

Establishment of an immunohistochemical method to detect West Nile virus in formalin-fixed paraffin-embedded tissues

100 μm

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Abstract

With the emergence of West Nile virus in Northern America and the re-emergence of the virus in Europe, the relevance of West Nile virus as a pathogen has been underlined. In particular the quick spread on the American continent and the increase in virulence, compared to previous appearances, have led to healthcare concerns. Reliable and accurate laboratory methods to detect this virus are therefore necessary. One of them is immunohistochemistry, which is an important instrument in research and histological pathology. In order to retrieve reproducible and reliable results from tests, it is of utmost importance to work in a fixed order, or protocol. Protocols are also important since no two laboratories are the same in their preferences and working habits. The aim of this study was to establish such a protocol, in this case for the detection of West Nile virus in tissues. For the establishment of this protocol, a series of experiments were performed, in which different variables were tested, eventually resulting in a protocol that produces satisfactory results and can easily be reproduced. After the establishment of the protocol, several avian, equine and wildlife cases of a possible West Nile virus infection were tested. However, no positive cases were found.

Introduction to West Nile virus

With a massive outbreak in the United States of America at the end of the 20th century, West Nile virus has focused attention to its position as a pathogen of great relevance to both veterinary and human medicine. Targeting primarily birds, horses and humans, it also had and has a considerable social and economic impact. The recent re-emergence of West Nile virus in Central and Western Europe, though of a different lineage than the one in the United States, has also caused international concerns.

Epidemiology

West Nile virus is a virus spread by arthropod vectors. Until now the virus has been isolated from 43, largely bird-feeding, species. The majority of them are of the genus *Culex*, yet *Aedes* species can also function as a vector¹. Occasional West Nile has also been isolated from certain tick species, which are also haemophagous arthropods. Birds act as the principal and amplifying hosts of West Nile virus²³. When mosquitoes feed on an infected bird, in which viraemia reaches sufficiently high titres, the virus may be taken up by the mosquito and in this way be transferred to the next animal (bird or other) to be fed upon⁴. When these birds migrate, the virus can possibly migrate with these birds to other areas. During research, infectivity of West Nile virus to individual bird species varied greatly⁵. Passeriformes, Charadriiformes, Strigiformes and Falconiformes seem to be more likely to be infected. Of the Passeriformes (or singing birds), certain species like the common grackle, finches, sparrows and corvids such as jays, crows and magpies exert an even higher infectivity rate, and have mortality rates over 40%.

Since the virus does not lead up to sufficiently high viraemias in mammals, except for horses and lemurs, they do not serve as an amplifying host.

History

Phylogenetic studies have revealed that West Nile virus emerged as a single virus around 1000 years ago⁶. Later on the virus developed in two separate lineages. Lineage 1 has spread epidemically through an area including Africa, North-America, Europe and Southern Asia. On the other hand lineage 2 is considered as a more African zoonose and less virulent.

West Nile virus was first isolated in the West Nile district in Uganda, in 1937⁷. Later on the virus was also isolated from human patients, birds and mosquitoes throughout the continent in many countries including Algeria, Botswana, Egypt, South-Africa and Nigeria². The first time the presence of the virus was indicated in Europe was in 1958, when two Albanian men were seen to have neutralizing antibodies to West Nile virus⁸. After this primary detection the virus has been identified in several European countries, most of them in southern Europe and Eurasia in the 1960s and 1970s⁹.

The exact incidence of West Nile virus still remains largely unknown, though sporadic cases have been identified throughout the years, and several outbreaks in southern Europe and Northern Africa in the late 1990s have been observed as well. Although some outbreaks were accompanied with human fatalities, scientists did not consider West Nile virus to be a major threat to public health. This changed with the outbreak of West Nile virus (lineage 1) in the late 1990s and early 2000s¹⁰. After hitting the New York area the virus spread further into North-America and at this time has been identified in Canada and several countries in Central America as well⁶.

