ARTICLE

Despite advances in treatment, rabies remains endemic in many countries around the world. Here, Richard A Collins takes a close look at a disease that is the leading cause of mortality due to infection in China.

Rabies in China
Diagnosis, treatment and prevention

For centuries, rabies has been a terrifying disease associated with horrific symptoms and a high mortality rate. Although rabies was eradicated in the UK over a century ago, a fatal case of bat-derived rabies in Scotland in 2003 reminds us that we should not be complacent about the re-emergence of this disease.[1]

Such complacency is unknown in China, where rabies is responsible for over 1000 deaths per year[2] and is the leading cause of mortality from infectious disease. As China is becoming an increasingly popular tourist destination, the diagnosis and treatment of rabies is worth reviewing.

The disease

Rabies is an incurable, uniformly fatal viral encephalitis. There are 30,000–70,000 human deaths from rabies reported every year.[3] The vast majority of deaths occur in Asia and primarily India.[4] In the USA alone, some 25,000–40,000 people are treated annually for exposure to rabies.[3] In China, the number of deaths has fallen from 4000–6000 per year to around 1000 per year, but recently this figures has begun to rise again (Fig 1).

The cause of disease in most cases is a bite from a rabid dog, although any mammal may harbour and transmit the virus. Deaths from inhalation of aerosolised virus have occurred in potholers and cave explorers and researchers in places where deposits of bat faeces build up. In 2004, three people died after contracting rabies from the transplanted liver and kidneys of a man whose rabies infection had not been identified.[5]

The rabies virus replicates in the central nervous system (CNS), having reached it by travelling along the peripheral nerves closest to the site of infection. Five clinical stages of disease are recognised: incubation, prodrome, an acute neurological period, coma and death.

In general, the incubation period is inversely related to the size of the inoculum, the degree of innervation and proximity of the bite to the CNS. The incubation period is generally between 20–90 days (range: four days to several years).

Clinical symptoms are first noted during the prodromal period, which usually lasts for two to 10 days. The symptoms are often non-specific (general malaise, fever and fatigue) or suggest involvement of the respiratory system (sore throat, cough and shortness of breath), gastrointestinal system (anorexia, difficulty in swallowing, nausea, vomiting, abdominal pain, and diarrhoea) or CNS (headache, vertigo, anxiety, apprehension, irritability and nervousness). More severe symptoms (agitation, photophobia, priapism, increased libido, insomnia, nightmares and depression) may also occur, suggesting encephalitis or psychiatric disturbances. The acute neurological period begins with objective signs of CNS dysfunction.

Furious rabies is a rapidly fatal brainstem encephalitis characterised by hydrophobia or aerophobia (fear of fresh air or draughts), hyperactivity and fluctuating consciousness. Bizarre behaviour is a typical feature of this stage of the disease. The disease is almost always fatal and without intensive care the patient will die within a few days. Paralytic (dumb) rabies runs a less-dramatic course but the final outcome is the same.

‘A fatal case of bat-derived rabies in Scotland in 2003 is a reminder that complacency about the disease is unwarranted’

