and two humans. Current Australian recommendations are that any Australian bat that bites or scratches a person should be tested for ABL antigen and the person should be offered appropriate treatment. In addition, persons who are exposed to bats through their profession or recreation should undergo prophylactic vaccination against rabies virus and have the titre of their serum antibodies checked.

Introduction

Aetiology

ABL belongs to genotype 7 of the genus *Lyssavirus* within the family Rhabdoviridae. There are six other genotypes in the genus including: classical rabies virus (genotype 1), Lagos bat virus (2), Mokola virus (3), Duvenhage virus (4), and European bat lyssaviruses 1 and 2 (genotypes 5 and 6, respectively).[1,2] In addition, the genus has been subdivided into four serotypes (1, rabies virus; 2, Lagos bat virus; 3, Mokola virus; 4, Duvenhage virus).[3] Neutralisation studies suggest that ABL belongs to serotype 1.[2,4]

Currently, ABL is known to occur only in Australia where it has been associated with the deaths of fruit bats, insectivorous bats and two humans.[4-7] Because ABL is a potentially lethal zoonotic virus, current Australian recommendations are that any Australian bat that bites or scratches a person should be tested for ABL antigen and the person should be offered appropriate treatment. In addition, persons who are exposed to bats through their profession or recreation should undergo prophylactic vaccination against rabies virus, and have the titre of their serum antibodies checked.[8] A strategy for the control of ABL in domestic animals and captive bat colonies is described in AUSVETPLAN.[9]

Epidemiology

In Australia, bats, particularly the four major species of fruit bat (*Pteropus poliocephalus*, *P alecto*, *P scapulatus* and *P conspicillatus*) and an insectivorous bat (*Saccolaimus flaviventris*), appear to be the main reservoir of ABL.[4,5] Antigen has been demonstrated in the CNS of many individuals, but there are no published data on the prevalence of ABL antibodies in the sera of free-flying fruit bats. In addition, there is little information on the disease, or the prevalence of ABL, in insectivorous bats in Australia. The only recognised cases in terrestrial mammals have been in two humans.

Transmission of ABL from bats is not fully understood. Of the two human victims, one was a bat-carer who worked closely with bats, and the other was attacked by an aggressive, free-flying bat.[6,7] While the latter case suggests that transmission of ABL, as in rabies, may be from the bite of an infected animal, the means of transmission is unclear in the former case. It is relevant that, between 1980 and 1997, 21 human cases of rabies in the USA were shown to have originated from insectivorous bats and, in almost all cases, the manner of transmission was not identified.[10,11]

The significance of antibodies to ABL that are detected in any animal is unknown because of a lack of information about the dynamics of the immune response to ABL in any species. Seroconversion to the closely-related rabies virus does not occur in terrestrial mammals until disease is advanced and death seems inevitable. The presence of healthy, free-flying bats that are seropositive suggests that ABL may not kill all bats.[5]

Clinical Signs

Although not all bats infected with ABL develop clinical signs of disease, most that do show neurological signs.[4,5,12] Paralysis or paresis of the limbs are the most obvious findings, and affected bats may be depressed and dull. Clonic muscle spasms, aggression and altered vocalisation have also been reported.

Abnormal neurological signs and symptoms, including paraesthesia at the site of infection, headaches, dizziness, muscle weakness, depression and eventual loss of reflexes characterised the two human cases of ABL infection.[6,7]

Gross pathology

Although it appears that ABL, like rabies virus, is highly neurotropic, there is little information on the pathogenesis of the disease. There are no characteristic gross lesions in an infected animal, but microscopic examination of the brain, spinal cord, and cranial and spinal ganglia may reveal a nonsuppurative meningoencephalomyelitis and ganglioneuritis.[4,5,13] Intracytoplasmic inclusion bodies (Negri bodies) have been described in neurones of bats and humans. While lesions may vary from very severe to almost absent, there is no obvious correlation between the severity of

the lesions and the severity of abnormal clinical signs. Viral antigen may occasionally be detected in epithelial cells of salivary glands of bats, but microscopic lesions have not been seen in these tissues.