Another recent case considering West Nile virus is its re-emergence in birds in eastern Europe in 2003 and 2004¹¹. This re-emergence is considered different from

earlier outbreaks, because it involved both lineage 1 and lineage 2 viruses, with lineage 2 which was previously believed to reside only in sub-Saharan countries in Africa. Virus strains of this lineage were also believed to be less pathogenic and not neuroinvasive, which in this case they were¹². Since this lineage 2 virus was identified in a goshawk, which is a non-migratory species, one may assume that this virus has adapted to the ecologic circumstances in Europe and may very well spread to other areas in Europe.

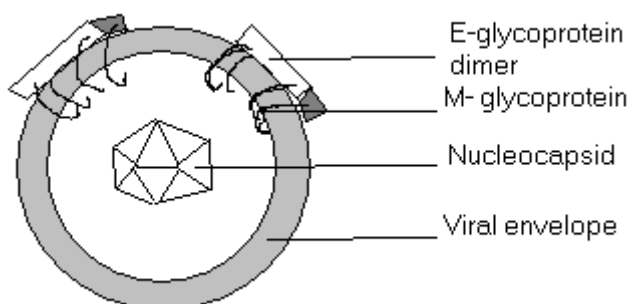
Taxonomy

As said West Nile virus belongs to the family of *Flaviviridae*. This family is subdivided in three genera, namely *Flavivirus*, *Pestivirus* and *Hepacivirus*. These three genera share a similar gene order but furthermore are only distantly related¹³. The genus *Flavivirus* to which West Nile belongs, is further divided in twelve antigenic serogroups. West Nile virus belongs to the Japanese encephalitis group. This group includes among others Murray Valley encephalitis (MVEV) and St. Louis encephalitis viruses (SLEV). West Nile virus serotypes are subsequently divided in two lineages (1 and 2), based on genetic characteristics. Lineage 1 isolates have been associated with outbreaks with human disease, whereas lineage 2 isolates are more restricted to endemic enzootics on the African continent. Lately this distinction has become less clear with the emergence of a lineage 2 isolate in Eastern Europe¹².

Viral structure

West Nile virions have a host-derived envelope with a diameter of about 50 nm, and located within this envelope is the spherical nucleocapsid, which comprises around 25 nm¹³. The envelope, derived from the host cell membranes during replication, is modified by the insertion of two membrane glycoproteins, namely the E (envelope) and prM (precursor membrane) glycoproteins. The prM protein is cleaved by a protease later in virion maturation, thus forming the M protein. The E-glycoproteins form homodimers, and associate with the M-glycoprotein.

Figure 1. Schematic diagram of a flavivirus virus, like West Nile virus. The nucleocapsid encloses the viral RNA. The E-glycoproteins form homo-dimers, and associate with the M (cleaved product of prM) glycoprotein.



The viral genome encodes 10 proteins, of which three are structural (E, C and prM) and 7 are non-structural¹⁴. The non-structural proteins form the replication machinery of the virus. The E protein, which is situated partially outside the envelope, is the immunologically most important protein and mediates the virus-host cell binding.

Replication

The replication cycle of West Nile virus has not yet been fully elucidated, but it is assumed that it resembles that of other flaviviruses, such as dengue virus and Murray Valley virus¹³. First of all the virion binds to a yet unknown cell receptor or

receptors. Glycosylation of the envelope (E) protein has been shown to be very important to this process¹⁵. After binding to this receptor or receptors, the virion is internalized through endocytosis of the virion-receptor complexes. With the fusion of the viral and endosomal membranes, the nucleocapsid is released into the cytoplasm¹⁶. With the disposal of the nucleocapsid, the RNA is subsequently translated, forming a single polyprotein. This polyprotein is cleaved by a complex of two non-structural viral proteins (NS2B-NS3 complex) at several sites, leading up to the mature viral proteins. Another non-structural viral protein (NS5), in cooperation with other non-structural viral and possibly host cell proteins, acts like an RNA polymerase, copying complementary negative strands from the initial RNA template. In turn, these negative strand RNAs serve as a template for the production of new positive strand viral RNAs. These positive strands can be used either for the production of viral structural and non-structural proteins or can be transported to the rough endoplasmic reticulum of the cell in cytoplasmic vesicle membranes. The process of transport to the rough endoplasmic reticulum and the subsequent assembly of the virion are not well understood. After the formation of the nucleocapsid and the envelope, the vesicles with the mature virions move to the cell surface, where they exit the cell through exocytosis¹⁷. Newly produced virions are released from an infected cell approximately ten to twelve hours after primary infection.