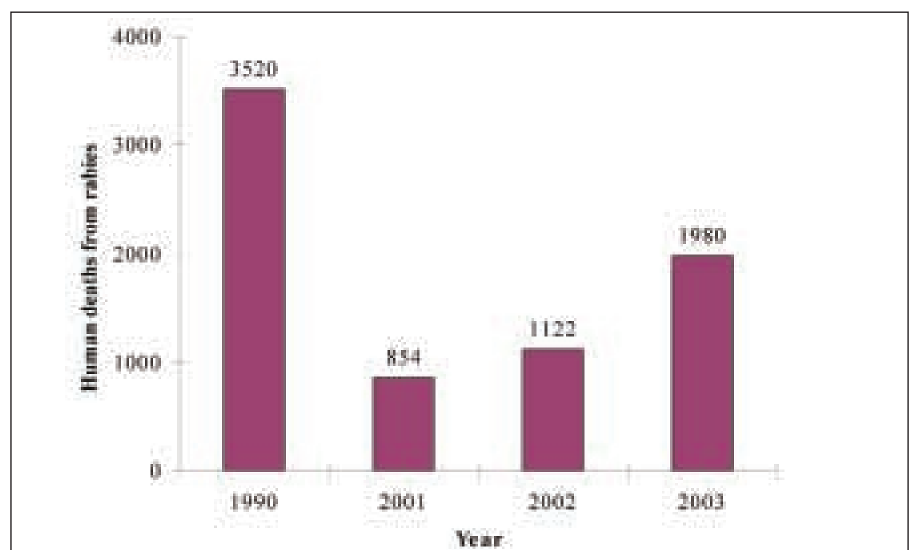

Fig 1. Number of human rabies deaths in China. Data extracted from references 2, 18 and 19.

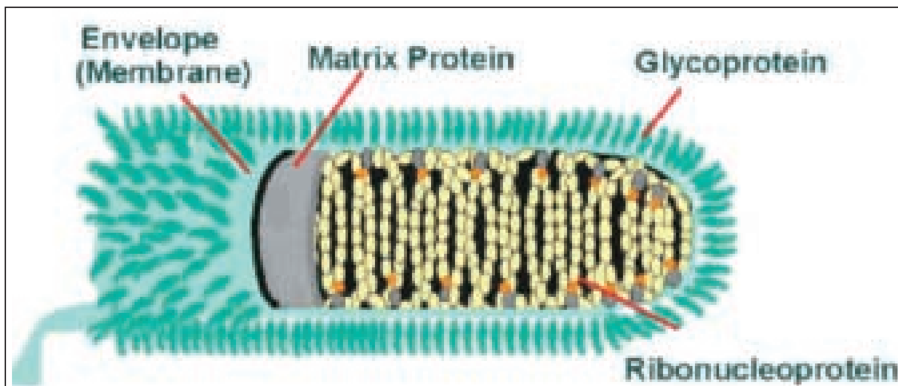

Fig 2. Diagrammatic representation of the rabies virion.

‘Rabies virus replicates in the central nervous system after travelling along the peripheral nerves from the site of infection’

Flaccid ascending paralysis with pain and twitching in the affected muscles and mild sensory disturbances precede death from bulbar and respiratory paralysis. At the end of the acute neurological phase, periods of rapid, irregular breathing may begin, but paralysis and coma soon follow.

Although life-support measures can prolong the clinical course of rabies, rarely will they affect the outcome of the disease. Only six cases of human ‘recovery’ have been documented, and all but one followed administration of pre-or post-exposure immune therapy.

The virus

Rabies virus is a member of the *Rhabdoviridae* family, genus *Lyssavirus*. Seven distinct genetic lineages can be distinguished within the genus by cross-protection tests and molecular biological analysis. The lineages are classical rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssaviruses 1 and 2 and the Australian bat lyssavirus.

The rabies virion is bullet-shaped (Fig 2), has a mean length of 180 nm (range: 130–300 nm) and a diameter of 75 nm. Spike-like projections (10-nm long) in the viral envelope can often be seen by electron microscopy.

The rabies genome comprises a single-stranded, non-segmented negative-sense RNA molecule about 11,900 nucleotides long. After penetrating the host cell, the genomic RNA must be transcribed into a complementary positive-sense molecule to permit the production of five viral proteins (Table 1).

Diagnosis

Several tests are required for the ante-mortem diagnosis of rabies in humans; no single test is sufficient. Tests are performed on samples of saliva, serum, spinal fluid and skin biopsies of hair follicles at the nape of the neck. Saliva can be tested by virus isolation or a reverse transcription polymerase chain reaction (RT–PCR) technique. Serum and spinal fluid are tested for antibodies to rabies virus. Skin biopsy specimens are examined for rabies antigen in the cutaneous nerves at the base of the hair follicles.