Diagnostic Tests

Although abnormal clinical signs and/or microscopic lesions may suggest ABL infection, definitive diagnosis depends upon detection of virus, viral antigen or viral genome in affected tissues. Specimens of choice are brain, spinal cord, and cranial or spinal ganglia.

Seroconversion may also be a useful indicator of ABL infection of bats, although FAT-positive, seronegative bats have been identified. In humans, seroconversion did not occur until clinical disease was advanced, at which stage, in rabies, there is usually a very poor prognosis.

Shipment of samples

For small domestic and wild animals, the whole carcase should be transported to the laboratory. For large animals, the head may be removed. There should be no risk of contamination of humans during the shipment of suspected material for diagnosis. Samples must be transported in a plastic bag in an insulated box, with refrigerant, in a container that is leak-proof and rigid. Transport regulations for dangerous materials must be observed.

If fresh tissues cannot be submitted to the laboratory, then formalin-fixed samples may be sent. Since formalin inactivates ABL, diagnosis can then be attempted only by using a FAT or microscopic examination.

Collection of samples

Usually the brain is removed from the skull in a necropsy room. In most cases, the brain is then divided along the midline, with half being fixed in 10% buffered formalin, and half remaining unfixed for fresh impression smears and for virus isolation. Tissues that are sectioned and examined include the parietal cortex, the hippocampus, the cerebellum and the brainstem at the level of the caudal cerebellar peduncles. When the skull cannot be opened safely, there are two methods for sampling the brain which are used in rabies cases and that may be used in suspect cases of ABL:

i) Occipital foramen route – A 5 mm drinking straw[14] or a 2 mL disposable plastic pipette[15] may be introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex and medulla oblongata.

ii) Retro-orbital route –The posterior wall of the orbit is punctured with a trocar and a plastic pipette is introduced through the hole.[16] The sampled parts of the brain are the same as in the first method, but in the reverse order.

Routine laboratory tests

When samples are submitted for exclusion of ABL, the minimum screening procedures should include the FAT and microscopic examination. Virus isolation may be reserved for samples that have given positive results in preliminary tests. Polymerase chain reaction (PCR) for the identification of virus strains is generally restricted to specialised laboratories.

Microscopic identification of characteristic lesions

Characteristic microscopic lesions in the CNS are suggestive, but not definitive, of an ABL infection. Intracytoplasmic, eosinophilic inclusion bodies in neurones (equivalent to the aggregates of viral protein known as Negri bodies in rabies virus-infected cells) also provide evidence of infection. The current immunohistochemical test detects a number of lyssaviruses, including ABL (and rabies virus).[4]

Immunochemical identification of ABL antigen

i) Fluorescent antibody test

The most widely used test for ABL diagnosis is the FAT.[17] This test uses a fluorescein-conjugated antibody preparation that specifically reacts with some lyssaviruses, including ABL. It may be used directly on an impression smear (of brain usually) or in cell culture to confirm the presence of ABL antigen. The FAT gives reliable results on fresh specimens within a few hours. For direct ABL diagnosis, impression smears are fixed in analytical grade acetone at –20°C, and then stained with the specific conjugate. The FAT is described in Appendix 1.

If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with an enzyme.[18,19]

ii) Immunochemical tests
A monoclonal antibody (such as HAM) may be used in an indirect immunoperoxidase method for the identification of lyssavirus antigens on sections of formalin-fixed tissue.[20]

Detection of the replication of live ABL after inoculation

These tests detect the infectivity of a tissue suspension for tissue culture or an animal.

i) Cell culture test
A neuroblastoma cell line from the American Type Culture Collection (ATCC) can be used for routine diagnosis of ABL.[21,22] This cell line is sensitive to isolates without any adaptation. Replication of ABL in the cells is shown by the FAT after incubation for 48 to 72 h.