Along with the virions, subviral particles called slowly sedimenting hemagglutinins (SHA) can be produced. These particles consist out of cell membranes inserted with the M and E and sometimes prM structural proteins. These particles are obviously non-infectious, but are antigenic.

Pathogenesis

An infection with West Nile virus can cause a large spectrum of neurological disorders and possibly death in several species. The cellular pathogenesis is a very complex process and has not yet been fully elucidated, though recently several pathways have been researched. An infection starts with the introduction of the virus into the host via the bite of an infected arthropod. After this inoculation, an initial viral replication is believed to take place in Langerhans dendritic cells of the skin¹⁸. These cells migrate to and seed draining lymph nodes. This way the primary viraemia results in the infection of peripheral tissues such as the spleen and kidneys.

Subsequently the virus spreads haematogenously to the central nervous system. Other spreading mechanisms include infected immune cells or retrograde axonal transport from infected peripheral neurons. How the virus crosses the blood-brain barrier remains largely unknown, however researchers suspect tumour necrosis factor-alpha (TNF- α) to play a role by mediating changes in the endothelial cell permeability¹⁹. After spreading to the central nervous system, the virus tends to cause cell degeneration and cellular death. Basically there are two distinct categories of cellular degradation, necrosis and apoptosis. Research showed that which one appears is dependent on the infectious dose that is taken up by the host²⁰. When a large dose is taken up, cells tend to show necrosis. When the infectious dose is low, cell tend to show apoptosis. West Nile virus can cause necrosis by the superfluous budding-off of virions during infection, which reduces the integrity of the cell plasma membrane and eventually causes lysis of the cell²⁰.

A possible pathway to apoptosis is associated with the capsid protein of the West Nile virus²¹. In a study, cells transfected with this protein showed a disrupted mitochondrial membrane potential. This disruption implies an increase of the

permeability of this membrane for cytochrome c, better known for its role in mitochondrial respiration. In the cytoplasm cytochrome c forms a complex with a protein called apoptosis activating factor 1 (apaf-1). This complex recruits and activates pro-caspase 9, which in turn is one of the initiating stages in the caspase cascade in apoptosis. Another mechanism of inducing apoptosis is associated with endoplasmic reticulum stress genes. E.R. stress is induced either by aggregation of unfolded protein or in case of excessive protein traffic, which may be the case in a viral infection, such as West Nile virus²². If this happens, a number of cellular mechanisms are set into action to prevent this from happening. If, however, the cell fails to restrain the cause of the accumulation of protein, it leads to apoptosis. The exact processes leading to apoptosis are yet unclear, however researchers showed that during an infection with West Nile virus, the transcription of one of the proteins involved in this process in astrocytes, called old astrocyte specific induced substance (OASIS), was decreased²³. This showed that West Nile virus is able to cause apoptosis in astrocytes.

Pathologic and clinical manifestations

West Nile virus has been associated with clinical disease in a large number of animal species. However avian species, horses and humans seem to be the most affected species¹⁰. Researchers also showed that for some species the virus' cellular tropism is nonrestricted, thus potent of infecting essentially all major organ systems²⁴. Logically this infers that the clinical manifestations of an infection with West Nile virus can be of varying nature.

Among avian species, the brain was a significant target of infection. In particular, the cerebellum is often specifically targeted by the virus. Macroscopical lesions include hepatomegaly, splenomegaly, meningeal congestion and calvarial haemorrhage²⁵. Other often observed lesions are necrotizing lesions in the heart, pancreas and adrenal glands. Microscopically often all tissues can be seen to be affected by the virus. In the CNS perivascular and diffuse inflammatory infiltrates of mostly lymphoplasmacytic origin, focal gliosis and neuronal degeneration are the most prominent lesions observed²⁶. Neural lesions can be found both in the central nervous system as well as in peripheral ganglia. The most frequently observed clinical symptom were those of neurological origin, including ataxia, tremors, circling and convulsions²⁴. Other often observed symptoms are weakness and sternal recumbency, probably to be accounted by the large number of organ systems affected by the infection.