Table 1. Properties of rabies virus proteins.

PROTEIN	NAME	LENGTH (AA)	REMARKS
N	Nucleoprotein	450	Closely associated with viral RNA.
NS, P	Non-structural protein (NS), phosphoprotein (P)	297	Binds to N protein to regulate virus transcription and replication and virus assembly.
L	RNA-dependent RNA polymerase	2142	Transcription of viral genome.
M	Matrix protein	202	Located on virion inner membrane. Regulates RNA synthesis.
G	Glycoprotein	524	Inserted into virion phospholipid membrane. Primary structural component of surface spikes. Internal portion associated with M protein. Responsible for the induction of virus-neutralising antibodies and T-cell stimulation. Heavily glycosylated.

Antigen detection

The standard diagnostic test in use currently is the direct fluorescence antibody (dFA) test. This test uses post-mortem brain tissue from animals suspected of being rabid. The dFA test has been thoroughly evaluated for more than 40 years and is recognised as the quickest and most reliable of all the tests available for routine use. Other tests for diagnosis and research, such as electron microscopy, histological examination, immunohistochemistry, RT-PCR and isolation in cell culture are useful tools for studying virus structure, histopathology, molecular typing and virulence of rabies viruses.

The dFA test is based on the observation that animals infected by rabies virus have rabies virus antigen present in their tissues. As the rabies virus is only present in nervous tissue (unlike many other viruses that are present in blood), the ideal tissue to test for rabies antigen is brain. The most important component of the dFA test is the fluorescence-labelled anti-rabies virus antibody. The rabies antibody used in the dFA test is directed mainly against the virus nucleoprotein.

When labelled antibody is incubated with brain tissue suspected of being infected with rabies, it will bind to the rabies antigen. Unbound antibody is washed away and the antigen is visualised as apple-green fluorescent areas under a fluorescence microscope (Fig 3a). Absence of staining is apparent if rabies virus is not present (Fig 3b).

Antibody detection

Mouse neutralisation test

In the mouse neutralisation test (MNT), serum is diluted over a known range and a constant amount of challenge virus is added. The virus/serum mixtures are then inoculated intracerebrally into groups of eight young adult mice. The percentage of mortality is determined and the serum dilution that protects 50% of the animals is calculated. In the absence of serum antibodies, mouse mortality will be high even at the lowest dilution tested.

Rapid fluorescence focus inhibition test

In the rapid fluorescence focus inhibition test (RFFIT), serum samples are diluted over a known range and added to 96-well

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microplates to which is added a constant dose of challenge virus. The sera/virus mixtures are incubated at 37°C for an hour, then rabies virus susceptible cells (baby hamster kidney [BHK] cells) are added and incubated for a further 24 hours. The BHK cell monolayer is fixed with acetone and stained with a fluorescence-labelled antibody in order to detect the presence of non-neutralised virus (foci of fluorescence). In the absence of serum antibodies, the challenge virus will bind to the BHK cells and give rise to an intense fluorescence.

Latex agglutination test (LAT)
In the latex agglutination test (LAT), latex beads are coated with purified rabies glycoprotein. The sensitised beads are then used to detect anti-glycoprotein antibodies in the sera of people who have taken a course of post-exposure vaccine. A positive reaction results in visible agglutination of the beads. This simple test shows 100% specificity and 95% sensitivity compared with the standard MNT. The LAT could be useful in monitoring seroconversion after vaccination.[6]

Enzyme-linked immunosorbent assay
An enzyme-linked immunosorbent assay (ELISA) to detect rabies antibodies from immunised humans was developed in 1977.[7] The antigen used was derived from a semi-purified tissue culture virus. An ELISA method for the quantification of the rabies glycoprotein was demonstrated in 1981 and was used for quality control purposes for the laboratory production of inactivated rabies vaccine.[8] Commercial ELISA tests to quantify the anti-glycoprotein antibody titre in serum or non-haemolysed plasma from immunised humans and animals are readily available.

