UTILITY OF IMMUNOHISTOCHEMICAL STAINING TECHNIQUE AS A ROUTINE TOOL IN RABIES DIAGNOSIS

by
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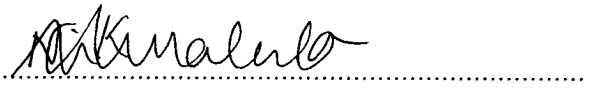
DECLARATION

I declare that all the work presented in this dissertation was done by myself and has not been previously submitted for a degree to this or any other University.



Mukatimui Namangale Kalima-Munalula.

Dedication

I would like to dedicate this work to my dear husband and friend, Thembinkosi;

and to my parents and siblings.

APPROVAL

This dissertation of DR. MUKATIMUI KALIMA-MUNALULA has been approved as fulfilling part of the requirements for the award of the degree of Master of Veterinary Medicine by the University of Zambia.

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Utility of Immunohistochemical Staining Technique As A Routine Tool in Rabies Diagnosis.

ABSTRACT

Rabies is one of few diseases that cause anxiety to both the exposed individual and the health authorities responsible for its prevention and control. Diagnostic procedures in rabies have involved histopathological detection of the Negri body, immunofluorescence and mouse inoculation.

Brain specimens from suspected and confirmed rabies cases were obtained from the University of Zambia, School of Veterinary Medicine, Lusaka. These specimens were taken from dogs, cats, cows and horses. The specimens were tested for rabies using the Direct Immunofluorescent Antibody Technique (DFAT) and the results compared to those obtained using the indirect avidin-biotin technique using Streptavidin alkaline phosphatase technique and routine haematoxylin eosin (HE) staining for Negri bodies.

Fixation in formalin was done for varying lengths of time from 24 to 72 hours. Sections from the paraffin blocks were cut using a sliding microtome at a thickness of 3 micrometers and fished onto clean slides coated with Poly-L-Lysine to avoid detachment during the incubation and rinsing steps. Enzyme digestion using trypsin was applied to sections to unmask antigenic sites concealed during formalin fixation. Fast red TR salt was the chromogen used.

Of the 39 cases tested the DFAT gave positive results in 29 cases and negative results in 10 cases. The immunohistochemical test gave positive results in 25 cases and negative results in 14 cases. The HE staining gave positive results in 16 cases and negative results in 23 cases. Only in one case did the DFAT give a negative result and the immunohistochemical technique give a positive result.

Using DFAT as the gold standard, the sensitivity and specificity of the immunohistochemical test and histopathology were calculated. However, these two means of comparison were not sufficient so the level of agreement between the methods was also calculated and a kappa value for each obtained. The sensitivity of the immunohistochemical technique was found to be 82.7%, specificity was 90%. The level of agreement was 85% with a positive predictive value (PPV) of 0.96, and negative predictive value (NPV) of 0.64. The calculated kappa value was 0.64. The histopathological test gave sensitivity of 55.1% and

specificity 100%. The level of agreement was 67% with PPV = 1.00, NPV = 0.43. The calculated kappa value was 0.39.

It was possible to show that the indirect immunohistochemical technique using Streptavidin alkaline phosphatase can successfully be used to diagnose rabies. This technique is an adjunct to DFAT, and is an alternative in places where it is not possible to keep specimens frozen or to get them to a diagnostic laboratory in a fresh state. Other instances where the immunohistochemical technique can be applied are those where the dark field microscope with an ultraviolet source is not available, or in retrospective studies.

It was also shown that trypsin digestion greatly improves the staining quality of the sections. However, digestion beyond 90 minutes was found to result in unacceptable tissue destruction and loss of antigenicity. Thus it was important to establish a time limit for the optimal digestion time. In this work, this was found to be between 30 and 60 minutes using a final concentration of 1 mg/ml in Tris buffer and at an incubation temperature of 37^0C.

Another aspect that this work attempted to cover was the effect of fixative and fixation time on the results obtained by the immunohistochemical technique. This was done by experimentally infecting mice with rabies virus and the brain collected and fixed in four different fixatives for varying lengths of time. It was possible to obtain positive reaction from tissues fixed in all four fixatives. However, the number of samples obtained was limited because of high number of deaths caused by trauma. Also the mice included in this part of the study were collected at a late time of infection. The virus titres are thus expected to be high. There seemed to be no difference following fixation for different time periods.

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CHAPTER I

INTRODUCTION

Immunohistochemical techniques are looked upon by pathologists as relatively young branches on the tree of Histochemistry. In 1942 Coons and co-workers were able to use fluorescein labelled antibodies to demonstrate pneumococcal antigen in tissues. This method was originally applied to investigative pathology. The development of enzyme-labelled antibodies gave new possibilities for immunohistochemistry in diagnostic pathology (Nakane and Pierce, 1967).

When compared to immunofluorescence, it was found that the immunoenzyme techniques provided preparations that were permanent and could be examined with ordinary light microscopes (Nakane and Pierce, 1967).

Immunoenzyme techniques have found widespread use in human diagnostic pathology. This has resulted in: a) the development of new criteria for diagnosis, prognosis and treatment, b) the possibility of aetiologic diagnosis, c) the recognition of previously unrecognised disease entities, and d) functional analysis of tissue components (Bosman and Kruseman, 1979).

In the field of Veterinary medicine enzyme labelled antibodies have also played a role in both investigative and diagnostic pathologies. The method has been used for identification of micro-organisms in processed tissues (Miller *et al.*, 1989; Evensen *et al.*, 1991; Massone *et al.*, 1991). Some workers have also used this method to localise neural proteins (Jeffrey *et al.*, 1990). Tumours of various origins have also been identified using enzyme labelled antibodies (Sandusky *et al.*, 1987; Kyriazidou *et al.*, 1989), and occasionally the Enzyme Linked Immunosorbent Assay (ELISA) technique.

The immunohistochemical demonstration of rabies virus antigen has been reported by several workers who have been able to identify rabies virus in various tissues like cardiac ganglia (Feiden and Metye, 1991) and salivary glands (Fekadu *et al.*, 1988).

Several workers have also reported the usefulness of enzyme labelled antibodies for rabies diagnosis using formalin fixed, paraffin embedded brain tissues (Feiden

et al, 1985; Bourgon and Charlton, 1987; Feiden *et al.,* 1988; Zimmer *et al.,* 1990; Hamir *et al.,* 1992; Hamir *et al.,* 1995;).

Diagnosis of rabies in Zambia is carried out using three different techniques namely the Direct Fluorescent Antibody Technique (DFAT), mouse inoculation and histopathology. ELISA is also occasionally used. Diagnostic capability is sometimes limited by poor transport network, particularly in rural areas; inadequate diagnostic reagents, personnel drain and inaccurate reporting of cases (Sinyangwe, 1992). Disadvantages of this method include the risk of exposure to humans handling rabies-suspect material during transportation, and the inability to keep materials fresh or frozen in the rural and outlying areas of the countries.

It has also been shown that acetone-fixed impression smears used for the fluorescent test remain infective (Fischman and Ward, 1969). This is in addition to the following: that naturally occurring fluorescence may obscure the fluorescence of specific reactions, the preparations are not permanent and fade with time, and the expense of using dark field ultraviolet microscopy (Nakane and Pierce, 1967).

The present work attempted to determine the utility of immunohistochemistry using the indirect avidin-biotin technique as a routine tool for rabies diagnosis. The method was compared to immunofluorescence and histopathology. Tests were also carried out by experimental inoculation of mice in order to determine the effect of fixative and fixation time on the efficiency of the immunohistochemical procedure.

Immunohistochemical staining is the detection of antigens in tissue sections through the use of specific antibodies that are labelled so that the sites of antigen-antibody reaction become microscopically visible (Haines and Chelack, 1991). Immunohistochemistry is highly dependent on specific antibody-antigen binding, a system of detection that allows permanent localisation and, a clear visualisation of the reaction (Pettigrew, 1989).