In horses, West Nile virus exhibits a viral distribution pattern that distinctly differs from that observed in avian species. West Nile virus shows a pronounced tropism for central nervous system related tissues²⁷. During histological research, extraneural tissues showed a lack of viral antigen and virus-associated lesions, therefore one may assume that the virus does not or only at a very modest degree infects these tissues. Viraemias also do not reach sufficiently high titres in horses to function as an amplifying host. Macroscopically visible attributes of the infection are usually restricted to redness of parts of the CNS, caused by congestion or haemorrhaging. Microscopically, an equine infection is often characterized by haemorrhaging and a mild to moderate, multifocal and lymphocytic to monocytic polioencephalomyelitis. Involvement of the ventral and lateral horns of the thoracic and lumbar spinal cord is also frequently seen. Focal gliosis and perivascular cuffs comprised of lymphocytes and to lesser extent macrophages, sometimes with infiltration of the adjacent neuropil, can also be observed. More common to West Nile

virus infections, neuronal degeneration can also be seen. Frequently observed clinical signs in horses include ataxia, weakness of limbs and difficulties with rising from recumbence. Other possible symptoms are fever, muscle fasciculation, facial paralysis and blindness²⁸.

In humans, West Nile fever is often the term used to describe an infection with West Nile virus. Histologic findings of West Nile virus infections include perivascular inflammation, microglial nodules, variable necrosis, and loss of neurons²⁹. These lesions occur most often in the deep gray nuclei, brainstem and spinal cord. As in horses, non-neuronal tissues are not affected in human cases of West Nile virus infection.

About eighty percent of human infections are apparently asymptomatic³⁰. Of the persons that do develop clinical signs, most develop West Nile fever. West Nile fever is clinically characterized as generally a mild, acute complex of symptoms, consisting of fever, headaches, malaise, nausea, vomiting and less often rash³¹. Fatigue, sometimes long-lasting (up to sixty days) is also a common complaint of patients. In less than one percent of person infected with West Nile virus, neuroinvasive disease develops. This percentage however might be lower, since neuroinvasive disease is more likely to be reported than an asymptomatic infection³⁰. Symptoms accompanied by a neuroinvasive West Nile virus infection may vary from mild disorientation to coma and even death. Flaccid paralysis and other movement disorders such as tremors however, are the most observed complications.

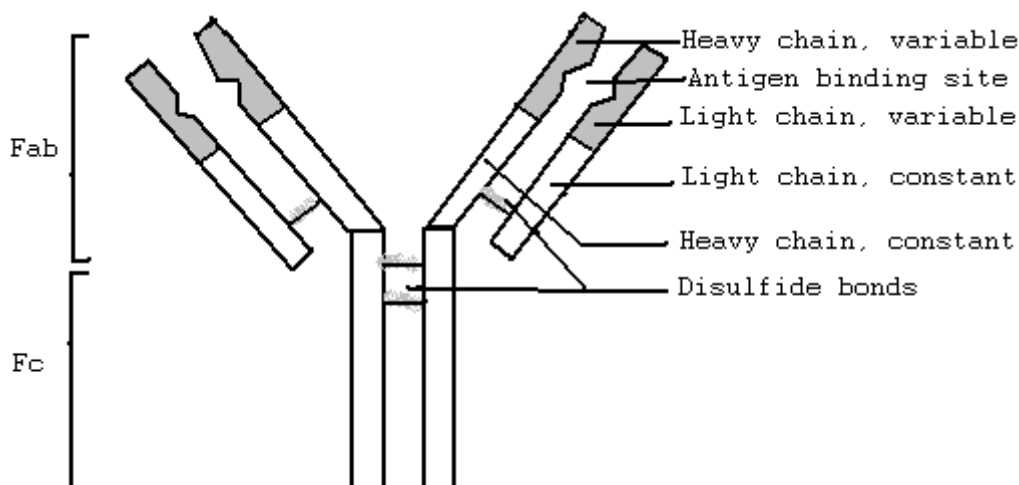
Introduction to immunohistochemistry

Immunohistochemistry is a combination of three separate scientific disciplines, histology, immunology and chemistry. Together they offer the possibility to simultaneously observe both histological irregularities and possible causes of these irregularities. In immunohistochemistry, antigens in tissues are made visible by attaching an antibody to it and subsequently attaching a label (often an enzyme) to the antibody. After the addition of a substrate for the enzyme, a chromogenic reaction has to take place, in order for it to be observed under the light microscope. Over the years, many improvements have been introduced, which have enlarged the specificity and diagnostic power of IHC. These improvements, such as antigen retrieval, blocking of background staining and signal amplification techniques have greatly increased the possibilities of researchers and improved their results. The basic 'ingredients' and techniques of IHC are discussed below.

