

## Application of immunoperoxidase techniques to formalin-fixed brain tissue for the diagnosis of rabies in southern Africa

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### ABSTRACT

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Two immunoperoxidase techniques, viz. avidin-biotin complex (ABC) and peroxidase-anti-peroxidase (PAP) procedures, were applied to paraffin-wax-embedded brain-tissue sections, from brains which had been fixed in 10 % formalin, to demonstrate the presence of rabies-virus antigen by light microscopy. These techniques positively identified both "viverrid" and "canid" rabies-virus antigen in tissue sections of species commonly infected with rabies virus in southern Africa, viz. the domestic dog (*Canis familiaris*), yellow mongoose (*Cynictus penicillata*), black-backed jackal (*Canis mesomelas*), bat-eared fox (*Otocyon megalotus*), cattle (*Bos taurus*), sheep (*Ovis aries*) and humans.

With both of these techniques rabies-virus antigen stained as sharply demarcated, brown precipitates within the cytoplasm of neurons. The virtual absence of background staining enabled identification of fine granules of viral antigen, often referred to as "virus dust", within axons, dendrites and cytoplasm of the nerve cell body. Staining with the ABC procedure produced clearer, more deeply-coloured precipitates than the PAP method.

### INTRODUCTION

Prior to 1804, all diagnoses of rabies were based on the clinical signs. According to Baer (1991), the first crude biological tests used to establish a diagnosis were conducted by Zinke in 1804, when he transmitted the disease experimentally to dogs and rabbits by

infecting cutaneous wounds with the saliva of rabid animals. Through the nineteenth century various refinements were made to these biological tests in an attempt to improve their diagnostic efficiency. These included the intramuscular and intracerebral inoculation of laboratory animals with brain material from animals suspected to have had rabies, as introduced by Galtier in 1881 and Pasteur in 1884, respectively (Baer 1991).

The first specific diagnostic test for rabies, described by Adelchi Negri in 1903, was dependent on the detection of inclusion bodies (Negri bodies), within the cytoplasm of large neurons of the central nervous system (Baer 1991). The histopathological identification of intracytoplasmic Negri bodies, a relatively insensitive diagnostic technique with an overall efficiency of only 66 % (Tustin & Smit 1962), was used extensively for the confirmation of rabies until 1958

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when Goldwasser and Kissling developed a direct immunofluorescent method for the demonstration of rabies virus antigen in squash preparations of the brain of infected animals. This fluorescent antibody technique (FAT) for rabies remains the most widely used diagnostic test for the confirmation of the disease, due to the speed with which results can be obtained and its excellent specificity and sensitivity (Baer 1991; Bradley 1979; WHO 1984).

There are several advantages in using formalin-fixed specimens rather than fresh brain or glycerol-saline-preserved material (used for the FAT) which may have very high concentrations of viable virus in a rabid animal (WHO 1984). There is therefore a danger of human exposure, not only during collection and processing of specimens, but also in transit to the laboratory. Formalin-fixed specimens, on the other hand, pose a threat only during collection, as the virus is rapidly inactivated by formaldehyde, making transport and laboratory processing of formalin-fixed specimens safe.

Another advantage of formalin-fixed samples is that histological lesions can be evaluated, a rabies-negative brain can be further evaluated for other lesions which might explain the nervous signs described. Fresh brain, or that immersed in glycerol-saline, does not keep well, even at low temperatures, and there is a limited time of about 6 months at 4 °C (Meredith, Foot-and-Mouth Disease Laboratory, Onderstepoort, personal communication 1994), for which this material can be utilized for immunofluorescence purposes. Additional drawbacks are the expensive equipment, such as an ultraviolet microscope, which is required to read the FAT; and the lack of permanence of the preparations due to fading of fluorochromes (Baer 1991; Haines & Clark 1991).