The immunohistochemical detection of ribonucleoprotein of rabies virus presents a much wider distribution of rabies virus within the central nervous system than would be expected by merely detecting the presence of Negri bodies in haematoxylin and eosin stained sections. It is also possible to use the test for diagnosis of rabies in tissues that have been fixed in formalin for several years (Fekadu *et al.,* 1988). The results obtained using the immunohistochemical technique are not adversely affected by freezing prior to formalin fixation, and the same sections can be used for conventional light microscopy and immunohistochemical studies (Suter *et al.,* 1984). Another advantage of the

immunohistochemical method is that the staining reactions are more or less permanent and fade very slowly with time (Nakane and Pierce, 1967), with some exceptions.

The first part of the work involved the processing of brain samples from rabies suspect animals through formalin fixation and paraffin embedding. These tissues were then stained using a modified immunoenzyme technique. The modifications involved the use of Streptavidin alkaline phosphatase as the enzyme rather than horseradish peroxidase and also the use of trypsin as a digestive enzyme. Results obtained from the procedure were then compared to those obtained from the fluorescent test and the routine histopathological staining.

The second part of the work involved the use of laboratory mice. These were experimentally inoculated with infective rabid brain tissue. Brains were harvested from these mice at different times and fixed in four different fixatives for various lengths of time. This was followed by staining with the immunohistochemical technique as described above. A comparison of the staining reaction was then made in order to determine which fixative and time gave the best results.

CHAPTER II

LITERATURE REVIEW

2.1 Historical Review

Throughout the recorded history of Western civilisation, rabies virus infection of humans and animals has been attributed to the bite of a "mad dog" or other animals. At the present time rabies is endemic in many parts of the world including Zambia.

Among the wild animal populations that harbour rabies, skunks and bats are the most commonly infected (Steele, 1975). In Zambia, rabies has been detected in jackals, foxes, duicker, ant bear, bushbaby, honey badger, wild dogs, wild rats and mongoose (Hussein *et al.*, 1983).

Rabies has been known in Europe and Asia for a long time. The first mention may be that found in the law tables of Mesopotamia: "If a dog is vicious and the fact has been brought to the knowledge of its owner, (if nevertheless) he does not keep it in, it bites a man and causes (his) death, then the owner of the dog shall pay two-thirds of a mina of silver. If it bites a slave and causes (its) death, he shall pay 15 shekels of silver" (Goetze, 1955). It was also known among ancient Greeks like Democritus, Xenophon, and Aristotle who knew it as 'lyssa' or 'lytta' meaning madness. Celsus the Roman recognised the role of wildlife in rabies transmission and recommended treatment by excision of bite wounds (Timoney *et al.*, 1988). The Latin word 'rabies' comes from the Sanskrit 'rabhas' denoting madness or rage (Nicholson, 1987).

The nineteenth century saw important developments in the knowledge of the disease: the experimental transmission of rabies using saliva from animals to man; and the growing awareness of the neurotropic nature of the virus. In the 1880s, Pasteur made significant findings in his experiments on postexposure treatment. He coined the term 'fixed' in reference to the highly reproducible biological properties which appeared following passage of street rabies in rabbits.

Still little was known about the disease until 1903 when Negri visualised darkly staining nerve cell inclusions which he wrongly thought were protozoa. His discovery was of major diagnostic importance, with the identification of Negri bodies in neurones being considered diagnostic for rabies. In 1904, Banat confirmed Remlinger's observation that the agent was of ultramicroscopic proportions. The pathogenesis of rabies was also studied during this era by increasingly sophisticated techniques until 1960 when the precise axonal pathway of the virus was established (Nicholson, 1987).

By 1963 the virus was recognised as a Rhabdovirus: enveloped, bullet-shaped, lipid-containing, single-stranded RNA (Hummeler and Koprowski, 1969). Its propagation in tissue cultures had also led to the ability to produce high standards of potency and safety during vaccine manufacture and also helped reveal many physicochemical, biological and antigenic properties of the virus. It is sensitive to lipid solvents, 45-70% ethanol iodine preparations, and quartenary ammonium compounds (Kaplan *et al.*, 1966). The nucleic acid is readily inactivated by β-propiolactone.

2.2 Scientific Review

Rabies is one of the few diseases that cause anxiety both to the exposed human individual and the health authorities responsible for its prevention and control. Laboratory diagnosis plays a prominent role in the control of rabies because decisions on whether or not to treat patients depend on results obtained from the laboratory. Precise and rapid diagnostic methods to identify rabies virus or rabies viral antigens are of paramount importance because diagnosis of rabies in a biting animal allows prompt and effective postexposure prophylactic measures. However, commencement of treatment of exposed individuals should never await laboratory results. Detection of rabid wild or domestic animals is essential in rabies surveillance and control programs.

Clinical signs

Clinical signs of rabies are similar in the various animal species but may vary widely in individual animal cases. Two main forms are recognised: (1) an excitatory, furious form and (2) a paralytic dumb form (Fenner *et al.*, 1987; Timoney *et al.*, 1988). Most cases will exhibit both forms to different degrees. In rare cases, rabid animals will die suddenly without any recognisable signs of illness (Timoney *et al.*, 1988).

In the excitatory form, most animals are violent, aggressive and dangerous. The paralytic form lasting a day or two, before ending in death follows this stage. Infected humans exhibit an early prodromal stage with vague changes in temperament accompanied by a feeling of unease, restlessness and apprehension (Timoney *et al.*, 1988).

Amongst the animal species, dogs frequently exhibit both the excitatory and paralytic forms of the disease. The excited dog will be restless, nervous and vicious (Fenner *et al.*, 1987; Timoney *et al.*, 1988). Restrained animals chew at metal chains and bars, breaking their teeth and lacerating their mouths and tongues. The dog may swallow pieces of wood, stone and other foreign bodies.

Hydrophobia is not as marked as in humans. The paralytic stage is less spectacular and may be misdiagnosed. Paralysis appears first in muscles of the head and neck and the dog has great difficulty in chewing and swallowing. It drools ropy saliva from its mouth. This may often be mistaken for foreign body lodged in the throat (Timoney *et al.*, 1988).

Cats usually show the furious form of rabies and are extremely dangerous to human attendants and owners because of the viciousness and quickness of action. Clinical signs in rabid horses are very variable. Genital excitement may be evident. Hyperaesthesia, ataxia, paralysis, fever and behavioural changes may all be seen. In cattle, signs are vague. The provoked animal may attack other animals or humans. The animal also bellows. Salivation and drooling are seen in cases with pharyngeal paralysis. Anorexia and cessation of lactation are also seen.

Epizootiology and Pathogenesis

The virus infects most warm-blooded animals and occurs worldwide. Several countries including Japan, the United Kingdom, Iceland, and the Scandinavian countries have eradicated rabies by implementing strict control and quarantine measures. The virus is mostly transmitted by the bite of an infected animal that introduces the virus-laden saliva into the wound of the bitten victim. Humans have often contracted the disease while probing the mouth of a rabid animal for suspected foreign body. It has also been shown that rabies is transmitted by inhalation of virus-contaminated aerosols (Constantin, 1967; Winkler, 1968)

It has been shown by experimentation that the virus replicates in myocytes at the site of inoculation before reaching peripheral nerves (Charlton and Casey, 1979, 1981). From the bite site the virus is transported centripetally in the axoplasm and spinal cord up to the brain (Johnson, 1965). Invasion of the central nervous system is followed by centrifugal spread of the virus via nerve axons to a wide variety of tissues in respiratory, gastrointestinal and urogenital tracts. The greatest significance of this spread is the presence of virus in the saliva that may be seen as early as 1 to 2 weeks *before* onset of clinical signs in a number of species (Baer, 1985; Fekadu *et al.*, 1982).

Treatment

Treatment of animals is strongly discouraged because of the potential risk of human exposure to such animals. Exposed humans are treated aggressively because once clinical signs occur, recovery is rare. Post-exposure treatment is in 3 forms:

Local wound treatment

Administration of rabies immunoglobulin along with vaccination

Human diploid cell vaccine.