This test is as sensitive as and cheaper than the mouse inoculation test, which takes longer.

ii) Mouse inoculation test
Specific pathogen-free (SPF) mice are the animals of choice for this test. Five to ten mice, 3- to 4-weeks-old, or a litter of 2-day-old newborn mice, are inoculated intracerebrally. The inoculum is the clarified supernatant fluid of a 20% (w/v) homogenate of brain material in an isotonic buffered solution containing antibiotics. Inoculated mice are observed for 28 days, and every dead mouse is examined for ABL using the FAT.

Other identification tests

The preceding tests may be complemented in specialised laboratories by the PCR followed by DNA sequencing of the PCR product for typing the virus. Typing enables isolates from insectivorous bats to be differentiated from those in fruit bats.[5]

Serological tests

A serological test was used to confirm a tentative clinical diagnosis in both human cases of ABL infection.[6,7] However, such a test is currently of little value for infections of domestic or wild animals because, as already mentioned, there is insufficient information about the dynamics of the immune response to ABL in these species.

A virus neutralisation test, based on a similar test to that used in rabies diagnosis,[23] is recommended.

Virus neutralisation test in cell culture – rapid fluorescent focus inhibition test

The principle of the RFFIT is the neutralisation in vitro, by serum anti-ABL antibodies, of a constant amount of rabies virus (CVS strain adapted to cell culture) before inoculating onto cells susceptible to rabies virus (Neuro 2a or BHK-21).

The serum titre is the dilution at which the virus is neutralised in 50% of the wells. This titre is expressed in International Units by comparing it with the neutralising dilution of a standard serum under the same experimental conditions.

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Appendix 1

Fluorescent Antibody Test (FAT) for lyssavirus antigen

Materials

Sterile instruments, clean glass microscope slides, slide jars, tissue homogeniser, tongue depressor sticks, 0.2 µm filter, Analytical Reagent Grade acetone (at -20°C), PBS 7.2, buffered neutral formalin.

Lyssavirus positive and negative control smears.

Commercially available FITC-anti-rabies conjugate, diluted for use according to manufacturers' instructions.

Procedure

Consult the reference for more detailed descriptions.[17]

All procedures are to be carried out by appropriately trained personnel who have been vaccinated for rabies.

All potentially infectious work is to be carried out in a Class II biosafety cabinet.

All waste materials are appropriately decontaminated before disposal.

(a) Unpack specimen in the biosafety cabinet and discard packing materials for autoclaving. Note general condition of sample. Label slides, centrifuge tube and formalin container.

(b) Remove the brain from the cranial cavity in a necropsy room. In most cases, the brain is then divided along the midline, with half being fixed in 10% buffered formalin, and half remaining unfixed. Tissues that are sectioned and examined include: the parietal cortex, the hippocampus, the cerebellum and the brainstem at the level of the caudal cerebellar peduncles. Excise portions of the following tissues:
  - medulla (brain stem)
  - cerebellum
  - hippocampus
  - cerebral cortex

(c) Make impression smears of each tissue onto clean, labelled slides. Place the tissue onto a tongue depressor with a cut surface facing upward. Touch the surface with a second tongue depressor to dry and remove debris. A clean microscope slide is gently touched to the cut brain surface to form a thin tissue impression on the slide. An alternative 'smear' method has been described[17] that is useful for small brain samples, or when post mortem degeneration has caused the brain tissue to soften.

(d) Allow the slides to dry in air in the cabinet, and fix in acetone chilled at -20°C for 30 min.

(e) Store portions of the samples of each tissue at -80°C. A second portion of each brain tissue is pooled, homogenised as a 10% suspension in PBS and clarified for virus isolation and PCR. For small brains (for example, bats), fix half the brain in buffered formalin for microscopic examination and immunohistochemistry. For larger brains, fix portions of the selected regions.