Many of these disadvantages of the FAT can be overcome by immunoperoxidase-staining procedures. Counterstaining with haematoxylin enables simultaneous assessment of viral antigen distribution and histopathological lesions. Furthermore, a permanent record of each case may be kept as the staining does not fade, making retrospective studies feasible. In addition, interpretation of stained slides is carried out under a standard light microscope. The methodology for immunoperoxidase staining for rabies is well known, but these procedures still need to be assessed in terms of their sensitivity and specificity when compared to the FAT.

Because of the problems associated with the FAT and the insensitivity of histopathological identification of Negri bodies as a routine diagnostic procedure (Reid, Hall, Smith & Baer 1983; Tustin & Smit 1962), it was decided that alternative techniques which could be applied to formalin-fixed brain specimens should be investigated for local diagnostic purposes. Two immunoperoxidase procedures, namely the peroxidase-anti-peroxidase (PAP) technique and the avidin-biotin complex (ABC) technique, were selected for

application. The immunoperoxidase staining methods are based on the ability of a primary antibody to bind to a specific antigen within a tissue section (Haines & Clark 1991).

Both immunoperoxidase procedures have been successfully applied in North America and the Far East to demonstrate the presence of rabies-virus antigen in formalin-fixed, paraffin-embedded brain sections (Bourgon & Charlton 1987; Fekadu, Greer, Chandler & Sanderlin 1988; Hamir, Moser & Rupprecht 1992; Palmer, Ossent & Suter 1985; Sinchaisri, Nagata, Yoshikawa, Kai & Yamanouchi 1992). Bourgon & Charlton (1987) used the PAP technique to confirm the presence of rabies-virus antigen in formalin-fixed brains of experimentally infected mice, skunks and foxes. Fekadu, Greer, Chandler & Sanderlin (1988) successfully employed the ABC system in formalin-fixed tissues of experimental cases, to confirm the presence of rabies-virus antigen in FAT and biologically positive brains. Hamir, Moser & Rupprecht (1992) used the ABC to study the neurological lesions in naturally acquired rabies of raccoons. Sinchaisri, Nagata, Yoshikawa, Kai & Yamanouchi (1992) used the ABC technique for studying time-sequential virus spread in the CNS of mice. Further refinements to the ABC and PAP procedures for the diagnosis of rabies in formalin-fixed tissue sections followed (Animal Disease Research Institute 1993; Western College Veterinary Medicine, Department Veterinary Microbiology 1993).

Species commonly infected with rabies virus in southern Africa include the domestic dog, yellow mongoose, black-backed jackal, bat-eared fox, cattle, sheep and humans (King, Meredith & Thomson 1993; Swanepoel 1994). Monoclonal antibody studies on the nucleoprotein components of southern African lyssavirus serotype 1 variants indicated antigenic differences in rabies-virus isolates of mongoose or "viverrid" origin and those of dogs or "canid" origin (King, Meredith & Thomson 1993). Viverrid strains were found predominantly in mongooses, and in domestic and wild cats with spill-over into domestic livestock (predominantly cattle and sheep). Canid strains, on the other hand, occurred mainly in domestic dogs, black-backed jackal and bat-eared foxes, with spill-over into mainly cattle and humans. Some spill-over of viverrid virus into canine species and *vice versa* does occur (King, Meredith & Thomson 1993; King & Turner 1993). The aim of this work was to establish whether these two immunoperoxidase techniques would successfully identify southern African lyssavirus serotype 1 variants in mammalian species commonly infected with rabies virus in southern Africa, that had been confirmed by FAT.