Prevention and control

Effective rabies vaccines are available for prevention of rabies infection in domestic animals and humans. Mass immunisation of dogs has been used for many years as a control measure. Some progress has been made with attempts to vaccinate reservoir wildlife species with various oral and parenteral virus preparations (Baer, 1985). Elimination of stray dogs and cats is also another method used for the control of the disease. Surveillance to measure the effectiveness of all control measures and public education programs to ensure co-operation should also be undertaken (Fenner *et al.*, 1987).

Diagnosis

Rapid and accurate diagnosis of rabies is of paramount importance because this determines the course of action to be taken. In situations of negative diagnosis, it saves the individual from the risk of hypersensitive reaction to the antirabies serum. In areas where the transport network is not well organised, conveyance of rabies suspect materials to the diagnostic laboratory becomes difficult and can result in deterioration of material making it unsuitable for the fluorescence technique. It may also result in delayed implementation of the necessary action.

Current diagnostic procedures in rabies may be divided into the following:

(a) *Histopathology*

This involves the staining of either brain impression smears or paraffin-embedded sections with appropriate stains such as Seller's stain to examine for Negri bodies. It has been found that Negri bodies when present are most readily shown in Ammon's horn (hippocampus major) of the brain and pyramidal cells of cerebral cortex and Purkinje cells of the cerebellum. They are also found, to a limited extent, in the neurones of thalamus, pons, medulla, spinal cord and sensory ganglia (Tierkel, 1973). Routine formalin-fixed and paraffin-embedded histological sections stained with haematoxylin eosin provide excellent morphological detail. However, 10-30% of rabies-positive material lacks Negri bodies that are visible by light microscopy (Swoveland and Johnson, 1982). On the other hand, the presence of pseudo-Negri bodies may also mislead the diagnosis with false positives (Innes and Saunders, 1962).

The Negri body is generally rounded but may assume any shape. It is characteristically acidophilic and takes on a pink to purplish colour in differential stains that use basic fuchsin or eosin with methylene blue as their base. It contains small inner bodies that stain dark blue to black. It is intracytoplasmic in histological sections. In impression smears or rolling technique, the histological pattern is disturbed and Negri bodies may be seen clearly outside the neurone.

The Negri body is specific for rabies when fully formed and can not be easily confused with any other inclusion. However, other forms of inclusions may be encountered especially in dogs, foxes, cats and laboratory mice. These inclusions are found in canine distemper, canine infectious hepatitis and fox encephalitis. Brains of non-rabid cats and laboratory white mice may contain non-specific acidophilic inclusions that can be differentiated by Sellers stain from Negri bodies as indicated in the table below.

Table 1. Differentiation of Negri Bodies from Non-rabies Inclusions

Negri bodies	*Non-rabies inclusion bodies*
Presence of basophilic inner granules	Absence of internal structure
Heterogeneous matrix	Homogenous matrix
Less refractive	More refractive
Magenta tinge	Colour more acidophilic

(Reproduced from Laboratory Techniques in Rabies. p50 WHO, 1973)

Other features of rabies that may be seen in histological sections are meningo-encephalitis, meningeal infiltration, perivascular cuffing, formation of encephalitic nodules (Babés nodules) and ganglion infiltration with satellotosis and neuronophagia (Lépine, 1973).

(b) *The direct fluorescent antibody technique*

This is the most accurate microscopic test at present and is employed in all laboratories undertaking rabies diagnosis. It has the advantage of being fast, comparatively inexpensive and the epidemiological sensitivity[1] is higher than histological examination and mouse inoculation (Timoney *et al.*, 1988).

The procedure consists of labelling antibodies with a fluorochrome, allowing the labelled antibody to react with specific antigen and observing the product of reaction under a fluorescent microscope. Fluorescein isothiocyanate is the most frequently used fluorochrome in rabies diagnosis. Antibody-antigen reactions appear brightly coloured apple-green or greenish-yellow against a dark background.

[1] expressed as positive individuals that test positive (Altman, 1993).

However, the fluorescent antibody technique has the following disadvantages:

It requires specialised equipment which might not be affordable by all the laboratories in a country like Zambia.

There is risk of exposure to humans handling rabies-suspect diagnostic specimens that may not be appropriately packed during transportation to the laboratory.

The test requires the use of fresh or frozen specimens therefore those places that are unable to keep materials in this condition may not perform the test.

(c) *Mouse inoculation test*

This is a simple procedure but results are highly dependent on how well it is performed. The experimenter should ensure that the test has been done as accurately as possible to avoid death of the mice from trauma. White mice of any breeding strain are suitable for this since no genetically resistant strain has been found. Brain or salivary gland tissue of the suspected rabid animal may be used for inoculation into the intracranial cavity. The mice are then observed daily and the following signs may be noted: ruffled fur, lack of co-ordination of hind limbs, paralysis and prostration. Death within 24- 48 hours of inoculation should not be attributed to rabies virus; rather, this is attributable to causes like trauma, bacterial contamination, shock or other viruses. The observation period should extend up to a minimum of 21 days following inoculation because the virus is rarely detectable in the inoculum after 21 days (Koprowski, 1973). Sacrificed mice can then be examined for Negri bodies or by immunofluorescent antibody staining. This method is therefore unsuitable for areas where live virus can not be adequately handled or where the direct fluorescent antibody technique facilities are not adequate.

Other tests that are available for rabies diagnosis include:

Virus neutralisation index test: This involves testing of rabies suspected material in known rabies-immune serum to check for neutralisation. Any material that is neutralised by the antiserum is then said to be positive for a rabies strain.

Complement fixation test, which has little routine diagnostic use because of its less reliable results.

Isolation of rabies virus in various tissue culture systems and electron microscopy of brain materials.

(d) *Immunohistochemistry*

Recently, immunohistochemical staining has been used for rabies diagnosis. Several workers have reported favourable results following staining of paraffin sections by the immunoperoxidase method. However, more work needs to be

done before this can be used as a routine diagnostic test. Immunohistochemical staining is performed on tissue sections of formalin-fixed, paraffin-embedded tissues.

Immunohistochemical staining is the detection of antigens in tissue sections through the use of specific antibodies that are labelled so that the sites of antigen-antibody reaction become microscopically visible (Haines and Chelack, 1991). Immunohistochemistry is highly dependent on specific antibody-antigen binding, a system of detection which allows permanent localisation and, a clear visualisation of the reaction (Pettigrew, 1989). The ultimate aim of immunohistochemistry is to achieve a quantitative measurement of tissue antigens (Petrusz, 1983).

In recent years, numerous studies have indicated that many proteins, especially viral ones, remain detectable by immunohistochemical methods even after formalin fixation and paraffin embedding. Detection of rabies-virus antigen in neurones, glandular acinar cells and vascular endothelial cells was enhanced using the avidin-biotin peroxidase method in comparison to the immunofluorescence technique. It was even possible to detect rabies antigen in tissues fixed in formalin for many years (Fekadu *et al.*, 1988).

There are several other reports on the demonstration of rabies viral antigens using the immunofluorescent techniques in routinely fixed paraffin-embedded brain tissue sections following digestion treatment (Johnson *et al.*, 1980; Umoh and Blenden, 1981; Budka and Popow-Kraupp, 1983). The immunoenzymatic staining techniques using polyclonal antiserum to the virus nucleoprotein have also been used (Palmer *et al.*, 1985; Bourgon and Charlton, 1987).

Feiden and others (1988) have found that immunoenzymatic labelling techniques are sensitive and very reliable for demonstrating rabies ribonucleoprotein in routinely fixed paraffin-embedded brain tissue sections from naturally infected wild and domestic animals. Similar results were also obtained in 3 human cases of rabies encephalitis (Feiden *et al.*, 1985), while Reid and others (1983) observed an increase in immunofluorescent staining following pepsin and trypsin digestion. Another advantage of immunoenzyme methods which was previously unknown is that results may not affected by freezing prior to formalin fixation, but would most likely depend on the type of antigen. The same formalin-fixed tissues can be used for conventional light microscopy as well as for immunohistochemical studies (Suter *et al.*, 1984).