Subsequently, a variant ELISA known as the rapid rabies enzyme immunodiagnosis (RREID) test, based on the detection of purified rabies virus nucleocapsid antigen in brain tissue, was developed.[9] This technique was not as sensitive as dFA, although it was simple and relatively cheap to perform. The endpoint of the enzyme reaction can be visualised with the naked eye, eliminating the need for an expensive fluorescence microscope. However, it cannot be used with formalin-fixed samples.

Fig 3. Direct fluorescence antibody (dFA) test for rabies antigen. Source: National Center for Infectious Diseases, CDC, Atlanta, USA.

Fig 4. Bait labelled with rabies vaccine.

Improvements in ELISA are constantly reported in the literature. A qualitative indirect ELISA to measure rabies virus specific antibodies from vaccinated cats and dogs has been described.[10] The test can be completed in four hours, without the need for specialised laboratory containment.

Virus isolation

The reference method for rabies virus isolation in cell culture utilises mouse neuroblastoma cells. Cell culture studies using whole virus are useful for determining the kinetics of infection, pathogenesis, receptor binding, apoptosis and other properties of both fixed laboratory strains and new field isolates. Other cell types, for example McCoy cells (mouse fibroblasts), are equally effective for the isolation of rabies virus but have not been adopted widely.[11]

Nucleic acid detection

The first PCR technique for rabies virus detection was described in 1990.[12] Rabies virus RNA extracted from infected mouse brain was reverse transcribed and amplified. Further studies used the rabies nucleoprotein and glycoprotein genes as the targets for amplification. *In situ* RT-PCR has been used to detect rabies virus RNA in mouse neuroblastoma cells.[13] Taqman RT-PCR methods have been described and used to differentiate six established rabies genotypes and rabies-related viruses.[14,15]

Other amplification techniques such as nucleic acid sequence-based amplification (NASBA) have also been described, although they appear to be less sensitive than general RT-PCR techniques.[16] The NASBA technique was used to detect rabies virus RNA extracted from 10 mg infected brain

Table 2. Rabies vaccines.

SUBSTRATE	RABIES VIRUS STRAINS USED	REMARKS
Neural tissue vaccines		
Sheep/ goat brain	Pasteur virus Pitman-Moore	Serious adverse events due to presence of myelin. Lower protective potency than cell-derived vaccine. Post-exposure treatment may require 17–23 injections. Cannot be used for pre-exposure protection. Shelf life about six months.
Suckling mouse brain	Challenge Virus Standard (CVS) Strain 51 Strain 91	Serious adverse events. Lower protective potency than cell-derived vaccine. Post-exposure treatment may require 17–23 injections. Cannot be used for pre-exposure protection.
Non-neural tissue vaccines		
Chick embryo cell	Flury LEP (low egg passage) -25	Live attenuated vaccine. Neutralising antibody response comparable with human diploid cell vaccines. More than 30 million doses administered.
Duck embryo cell	Pitman-Moore	Inactivated vaccine. Virtually free of serious side reactions. Low potency (>20% of vaccinees fail to develop antibodies after pre-exposure vaccination). No longer manufactured
Chick embryo cell	Flury HEP (high egg passage)	Live attenuated vaccine. Available in Japan only.
Cell culture vaccines		
Rhesus monkey lung fibroblast	CVS-11 Kissling	Inactivated vaccine. Available in USA only. Lower incidence of allergic reactions.
Primary hamster kidney cell	Beijing	Inactivated vaccine. Available in China only. More than five million doses administered annually.
Vero cell	Wistar	Inactivated vaccine. Neutralising antibody response comparable with human diploid cell vaccines.
Human diploid cell	Pitman-Moore L503 Flury	Gold-standard inactivated vaccine. More than 1.5 million doses administered.

absorbed on to filter paper and stored at over 30°C for 222 days.[17] This extends the possibility of detecting rabies in remote areas where there are few freezers and no stable electrical supply.