## MATERIALS AND METHODS

### Production of the primary rabies antibody

Four 8-month-old, male, New Zealand white rabbits in good health were selected for the production of rabies

antibody. They were housed in commercial-wire rabbit cages (Labotec, P.O. Box 5103, Durban, 4000 South Africa), which were in a fully ventilated brick building at Allerton Regional Veterinary Laboratory. Nutrition consisted of Epol (Epol, P.O. Box 1299, Pietermaritzburg, 3200 South Africa) commercial rabbit pellets, and municipal water was supplied via automatic drinkers attached to the cages. These rabbits were immunized by intramuscular inoculation of 1 ml of high egg passage (HEP) Flury strain, an attenuated, live virus vaccine (Onderstepoort Veterinary Institute) batch G12 OP/53). Thirty days later these rabbits were boosted with rabies nucleoprotein expressed by a baculovirus in insect cells (Prehaud, Harris, Fulop, Koh, Wong, Flamand & Bishop 1990). An aliquot of 8 ml of this baculovirus-expressed vaccine was emulsified with an equal volume of Freud's incomplete adjuvant, creating a final volume of 1,6 ml which was subdivided and injected intramuscularly into the following four sites: left and right popliteal areas, and the left and right gluteal muscles.

Fourteen days after the nucleoprotein booster, all four rabbits were anaesthetized and exsanguinated. The 550 ml of blood collected, yielded 220 ml of serum which was then filtered through a sephadex G25 column to remove globular proteins of 1000–5000 MW from the antiserum. The antibody titre of this antiserum was established at the Foot-and-Mouth Disease Laboratory, Onderstepoort, utilizing a blocking enzyme-linked immunosorbent assay and was measured at 1:2353. This antiserum was utilized as primary anti-rabies antibody for the immunoperoxidase staining techniques.

### Selection of positive cases and controls

The brains selected for the application of the immunoperoxidase staining techniques included three naturally infected dogs, a human from Natal, a naturally infected yellow mongoose, a black-backed jackal from the Transvaal and a bat-eared fox from the northern Cape Province, as well as experimentally infected sheep and cattle. "Viverrid" rabies viruses had been administered to the experimental cases by intramuscular injection as part of a separate set of experiments (Thomson & Meredith, Foot-and-Mouth Disease Laboratory, Onderstepoort, personal communication 1993). The above species were selected as they are commonly infected with rabies virus in southern Africa (Directorate Animal Health 1991; King, Meredith & Thomson 1993; Swanepoel 1994). All of these cases had been confirmed positive for rabies by the FAT applied to specimens of brain tissue. Specimens of the brains of two dogs, one of which had died of strychnine poisoning and the other of canine distemper, were used as negative controls.

### Immunoperoxidase procedures

ABC and PAP procedures were based on standard published methods (Bourgon & Charlton 1987; Fekadu, Greer, Chandler & Sanderlin 1988). Tissue blocks from the thalamus, hippocampus, brain stem and cerebellum of positive cases as well as negative controls, were fixed in 10 % buffered formalin and processed according to routine laboratory procedures for light microscopy. Pronase digestion and hydrogen peroxide quenching steps were omitted as they caused background staining on our material. The ABC procedures were carried out as follows: tissue blocks were processed in paraffin wax and sectioned routinely at 3 µm, affixed to poly-L-lysine-coated glass slides (Sigma Chemical Company, P.O. Box 14508, St Louis, Missouri, USA) and dried overnight in an incubator at 37 °C. The tissue sections were then dewaxed in xylene for 5 min, rehydrated through graded alcohols, and washed in distilled water for 3 min. After two consecutive 5-min washes in phosphate-buffered saline (PBS, pH 7,6) containing 0,1 % bovine serum albumin, sections were blocked with a 10 % normal goat serum (Labotec, P.O. Box 5103, Durban, 4000 South Africa) for 20 min. The serum on the slides was blotted and the slides incubated with a 1:100 dilution of primary antibody for 60 min at room temperature. Following two consecutive 5-min washes in PBS (pH 7,6), the sections were incubated for 30 min at room temperature, with a 1:500 dilution of biotinylated goat anti-rabbit antibody (Dako A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark).

After two further 5-min washes in PBS, flooding of sections for 30 min with ABC reagent (Sigma Chemical Company, P.O. Box 14508, St Louis, Missouri, USA) followed. Once again, sections were rinsed in PBS and treated for 8 min with the peroxidase substrate diaminobenzidine tetrahydrochloride (DAB) (BDH Laboratory Supplies, Poole, Dorset, England). Slides were then washed in distilled water, counterstained with Mayer's haematoxylin, dehydrated and mounted.