The immunohistochemical detection of ribonucleoprotein of rabies virus presents a much wider distribution of rabies virus within the central nervous system than

would be expected by merely detecting the presence of Negri bodies in haematoxylin and eosin stained sections.

The techniques of conjugating enzymes to antibodies and their use in antigen localisation were developed following the disadvantages observed with the fluorescent antibody technique (Nakane and Pierce, 1967).

Initial experiments involved conjugation with acid phosphatase but these produced variable results. Nakane and Pierce (1967) then developed the horseradish peroxidase method. These workers found that this method had the following advantages over the fluorescent antibody technique:

Peroxidase-antibody-antigen reaction products are more permanent whereas the fluorescent preparations tend to fade with time.

Peroxidase-antibody-antigen reaction products can be examined with ordinary light microscopes, avoiding the trouble and expense of dark field ultraviolet microscopy

It is possible to localise several antigens in a single specimen using appropriate antibodies labelled with a specific enzyme.

There are several ways in which the peroxidase method can be applied in immunohistochemistry: a) Direct method b) Indirect method c) Enzyme bridge d)Labelled antigen method (Heyderman, 1979).

The peroxidase-antiperoxidase technique was introduced nearly 20 years ago with the subsequent evolution of immunocytochemistry as a diagnostic discipline in surgical pathology.

Some work has involved the use of alkaline phosphatase instead of peroxidase. The work in this thesis is based on work done using the Streptavidin alkaline phosphatase method.

Since the test is performed on formalin-fixed, paraffin-embedded tissue sections, effect of fixation on the result of any immunohistochemical method should be borne in mind. The most commonly used fixative is formaldehyde, which is a cross-linking one. This chemical was 'accidentally' discovered by the German physician Ferdinand Blum in the late part of the nineteenth century (Fox *et al.*, 1985). He made this discovery whilst studying the antiseptic properties of formaldehyde and found that the skin on his fingers hardened when he worked with diluted solutions. It was then compared to the traditional fixatives, that is alcohol and was seen to induce little tissue shrinkage and distortion.

The cross-linking effect of formaldehyde tends to mask antigenic determinants and this is undesirable in immuohistochemical staining. Pepsin and trypsin are known to reverse the masking effect of formalin by breaking the strong bonds formed. The degree of unmasking depends on the concentration of enzyme, temperature and duration of digestion. It has been observed that prolonged digestion can lead to tissue destruction and loss of reactivity (Reid *et al.*, 1983). Thus, it is important to establish a working dilution, duration and temperature in order to obtain optimal unmasking or antigen retrieval with minimal destruction of tissue sections.

Immunohistochemical localisation of tissue antigens is being used with increasing frequency in many areas of modern biological research. The immunohistological results are, however, not quantifiable and thus the ultimate aim is quantitative measurement of tissue antigens (Evensen, 1995). It is therefore necessary to define criteria of reliability that will aid in designing and interpreting the results obtained in immunohistochemical experiments.

Definition of terms

Immunohistochemical staining is a complex procedure subject to many errors, and it is not always possible to obtain satisfactory results. Methods must thus be developed by which errors can be evaluated and their influence on final results determined. These criteria are efficiency, accuracy, precision, sensitivity and specificity.

Efficiency

This expresses the signal-to-noise ratio in the immunohistochemical preparation. This concept was introduced in order to distinguish changes in staining intensity due to dilution of antiserum or technical variations from that due to variation in antigen actually present in the tissue (Petrusz *et al.*, 1976).

The 'titration curve' obtained when assessing efficiency helps to compare techniques, to characterise the primary antiserum, and to determine method specificity (see below). It also helps eliminate dilution-dependent false negative results.

Accuracy and Precision

These terms are used interchangeably and thus incorrectly.

Every observation (O) is the sum of the 'true value' (T) and the error component (E),

$$O = T + E.$$

Error can thus be considered as the difference between the observed and the true value

$$E = O - T$$

Thus error is a measure of the ***accuracy*** of the observation.

Accuracy is then defined as the closeness of the observation to the true value. Error can not be stated if the true value is not known and true value cannot be obtained if error is unknown.

In contrast, ***precision*** refers to the variability of repeated observations (replications) made in the same system. Precision is independent of the magnitude of error and can be estimated by statistical parameters.

High accuracy can thus not be obtained without high precision, but high precision alone does not guarantee accuracy. To ensure the best achievable precision and accuracy, it is important to keep the controllable errors and random variations as low as possible by standardisation of all steps in the technique.

Sensitivity

This can be defined as the lowest amount or concentration of tissue antigen that can be detected by the immunohistochemical method expressed as the lowest staining intensity that can be distinguished from background (Petrusz, 1983). The confidence with which such a distinction can be made depends on the precision of the method. Thus high efficiency and high precision are essential for high sensitivity. One common error in the interpretation of immunohistochemical results is the inability to realise that even the most sensitive methods have a lower limit below which it is not possible to produce a detectable signal.

Sensitivity is best evaluated by use of artificial antigen media with known concentrations of antigen embedded in an inert matrix (Avrameas and Ternynck, 1969; Petrusz, 1983). Bovine serum albumin can easily be polymerised with glutaraldehyde; and after washing the gel is seen as a matrix that can be infiltrated with known concentrations of purified antigen. The detection sensitivity can then be determined by incubating parallel blocks with decreasing amounts of antigen. This detection sensitivity is thus defined as the lowest staining intensity that can be differentiated from background (Valnes *et al.*, 1984). The staining intensity can be measured by fluorescence intensity using a spectrofluorometer, light

microscope or by use of computer-assisted densitometry if analyses of immunoenzyme methods are used (Evensen *et al.*, 1994).

Specificity

Specificity is one of the most important criteria of validity and yet the most difficult to define. Specificity can be regarded as the ability of the antibodies to detect one antigenic determinant to the exclusion of all others. The nomenclature used to describe specificity can be both confusing and awkward. Non-specific or unspecified staining should be used to describe interactions that do not have an immunological basis. Unwanted staining might have an immunological or non-immunological basis, while cross-reaction refers to interactions with an immunological basis.

Specificity can be better discussed as method specificity and antibody specificity (Petrusz, 1983). Method specificity indicates the lack of staining due to interactions of tissue components with staining reagents other than the antibodies directed against the tissue antigen to be localised. Method specificity is, simply, the lack of any artefacts. A series of dilutions or the omission of each reagent one at a time can check Method specificity. The method should be standardised so that only the primary antiserum produces detectable staining (Petrusz *et al.*, 1976)

Antibody specificity in immunohistochemistry can be understood on the basis of the following:

Antibodies recognise relatively small regions or sites on the surface of larger antigenic sites and are incapable of identifying the molecules themselves. The detected site within the molecule may be contained not only within the intended antigen but also in precursors or fragments of the same molecule, within related molecules, and even unrelated molecules (cross-reaction). Thus it is important to realise that immunocytochemical specificity tests reflect only regional or site specificity and not molecule specificity (Petrusz, 1983).

CHAPTER III

MATERIALS AND METHODS

3.1 Comparison of Techniques for Identification of Rabies Virus.

Brain specimens from suspected and confirmed rabies cases were obtained from the University of Zambia, School of Veterinary Medicine, Lusaka. These specimens were taken from dogs, cats, cows and horses. The number of cases per species is indicated in the table below.