Antibodies

Immunohistochemistry localizes specific antigens by the use of antibodies that bind to a certain area of the antigen, called the epitope. Antibodies are produced in animals by B-lymphocytes as an immunological response to a presented substance.

Figure 2:
Schematic drawing of IgG class antibody.



There are five classes of antibodies, IgA, IgM, IgG, IgE and IgD, all with different functions. Both IgM and IgG can be used in immunoassays, however IgG is the most frequently used in immunohistochemistry. IgM eliminates pathogens in the early stages of B cell mediated immunity before there is sufficient IgG. The IgG molecule has two separate functions; to bind to the antigen that elicited the response, and to recruit other cells and molecules for destruction of the antigen, and the pathogen with which it is associated³². IgG molecules provide the majority of antibody-based immunity against invading pathogens, and it is one of the most abundant proteins in serum of adult animals. They constitute about 75 percent of all serum immunoglobulins, which makes them very suitable for the production of antibodies. IgG consist of two heavy or large chains and two light or small chains, that are linked by disulfide bonds. This way they form a Y-shaped molecule. The stem of the Y consists of amino acid sequences that are common to all IgGs, and therefore these regions are referred to as the constant region, or the abbreviation Fc (fragment crystallizable). The two arms of the Y, which are made up of both heavy and light chains, contain variable amino acid sequences and are thus referred to as variable region or the abbreviation Fab (fragment, antigen binding). This regions needs to be

variable as it contains the antigen binding site, which has to be chemically and topologically complementary to the antigen in order to bind to it³³.

Antibody production

When a researcher intends to perform immunohistochemistry, he or she must have an antibody that specifically targets the desired antigen that needs to be visualized. There are two ways to do so, namely the production of polyclonal and monoclonal antibodies. Both techniques have their pros and cons, however which one to choose largely depends on the nature of the research that is taking place.

Polyclonal antibodies

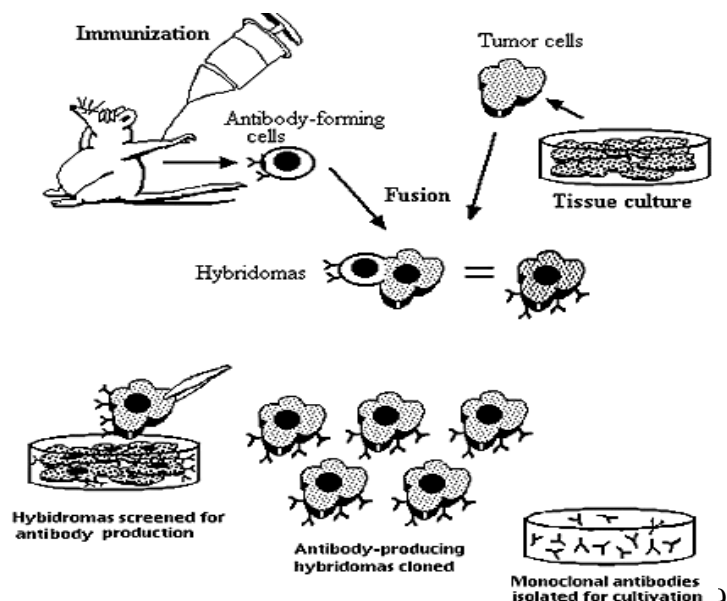
The antigen which one wants to visualize in immunohistochemistry is injected into a mammal, after which it comes into contact with the immune system of the mammal. The cell type of interest is, as said, the B-lymphocyte. The B-lymphocytes that are able to bind the antigen are stimulated to proliferate and to produce antibodies to the antigen. Subsequently the antibodies are purified from the serum³⁴. Antibodies that are produced by this method are derived from different types of immune cells, and hence they are called polyclonal. Polyclonal antibodies can be produced in virtually any animal; however goats, horses, guinea pigs, mice, rats, hamsters, chickens and sheep are more common. When large quantities of antibody are necessary, large animals are used. Chickens are also a frequently used production animal, since they transfer high quantities of IgG into the egg yolk. This leaves invasive bleeding procedures unnecessary.