The PAP staining procedure was identical in all respects, except for the steps following primary antibody incubation. Following two washes in PBS, the slides were incubated for 30 min with 1:400 dilutions of goat anti-rabbit secondary antibody. In addition, the ABC reagent was replaced with a 1:400 dilution of PAP reagent in PBS; otherwise the steps were the same as those described above.

### Specificity

Duplicate sections of positive brains were processed, substituting the primary rabies antibody with an unrelated antibody in order to evaluate specificity. Either *Neospora caninum* or canine distemper primary antibody was inserted in place of the rabies antibody, while in other sections saline was used *in lieu* of the primary antibody.

## RESULTS

Intense staining was achieved with the ABC procedure, viral antigen aggregates staining a deep golden-brown. These antigen aggregates were encountered as small granules (rabies virus dust) (Fig. 1), or large, discrete clusters (Negri bodies) (Fig. 2) within the soma, axons and dendrites of large neurons of the cerebrum, hippocampus, thalamus and brainstem, as well as in the Purkinje cells of the cerebellum. In addition, clumps of viral antigen within the dendritic processes of the Purkinje cells, enabled their clear demarcation (Fig. 3). Some of the larger viral aggregations had a slightly paler-staining centre.

Only small quantities of endogenous peroxidase were observed in red-blood cells, as indicated by light, yellowish-brown, granular staining. No non-specific background staining was observed in the neuropil. Staining with the PAP procedure produced paler, golden-yellow antigen aggregates within the cytoplasm of neu-

rons. With the PAP stain this viral antigen was not as clearly defined (Fig. 4), and positively stained viral antigen was not as easily detected in the dendritic processes of the Purkinje cells as it was with the ABC procedure. Rabies-virus antigen was detected in the sections from the naturally infected dogs, human, mongoose, jackal and bat-eared fox as well as the experimentally infected cattle and sheep. No positive staining with ABC or PAP was observed, either in sections where the primary rabies antibody had been substituted, or in the negative controls.

Morphological detail of the sections, with haematoxylin counterstaining, was well maintained, being comparable to that of routine haematoxylin and eosin-stained histological sections.

## DISCUSSION

Both the ABC and PAP detected viral antigen within brain sections of species commonly infected with ra-

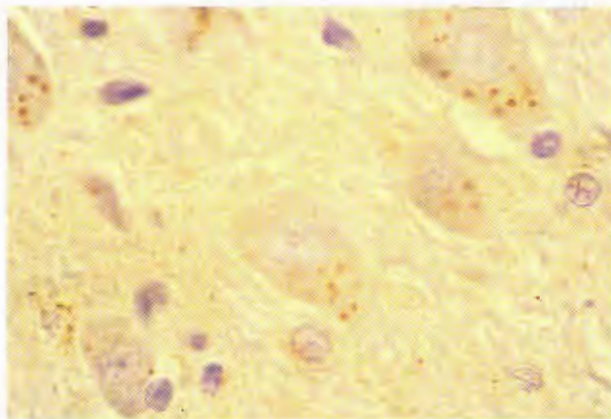


FIG. 1 Small granules of viral antigen, "virus dust", within the cytoplasm of neurons in the brainstem. Avidin biotin peroxidase complex method, Mayer's haematoxylin counterstain (630x)

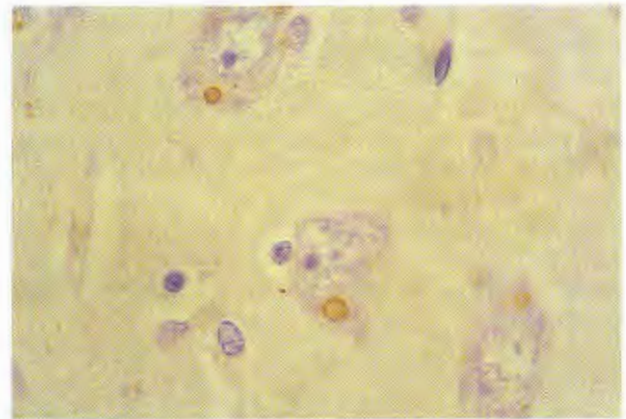


FIG. 2 Negri bodies with paler-staining central portion within the cytoplasm of neurons in the brainstem. Avidin biotin peroxidase complex method, Mayer's haematoxylin counterstain (630x)