Table 2. Number of Cases per Species

Species	Number of Cases
Bovine	6
Canine	30
Equine	1
Feline	2

Table 3. Table of Reagents Chemicals Used

Chemical	Source
Tris hydroxymethylamino methane	Sigma
Bovine Serum Albumin	Sigma
Aqueous mounting medium	BDH Supplies
Naphthol-AS-MX phosphate	Sigma
Poly-L-Lysine	Sigma
Levamisole	Sigma
Trypsin	Courtesy of Ms Randi Terland, Oslo, Norway
Fast Red TR. Salt	Sigma
N,N-dimethylformamide	Sigma
Streptavidin Alkaline Phosphatase	Boehringer Mannheim
Rabbit immunoglobulins to rabies	Courtesy of Prof. M. Reinacher, University of Leipzig, Germany
Biotinylated goat anti-rabbit immunoglobulins	Courtesy of Dr Ø Evensen, Central Veterinary Laboratory, Oslo, Norway

All brain samples that were received at the Diagnostic Laboratory were divided into two by medial section. One half was taken to the Virology Laboratory where the fluorescent test was performed, whilst the other half was trimmed at various levels and fixed in 10% buffered formalin. Brain specimens from suspected and confirmed rabies cases were trimmed and fixed in 10% buffered formalin. Trimming was done from the following areas: forebrain including olfactory lobe where this was intact, midbrain including the hippocampus, cerebellum including medulla oblongata area, and spinal cord.

Fixation was done for varying time periods from 24-72 hours. Some of the tissues had been in formalin for several months. These tissues were then processed through an automatic tissue processor before embedding in paraffin wax. The sections from these paraffin blocks were cut on a sliding microtome at a thickness of 3 micrometers. Clean slides had previously been coated with Poly-L-Lysine in order to avoid the problem of sections being washed off slides during the incubation and rinsing steps. The sections were then dried on a slide warmer overnight before being stained using the two methods as outlined below.

(a) Haematoxylin-eosin method.

(b) Indirect avidin-biotin technique with Streptavidin alkaline phosphatase.

Modifications:

In order to uncover antigenic sites concealed during fixation, Trypsin was applied to the formalin-fixed tissue sections. Trypsin was applied at a final concentration of 1 mg per ml in Tris acetate buffer pH 8.0. The trypsinisation was done at 37° C for 35 minutes. Trypsinisation was done because it has been observed that tissues fixed in formalin may yield negative or weakly positive results because of the masking effect it has on antigenic determinants (Brozman, 1978; 1980). Trypsinisation also included 30, 60, 90, and 120 minutes as a preliminary study to test the effect of unmasking.

(a) **Haematoxylin eosin method**

This is the routine method used for histopathological diagnosis of rabies by detection of Negri bodies. It involves the use of Mayer's Haematoxylin and the procedure was carried out as outlined in the table.

Table 4. Haematoxylin Staining Procedure

1	*Dewaxing through 2 changes of xylene*	*3 min each*
2.	*Hydration through graded alcohol*	*1 min each*
3.	*Rinse in water*	*2 min*
4.	*Haematoxylin*	*5-10 min*
5.	*Bluing in tap water*	*5 min*
6.	*Counterstain in eosin*	*30 sec*
7.	*Dehydrate through graded alcohol*	*10-30 sec each*
8.	*100% Alcohol I*	*1 min*
9.	*100% Alcohol II*	*3 min*
10.	*Absolute alcohol*	*5 min*
11.	*Xylene I*	*5 min*
12.	*Xylene II*	*5 min*
13.	*Mount with Eukitt*	

(b) **Indirect Avidin-Biotin Technique**

This technique involved the use of paraffin sections that were cut at 3 microns thick and were attached to the slides coated with Poly-L-Lysine. All incubations were carried out in a glass humid chamber.

Following trypsinisation, with Trypsin applied at a final dilution of 1 mg per ml in Tris acetate buffer pH 8.0 and temperature 37°C, the sections were incubated with 5% Bovine Serum Albumin (BSA) which blocked potentially non-specific binding sites. The primary antibody was then applied. In this work this was made of rabbit immunoglobulins to rabies. The primary antibody was applied either for 60 minutes or overnight as shown in the table below. Biotinylated secondary antibody, in this case goat anti-rabbit immunoglobulins, was then applied to the sections. Streptavidin alkaline phosphatase was then applied to the sections before being stained with the Fast red substrate. Counterstaining with Haematoxylin was carried out and sections observed under a normal light microscope.

Table 5. Indirect Avidin-Biotin Staining with Streptavidin Alkaline Phosphatase

1. *Pre-treatment*	
Dewaxing through xylene/graded alcohol series to water	*5 min each*
Treatment with Trypsin at 37°C	*30-60 min*
2.Blocking of non-specific binding sites with 5 % BSA in tris buffered saline (TBS)	*20 min*
3.Primary antibody diluted 1:25,000 in 2.5 % BSA in TBS	*60 min*
Or 1:100,000 in 2.5% BSA in TBS	*overnight*
4. Biotinylated secondary antibody diluted 1: 300 in 2.5 % BSA in TBS	*30 min*
5. Streptavidin alkaline phosphatase diluted 1:1000 in 2.5 % BSA in TBS	*30 min*
6. Fast red substrate	*20 min*
7. Counterstain in Haematoxylin	*2 min*
8. Wash in tap water and coverslip	*10 min*
*9. Between steps (except 2 and 3) wash in TBS**	*5 min*

*TBS (Tris-HCL pH 7.6, sodium chloride 0.8%)

(c) The Direct Fluorescent Antibody Technique
This was performed on fresh brain samples as follows:

Impression smears were made from fresh brains immediately on arrival at the Diagnostic Reception. These smears were then fixed in cold acetone at 4° C overnight. Fluorescein isothiocyanate conjugate diluted 1:12 in normal mouse brain was then applied to the smears. This was incubated for 30 minutes at 37° C, followed by rinsing in phosphate buffered saline (PBS-) on a micro shaker for 10 minutes. The smears were mounted with 50% glycerol and observed under a fluorescent microscope for fluorescence.

The differences in the results obtained from the three methods were described by sensitivity, specificity, positive and negative predictive values and agreements between tests expressed as the kappa parameter.

3.2 Evaluation of the Fixative Parameters and Their influence on Immunohistochemical Methods used for in situ *Detection of Rabies Virus.*

Mice that are susceptible to rabies were used for this part of the work. An infective rabies strain was obtained courtesy of the Disease Control Department, School of Veterinary Medicine, University of Zambia.

Preparation of Inoculum

Brains from mice that had previously been inoculated with a known virulent rabies virus strain, RAV 279 were crushed in a mortar and suspended in phosphate buffered saline to make a 20 % solution. Streptomycin and Penicillin were then added at a stock dilution of 1 million units penicillin G and 1000μg

streptomycin in 20 ml of double distilled water. The suspension was allowed to stand for 30 minutes before inoculation into the mice using 1.0-ml tuberculin syringes.

Inoculation Procedure

Laboratory mice (BALB/c) were inoculated intracerebrally with 0.03 ml of rabies virus coded RAV 279. The mice were then observed daily. Any of the following signs were noted: ruffled fur, lack of co-ordination of hind limbs, paralysis and prostration. Death within 24- 48 hours of inoculation was not considered *attributed to rabies virus. Rather, this was attributable to causes like trauma, bacterial contamination or other viruses.*

A total of 28 laboratory mice were infected with 0.03ml of the inoculum. These mice were from four different parental stocks and were put in cages accordingly.

Cage One	8 mice	7 inoculated
Cage Two	8 mice	7 inoculated
Cage Three	8 mice	7 inoculated
Cage Four	10 mice	7 inoculated

Out of the total number inoculated, 19 mice died from trauma. Eight of the mice survived beyond four days post-inoculation.

Two mice each were then sacrificed using diethyl ether at day 6, and 4 at day 11 post-infection, making a total of six mice included in this part of the study. It was not possible to sacrifice any mice beyond 12 days of inoculation because there was a high rate of death due to trauma. The brain was removed from the dead mice and parallel samples were fixed in four different fixatives for periods of lengths of time.

Ten per cent phosphate-buffered formalin for 12, 24, 36 hours, and 7 days.

Bouin's fixative for 24 hours

Periodate lysine paraformaldehyde (PLP) for 24 hours

Ethanol with 25 % glycerol for 48 hours at 4^0C.