(f) Dry the fixed smears in air. Using about 2.5 mL of conjugate and a syringe with a 0.2 µL filter, place drops onto the four test smears on slides and onto the positive and negative control smears on slides.

(g) Incubate the slides in a humid container at 37°C for 30 min.

(h) Gently rinse all slides with PBS twice. Rinse control smears separately and apart from the test slides.

(i) Drain the slides, dry in air and mount under a coverslip with glycerol mounting medium. Examine under UV transillumination.

Interpretation

Positive control smear and positive test samples will contain small, brilliantly fluorescing, apple green particulate points and larger cytoplasmic inclusions.

Appendix 2

Immunoperoxidase staining for lyssavirus antigen

Materials

Antibodies: Anti-rabies RNP monoclonal antibody (for example, HAM, which is in limited availability from AAHL), negative control monoclonal antibody.

Secondary antibody: Anti-mouse antibody conjugated with horse radish peroxidase (HRP).

Tissue sections: formalin-fixed paraffin-embedded test sample and infected (positive control) and uninfected (negative control) brain.

Buffers: PBS diluent (0.1% skim milk powder in PBS)

Stains and substrates: 3-amino-9-ethylcarbasole (AEC), haematoxylin and Scott's blueing solution.

Aqueous mounting medium.

Procedure

(a) Dewax slides of test sample, and positive and negative controls. Rehydrate to water. Rinse slides in PBS.

(b) Apply 200 µL of a 1:100 dilution of the anti-rabies monoclonal antibody to test and control slides. Similarly apply the negative control monoclonal antibody to slides of test sample slide and positive and negative controls slides. Cover and incubate at 37°C for 1 hour.

(c) Rinse slides in PBS. Treat slides with 200 µL of 3% H_2O_2 for 20 min at room temperature. Rinse in PBS.

(d) Apply 2 to 3 drops of anti-mouse antibody conjugated with HRP and incubate at 37°C for 20 min.

(e) Rinse in PBS and apply AEC substrate.

(f) Check control slides for sufficient staining, and, after 2 to 5 min, stop reaction by rinsing in distilled water.

(g) Counter stain in haematoxylin for 3 min, and rinse in tap water. Rinse in Scott's blueing solution if necessary, and then in tap water.

(h) Rinse slides in distilled water, then mount in aqueous mounting medium.

Interpretation

Read slides for focal or diffuse deposition of the chromagen indicating the presence of lyssavirus antigen.

Appendix 3

Virus Isolation

Materials

Cells: Mouse Neuroblastoma cells (for example, ATCC CCL 131 Neuro-2a) at 1×10^6 cells per mL in medium + 1% foetal calf serum.

Media: MEM, FCS

Cell culture plasticware: 25 cm^2 flasks

Tissue homogenate (see Appendix 1)

Procedure

(a) Add 0.5 mL of tissue homogenate to 1 mL of cells. Separately hold 1 mL of cells for seeding a control flask.

(b) Incubate at 35°C for 1 h with occasional shaking.

(c) Make control and inoculated cells up to 8 mL volumes by addition of MEM + 10% serum.

(d) Add to 25 cm^2 flasks and incubate at 35°C for 3 to 5 days.

(e) Dislodge cells into the supernatant medium by tapping flask and remove half of the medium to a sterile centrifuge tube.

(f) Gently centrifuge the suspension at 1000 g for 5 min, and decant the supernatant into the original flask.

(g) Resuspend the cell pellet in a small volume of PBS (about 200 µL) and smear about 50 µL on a clean glass slide.

(h) Dry the smear in air, fix in acetone and proceed with staining for lyssavirus antigen as described in Appendix 1.

(i) Positive isolates will show distinctive fluorescence. Store isolates at -80°C and re-seed infected cells with fresh Neuro-2a cells to further adapt the isolate to growth in cell culture.