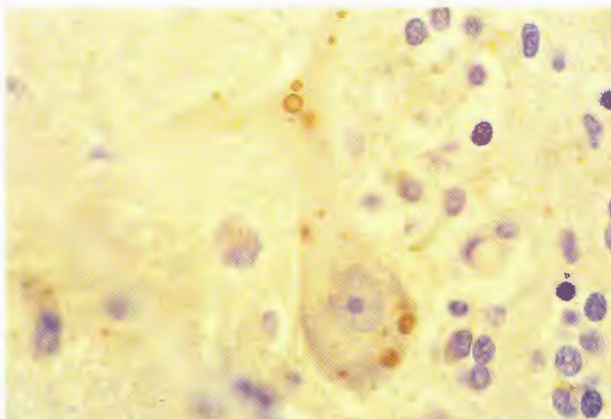


FIG. 3 Clumps of viral antigen within dendritic processes of Purkinje cells. Avidin biotin peroxidase complex method, Mayer's haematoxylin counterstain (630x)

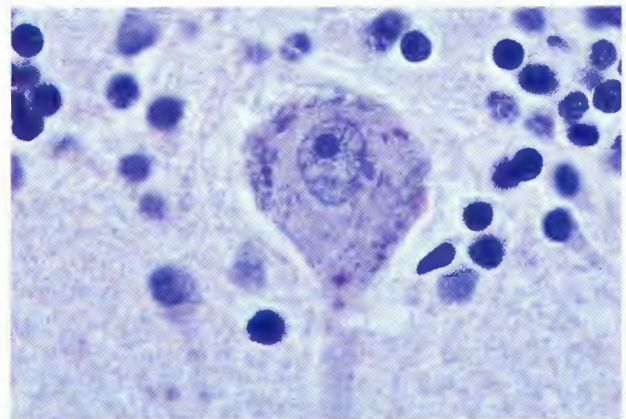


FIG. 4 Paler staining of viral antigen within the cytoplasm of a Purkinje cell. Peroxidase-anti-peroxidase method, Mayer's haematoxylin counterstain (630x)

abies virus in southern Africa, viz. the domestic dog, yellow mongoose, black-backed jackal, bat-eared fox, sheep, cattle and humans. This work documents the first record of the application of the ABC and PAP immunoperoxidase-staining procedures to formalin-fixed brain-tissue sections from species commonly infected with rabies virus in southern Africa.

Rabies-virus isolates from a given geographical location or animal species show unique reactivity patterns in both the glycoprotein and the nucleoprotein components of the virion (King, Meredith & Thomson 1993; King & Turner 1993; Swanepoel 1994). Thus isolates from Africa differ antigenically from those of North America as well as those from Europe (Baer 1991). Monoclonal antibody studies on southern African rabies isolates indicate that at least two major host species groups of the virus are involved, with "spill-over" to other species in the same area (King, Meredith & Thomson 1993). These host species groups are the viverrid group, with the yellow mongoose predominating, and the canid group, which includes the domestic dog, black-backed jackal and bat-eared fox (King, Meredith & Thomson 1993). Our techniques successfully identified both "viverrid" rabies virus antigen and "canid" rabies-virus antigen.

The results obtained with these two immunoperoxidase procedures indicate specific staining of rabies-virus antigen in paraffin-wax-processed tissue sections. The use of these methods for routine rabies diagnostics is being further investigated at present, especially as regards their sensitivity when compared to the FAT.

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