Periodate lysine paraformaldehyde was prepared fresh just before use as follows:

3% paraformaldehyde	12.5 ml
0.1M disodium hydrogen phosphate (PBS)	25 ml
Lysine	0.225g
Sodium periodate	0.0365g

Bouin's fixative was prepared thus:

Saturated picric acid solution	15 ml
Formalin	5 ml
Acetic acid	5 ml

Following fixation, the sections were rinsed in 70% alcohol before being embedded in paraffin wax. The specimens were then treated as outlined earlier.

MOUSE HISTORY CARD (a)

Cage No. 1 Date: 24/11/95 Virus: Rabies

Strain: RAV 279 Preparation: Suspension in PBS- with Penicillin and Streptomycin

Volume: 0.03 ml

Date	24/11	25/11	26/11	27/11	28/11	29/11	30/11
Day	0	1	2	3	4	5	6
1	Dead						
2	Dead						
3	Dead						
4	Dead						
5	Alive	Alive	Alive				Sacrificed
6	Dead						
7	Dead						
8	Control						

MOUSE HISTORY CARD (b)

Cage No. 2 Date: 24/11/95 Virus: Rabies

Strain: RAV 279 Preparation: As in Cage 1

Volume 0.03 ml

Date	24/11	25/11	26/11	29/11	30/11	1/12	2/12	3/12	4/12	5/12
Day	0	1	2	5	6	7	8	9	10	11
1	Dead									
2	Dead									
3	Dead									
4	Dead									
5	Alive	Alive			Sacrificed					
6	Alive	Alive								Sacrificed
7	Alive	Alive								Sacrificed
8	Control									

MOUSE HISTORY CARD (c)

Cage No. 3 Date: 24/11/95 Virus: Rabies

Strain: RAV 279 Preparation: As for Cage 1

Volume: 0.03 ml

Date	24/11	25/11	26/11	2/12	3/12	4/12	5/12	6/12	7/12	8/12	9/12	10/12	11/12
Day	0	1	2	8	9	10	11	12	13	14	15	16	17
1	Alive						Sacrificed						
2	Alive						Alive	Blind					Dead
3	Dead												
4	Alive						Alive	Dead					
5	Dead												
6	Dead												
7	Dead												
8	Control												

MOUSE HISTORY CARD (d)

Cage No. 4 Date: 24/11/95 Virus: Rabies

Strain: RAV 279 Preparation: As for Cage 1

Volume: 0.03 ml

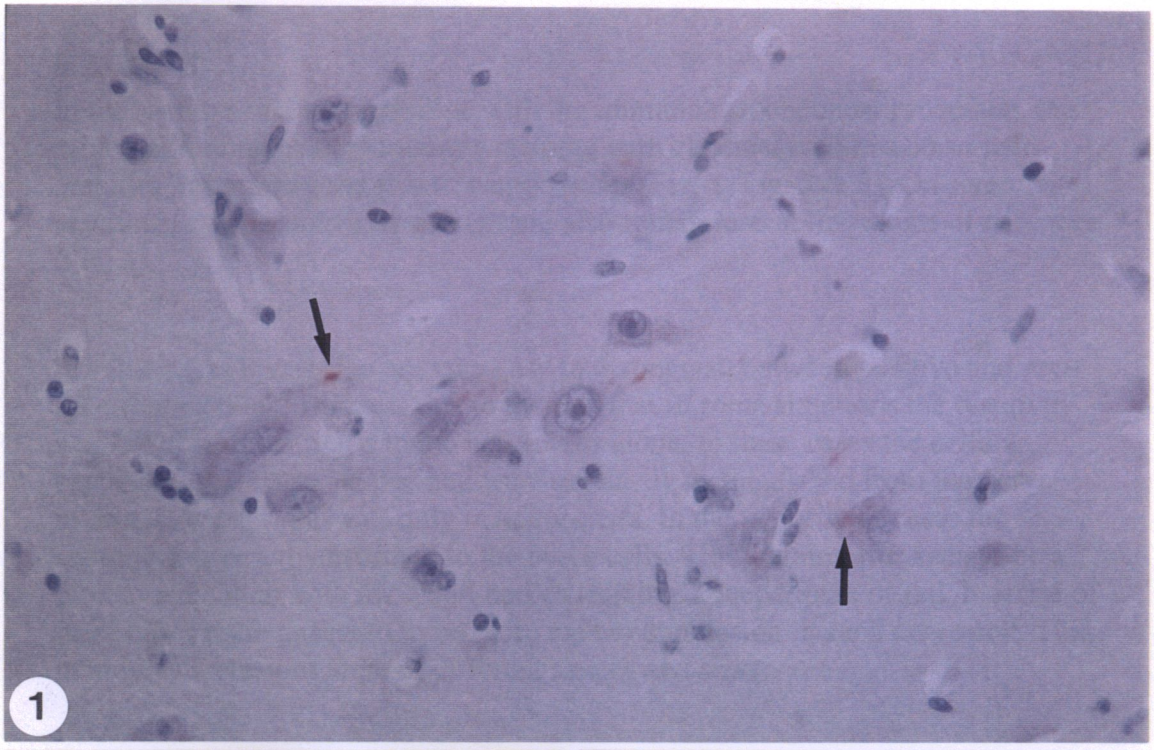
Date	24/11	25/11	26/11	4/12	5/12	6/12	7/12	8/12	9/12	10/12	11/12
Day	0	1	2	10	11	12	13	14	15	16	17
1	Dead										
2	Dead										
3	Alive				Sacrificed						
4	Dead										
5	Dead										
6	Dead										
7	Alive										Dead
8	Control										
9	Control										
10	Control										

Legends to Figures 1, 2, & 3.

Figure 1.
Horse, brain, hippocampal area. Immunostaining for rabies virus reveals small intracytoplalsmic granules in neurons (lower right arrow) and in axon (upper left arrow). Streptavidin alkaline phosphatase method with Mayer's haematoxylin counterstain. x533.

Figure 2.
Detail from same specimen as in Fig. 1. Note intracytoplasmic reaction resembling Negri body. Streptavidin alkaline phosphatase method with Mayer's haematoxylin counterstain. x833.

Figure 3.
Dog, brain, hippocampal area. Note strong intracytoplasmic staining in a neuron. Streptavidin alkaline phosphatase method with Mayer's haematoxylin counterstain. x833.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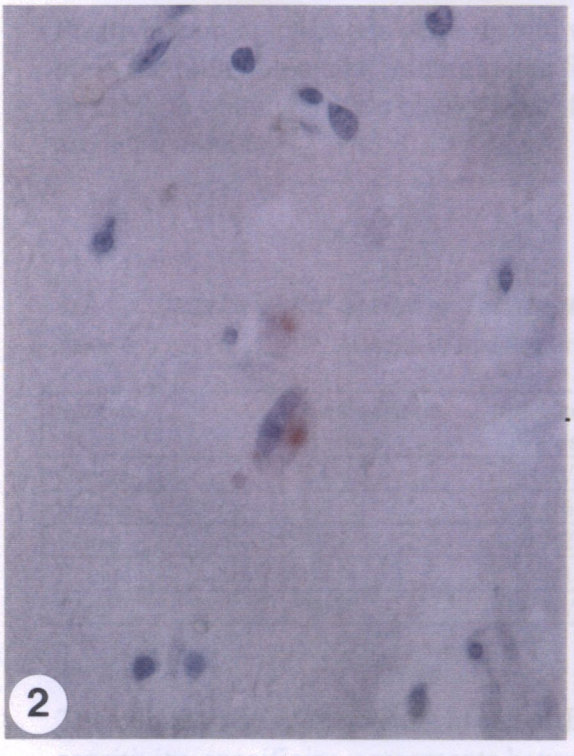
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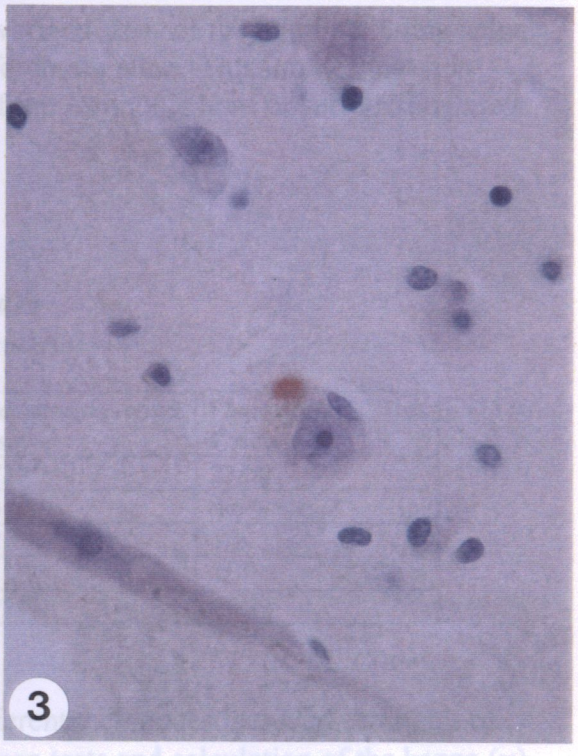
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CHAPTER IV