Treatment

Rabies, if left untreated, is always fatal. Current antiviral therapy is confined principally to interferon and rabies immune therapy. In the search for an effective treatment, a vast array of small molecule inhibitors, natural compounds and derivatives, and biological entities have been tested *in vitro* and *in vivo*.

Over the past 30 years, promising candidates have included tilorone hydrochloride (1971), ammonium picrate (1972), ribavirin (1972), adenosine analogues (1984), ammonium tungsten antimoniate (1985), gangliosides (1987), synthetic peptides (1988), desferrioxamine B (1990), ketamine (1992), reserpine (1993), lectins (1995), anti-sense DNA (1996), phosprenyl (1996) and plant-derived monoclonal antibodies (2003), to name just a few.

As a result of these studies, it is clear that an effective post-exposure antiviral drug for rabies has yet to be found. Thus, efforts must be made to reduce exposure and boost immunity through preventive vaccination.

Prevention

One way of minimising the risk of contracting rabies is to reduce the risk of exposure. Many countries, including the UK, have strict quarantine procedures. The aim is to keep the animal in isolation until any symptoms become apparent. Symptomatic animals are destroyed to prevent spread of the virus. Asymptomatic animals are released as probably virus-free. Culling of wild animals known to harbour the disease (eg foxes in Europe) is another means of control. In addition, release of bait labelled with vaccine is becoming an increasingly popular means of control (Fig 4).

Vaccine

Louis Pasteur developed the first effective rabies vaccine in 1885. He produced a live attenuated vaccine by air-drying infected rabbit spinal cord over potassium hydroxide. The vaccine was first administered to nine-year-old Joseph Meister on 5 July 1885, after being bitten by a rabid dog. Joseph received a total of 13 injections of Pasteur's new vaccine.

A second patient was given the same vaccine on 20 October 1885, six days after being bitten. By April 1886, Pasteur had treated more than 700 patients, the majority of whom survived.

As rabies is endemic across much of the globe it is not surprising that different governments and agencies have made extensive efforts to control the disease, and, as a result, a wide variety of rabies vaccines have been developed. The three main types are neural tissue vaccine (such as Pasteur's original vaccine), non-neural tissue vaccine (duck and chick embryo vaccine) and cell culture vaccine (human diploid cell vaccine) (Table 2).

Neural tissue-derived vaccines are still used extensively in some regions (eg India) because they are relatively cheap to produce; however, they can produce severe side-effects due to the unwanted presence of myelin basic protein in the vaccine preparation, which provokes an extreme immune response in the recipient. Human diploid cell vaccines are now regarded as the gold standard for inactivated vaccines and are associated with a much lower incidence of side effects.

The decision to use a vaccine should be taken after consideration of the nature of the exposure to rabies virus. Table 3 categorises the degree of exposure and indicates when vaccination is recommended.

Prophylaxis

Despite advances in treatment, rabies remains endemic in many countries around the world. Caution should be exercised in areas where there is a risk of exposure. Prompt treatment of wounds caused by wild animals is recommended. Pre-exposure prophylaxis is recommended for travellers spending long periods (>30 days) in areas where rabies is endemic. ■

Table 3. Categories of rabies exposure.

CATEGORY	TYPE OF EXPOSURE	TREATMENT REQUIRED
I	Touching or feeding animals; licks on the skin	None.
II	Nibbling of uncovered skin; minor scratches or abrasions without bleeding; licks on broken skin	Immediate washing and flushing of all bite wounds and scratches; immediate vaccination.
III	Single or multiple transdermal bites or scratches; contamination of mucous membrane with saliva from licks	Immediate washing and flushing of all bite wounds and scratches; immediate vaccination and administration of rabies immunoglobulin.

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