RESULTS

In the sections that were positive with the immunohistochemical procedure, the principal finding was cytoplasmic staining with classical well-circumscribed granules, resembling the size of Negri bodies (Fig. 1; Fig. 2 & 3). On some reactions, cytoplasmic extensions were also found, resembling axons of neurones (Fig. 1).

In some cases it was also not very easy to distinguish between positive and non-specific reactions. This was due to the fact that in some situations the reactivity was not always restricted to the nerve cells alone. In these cases the cellular extensions were also stained and were not easily distinguished from the nerve matrix. However, this was only in a few cases. In the majority of cases the staining was strictly restricted to the nerve cells. This staining was seen to be a pink to red colour with the Negri bodies staining a deeper pink or red. In some of these cases there appeared to be more than one inclusion in each nerve cell. These numerous inclusions were of different shapes and sizes.

Positive staining reactions were seen in various areas of the brain including spinal cord, medulla oblongata, midbrain and forebrain areas. This implies that it is possible to obtain positive results even if the Ammon's horn or surrounding areas are not available.

4.1 Results of the Staining Techniques

Table 6. Results of Staining Procedures

Species	Avidin-Biotin Positive	Haematoxylin-eosin Positive	Fluorescent Test Positive
Canine	20/30	10/30	23/30
Bovine	4/6	4/6	4/6
Feline	0/2	1/2	1/2
Equine	1/1	1/1	1/1
Total	25/39	16/39	29/39

The comparison of the results of the different techniques included estimates of sensitivity, specificity, agreements between tests and calculation of the kappa (κ) value. Positive and negative predictive values were also included.

The sensitivity of the techniques was determined as the proportion of the true positives that are detected by the test. In this case the true values were considered to be those obtained from the DFAT which is currently the best available test for rabies diagnosis. The specificity was the proportion of true negatives that are detected by the test.

True Status (immunofluorescent Test Result)

Test Status (other tests)	Positive	Negative	Total
Positive	A	B	A + B
Negative	C	D	C + D
Total	A + C	B + D	A + B + C + D

Thus the sensitivity can be calculated as

Sensitivity =A/A + C Specificity = D/B + D

For the Immunohistochemical test compared to DFAT

Test Status	Positive	Negative	Total
Positive	24	1	25
Negative	5	9	14
Total	29	10	39

Sensitivity = 24/29 = 82.7%

Specificity = 9/10 = 90%

Agreement was 85%

$\kappa = 0.64$

PPV = 0.96

NPV = 0.64

For histopathology compared to DFAT

Test Status	Positive	Negative	Total
Positive	16	0	16
Negative	13	10	23
Total	29	10	39

Thus Sensitivity = 16/29 = 55.1%

Specificity = 10/10 = 100%

Agreement was 67%

$\kappa = 0.39$

PPV = 1.00

NPV = 0.43

McNemar's test can also be applied to determine if the tests used are different. This test uses a Chi-square statistic and is calculated with the following formula:

$$X^2 = \frac{(|b-c| - 1)^2}{b + c}$$

Thus, for the Immunohistochemical technique, we have the following:

H_o: There is no difference between the Immunohistochemical technique and the immunofluorescent test.

H_a: There is a difference between the two methods.

At the 95% confidence interval, with 1 degree of freedom the Chi-square table gives a value of 3.486.

The calculated value for the Immunohistochemical technique is

$$X^2 = \frac{(|1-5| - 1)^2}{1+5}$$

$= 9/6$

$= 1.5$

This calculated value is less than the table value so we cannot reject the null hypothesis at the 5% level.

For the histopathological procedure

$$X^2 = \frac{(|b-c| - 1)^2}{b + c}$$

$$= \frac{(|0-13| -1)^2}{13}$$

$$= 11.1$$

This calculated value is higher than the table value so we can reject the null hypothesis.

4.2 Effect of Trypsinisation

It was difficult to obtain a fixed time of trypsin treatment because different specimens responded differently to this. It was possible to show that various time periods of trypsinisation gave similar results without any evidence of any one being better than the other. Preliminary tests had included trypsinisation for 30, 60, 90, and 120 minutes. However, in this study it was observed that trypsinisation with trypsin applied at a final concentration of 1 mg per ml in Tris acetate buffer pH 8.0 and temperature 37^0C, beyond 90 minutes resulted in tissue destruction and non-specific reactions. It was also possible to obtain positive results from samples that had been in formalin for several months following unmasking with trypsin.

4.3 Results of Fixative Comparisons

The mice which survived up to day 11 post-inoculation exhibited the following signs:

prostration, paralysis, exaggerated twitching when lifted by the tail, and trembling. Some seemed to have a cloudy discharge from their eyes that caused their eyes to remain shut. The two mice that were found dead on day 17 post-infection both had prominent post-mortem changes so it was not possible to use their brains for fixation.

Varying fixation time did not result in any differences with regard to immunohistochemical staining results. Bouin's fixative gave positive results for all brain sections and fixation times. A positive result was obtained from all fixatives and for formalin also at all fixation times.

CHAPTER V

DISCUSSION

5.1 Comparison of Techniques

It was possible to diagnose rabies positive material that had been processed in formalin and paraffin using the indirect avidin-biotin staining technique with Streptavidin alkaline phosphatase. This means that materials need not be fresh in order for a positive result to be obtained. It also gives the possibility of diagnosing rabies in areas where it is not possible to perform the fluorescent test due to limitations arising from poor transport infrastructure or lack of specialised equipment, or facilities for storage of frozen or fresh materials.

It is also possible to diagnose rabies in retrospective studies from stored paraffin blocks or from tissues that have been fixed in formalin for extended periods of time. This is facilitated by the use of proteolytic enzymes like pepsin, trypsin or pronase (Reid *et al.*, 1983; Bourgon and Charlton, 1987; Fekadu *et al.*, 1988). Trypsin has been widely used for various purposes, such as dispersing cell clumps in cell cultures and to improve haemagglutination. Huang and others (1967) were the first to use trypsin in an immunohistochemical procedure. It is known to catalyse the hydrolysis of arginyl and lysyl bonds of peptides (Mihalyi, 1972). Other workers have used enzymes like pronase (Fekadu *et al.*, 1988), pepsin (Bourgon and Charlton, 1987), or a combination of pepsin and trypsin (Reid *et al.*, 1983).

A limitation of the immunohistochemical technique observed in this study was that it was difficult to obtain a fixed time of trypsin treatment because different specimens responded differently to this. It was possible to show that various time periods of trypsinisation ranging from 30-60 minutes gave similar results without any evidence of any one time period of trypsin treatment being better than the other. However, in this study it was observed that trypsinisation with trypsin applied at a final concentration of 1 mg per ml in Tris acetate buffer pH 8.0 and temperature 37^0C, beyond 90 minutes resulted in tissue destruction and non-specific reactions. Some workers have reported that enzyme digestion time beyond three hours with 0.25 % concentration of trypsin led to the unacceptable tissue degradation (Reid *et al.*, 1983). The tissue destruction and non-specific reactions that were observed could be due to the fact that at the concentration, pH and temperature used, trypsin treatment resulted in 'overdigestion' of the specimens with negative influence on morphology and immunostaining.

It appears that the period of trypsinisation is dependent on the length of formalin fixation. Initial reactions of formalin with proteins are reversible and are lightly hydrolysed by simple washing. Prolonged formalin fixation, however, is strongly irreversible and involves formation of methylene bridges between aromatic hydrogen and ammonia (Brozman, 1978). Some of the specimens used had been in formalin for several months but it was still possible to obtain positive results from them following trypsinisation. This was one of the other advantages observed with this method in that it was possible to obtain positive results from both stored paraffin blocks and from tissues fixed in formalin for extended periods of time. However, as stated earlier, enzyme digestion beyond 90 minutes resulted in tissue destruction even in those samples that had been in formalin for several months. The optimal time period for trypsin treatment in this work was found to be 30-60 minutes at 37 °C with trypsin applied at a final concentration of 1 mg per ml in Tris acetate buffer pH 8.0.

The sensitivity, that is, epidemiological sensitivity of the immunohistochemical technique was calculated to be 82.7% and the specificity as 90 %. Kotwal and Narajan (1985), using the direct immunoperoxidase test reported sensitivity and specificity of 100 %. Other workers have reported similar results (Feiden *et al.*, 1988; Fekadu *et al.*, 1988; Hamir and Moser, 1994). The differences in the sensitivity and specificity could be attributed to any of the following factors:

Origin of material - The source of material was different in all the mentioned cases so it is expected that differences in results will be observed.

Primary antibody used is different so the reactivity may not be expected to be exactly the same.

Difference in procedure. The conditions used in the present work were not duplicated exactly as with other workers, thus differences in laboratory conditions are likely to results in differences in results obtained.

The animal species used could also affect the final results obtained.

False negative results may be obtained from known positive cases due to any of the following:

Few areas of the brain are examined. The chance of detecting positive cases increases when more brain areas are examined. (Hamir and Moser, 1994).

An irregular pattern of virus antigen (Zimmer *et al.*, 1990)

Antigen concentration below detection level (Zimmer *et al.*, 1990)

Further, it is important to ensure that the primary antibody that is used in the immunohistochemical procedures reacts with the local strains available because there are various strains of rabies which seem to be grouped in geographical areas (Wiktor and Koprowski, 1980; Rupprecht *et al.*, 1987; Botros *et al.*, 1988). It is

also important to obtain the correct antibody type because some are known not to work well for *in situ* diagnosis (Evensen, personal communication). The antibody used should also be

known to react with an antigenic determinant that can withstand fixation. Preliminary procedures in this work had involved the use of an α-G protein monoclonal antibody. It was observed that this gave negative results in known positive cases. This was attributed to the fact that this protein of the virus may be severely affected by the fixation process as to be irretrievable during trypsinisation. Later the α-N protein rabbit immune serum was tested and this proved to give distinct and strong immunostaining.

In one case the immunohistochemical procedure gave a positive result whereas the fluorescent test gave a negative result. This would usually thought to be a false positive result since the fluorescent test is seen as the 'gold standard'. However, it is possible that the immunohistochemical test was better able to detect this case than the 'gold standard'. Thus sensitivity and specificity may not necessarily be the most appropriate means of determining method efficiency. For that reason, agreement tests and κ-value statistics were also included. Immunohistochemistry versus DFAT showed an agreement of 85% and a kappa value at 0.64. This would indicate a good strength of agreement between the tests. For histology versus DFAT, agreement was moderate to poor (Altman, 1993).

Kappa values are greatly influenced by the proportion of subjects in each category, and interpretation of kappa values should include an inspection of the background material. Kappa values between different studies cannot be compared (Altman, 1993). Further, the positive and negative predictive values indicate the proportion of positive and negative cases that are correctly diagnosed using DFAT as indicating the true state (Altman, 1993). This would give an indication of the usefulness of a test in practice. However, one should be aware that positive and negative predictive values are influenced by prevalence. In the context of examination for rabies virus infection of clinically suspect cases, meticulous clinical assessment would theoretically increase the likelihood that cases presented for post-mortem examination are truly infected, i.e. this would increase the prevalence, and vice versa. The consequence is that the lower the prevalence, the less sure one can be that a negative result indicates no infection of rabies, and similarly for a positive result (Altman, 1993). Collectively, the predictive values depend on the prevalence of the infection of the cases being tested.

It was also observed that in two cases where Negri bodies were seen, the immunohistochemical technique gave negative results. The technique is expected to be able to detect the antigen material that is found within the Negri body. Therefore, in these two cases the failure to obtain a positive staining reaction could be attributed to poor technique or incorrect identification of the Negri body (false positive).

5.2 Comparison of Fixatives

The ideal fixative for immunohistochemistry should have the following properties:

It should not destroy immunoreactivity

it should prevent loss or extraction of antigen in subsequent processing and

It should give good morphological preservation (Hall *et al.*, 1987).

It was possible to obtain positive results from the mice sacrificed starting from day 6 after inoculation. The staining reactions did not seem to be influenced by the fixative used and duration of fixation. Other workers have found that the use of Bouin's fixative gave negative results even when known positive samples were tested (Bourgon and Charlton, 1987). The reason for this discrepancy could be that there are differences with regard to origin of material, primary antibody, animal species and procedures. Another factor could be that in the present work, there was a limited number of animals surviving the trauma of inoculation thus insufficient amount of brain material was available for examination.

There was apparently no difference in fixative or time of fixation possibly due to the fact that the brains were collected in the late stage of disease and a high antigen concentration is expected at this time. Therefore, it is difficult to correctly observe any differences in fixative effect.

All four fixatives may be used for the immunohistochemical procedure. The choice would probably be influenced by factors like ease of obtaining the chemicals and the expense that would be incurred. Periodate lysine paraformaldehyde has the disadvantage that it has to be freshly prepared before use.

Workers using periodate lysine paraformaldehyde for fixation of tissues for cryostat studies using immunohistochemistry have found that this has little detrimental effect on the immunoreactivity but that it appears to cause some antigen diffusion (Hall *et al.*, 1987). In this work it was found that using periodate lysine paraformaldehyde did not reduce immunoreactivity. However, there did not seem to be antigen diffusion.

CHAPTER VI

CONCLUSION

The present work has shown that the immunohistochemical staining technique using Streptavidin alkaline phosphatase can successfully be used as a tool for routine rabies diagnosis either as an adjunct to the DFAT or in those situations where it is difficult or impossible to perform the fluorescent antibody test due to factors like poor infrastructure and equipment, inability to keep materials in a fresh or frozen state as is required for the fluorescent test. There was no statistical difference between the two methods. It can also be successfully used in samples that have been fixed for long periods of time, or on stored paraffin blocks.

The immunohistochemical test as a tool for routine diagnosis of rabies can be successfully applied if the laboratory is able to establish its working criteria designed for the specific laboratory. These criteria would include the availability of suitable primary antibodies that are able to detect antigenic sites within the specimen.

Other criteria to be established are the optimal time for trypsin digestion, depending on the concentration of the enzyme. In this work, this was found to be between 30-60 minutes at 37^0C using a final concentration of 1mg/ml in Tris acetate buffer pH 8.0. The use of trypsin was found to be valuable for unmasking antigenic sites caused by the cross-linking effect of formalin fixation.

It should be borne in mind that it is possible to obtain negative results when known positive specimens are fixed in some fixatives. Thus, several fixatives should be tested in order to determine the best fixatives to use. The laboratory can then use the appropriate fixative depending on the availability, cost and ease of use.

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