Polyclonal antibodies are inexpensive to produce compared to monoclonal antibodies. In addition, large quantities can be produced more easily than when using the monoclonal technique. Finally, the process of developing an antibody with a very high affinity for its antigen takes less time (within 2 to 3 months is possible), which makes it more suitable for studies that need results on a short notice. Disadvantages of polyclonal antibodies are the fact that sometimes nonspecific antibodies are produced, leading to background staining in IHC, and that the multiple epitopes, to which polyclonal antibodies are targeted, can more easily lead to cross-reactivity with other antigens³⁵.

Monoclonal antibodies

In contrast to polyclonal antibodies that are produced by different types of immune cells that recognize the same antigen, monoclonal antibodies are derived from a single cell line (or clone)³³.

Figure 3: Schematic overview of monoclonal antibody production. Image can be obtained freely at <http://www.accessexcellence.org/RC/VL/GG/monoclonal.php>



The first step of the production of monoclonal antibodies is equal to that of polyclonal antibodies, with the immunization of an animal. Subsequently the spleen of this animal is harvested, and within it the antibody-producing lymphocytes. Thereafter these lymphocytes are mixed with tumour cells. If fusion of a lymphocyte and a tumour cell occurs, the lymphocyte is in this way 'immortalized' and called a hybridoma. Then the cells need to be screened, in order to obtain the hybridoma that produces the correct antibody. This takes place by growing the separate cell lines in culture and test both if antibody secretion is taking place and using among others an ELISA and Western blot to determine whether the antibody is targeted to the antigen. When the desired clone is identified, two possibilities exist for mass producing this antibody. Firstly, the hybrid cell can again be injected into the peritoneal cavity of a mouse, where after they will form a tumour. The antibody can now be obtained from the antibody-rich ascites fluids formed in the mouse by the tumour. Secondly, the clone can be grown in culture. The former technique however is now considered as somewhat unethical, since the ascites technique may be very painful for the animal. Monoclonal antibodies have several advantages over polyclonal antibodies. For instance, the hybridomas can be frozen and thawed whenever necessary, and therefore a constant and renewable source of antibody is available. The fact that the homogeneity of monoclonal antibodies is high also means that results are better reproducible³⁵. In addition, the chemical composition of monoclonal antibodies can be analyzed to a molecular level, due to its defined composition. A disadvantage of monoclonal antibodies is the fact that loss of epitope as a result of chemical treatments is more likely to happen, since monoclonal antibodies only bind to one epitope.

Tissue preparation

Immunohistochemistry is performed in tissues. In order for this technique to function and lead to satisfactory results, many factors have to be taken into account. Proper fixation and washing conditions, as well as correct incubation conditions are crucial for satisfactory results.

Tissue fixation

Fixation of the tissues is necessary to maintain cell morphology, tissue architecture and the antigenicity of epitopes, which are essential for both a functional IHC and interpretation of the results. Three main types of fixation exist, namely air drying, snap freezing and chemical fixation. For IHC, chemical fixation is the most often used fixating technique, with formaldehyde (in aqueous solution referred to as formalin) as the 'gold standard'³⁶. Other aldehydes can be used as well, though formaldehyde is used most frequently. Formaldehyde preserves peptides and the general structure of cellular organelles. Its fixating action is based on the formation of addition products between the formalin and reactive amino groups. These addition products can cross-link with other reactive groups, and thus profound conformational changes occur. The conformational changes can impede the antigen-antibody binding, and therefore lead up to false negative results. Hence overfixation of tissues should be avoided. The use of buffered formalin can also cause problems. Since amino acids are amphoteric substances, they are influenced by pH. During changes of pH, reactive groups can be formed, leading up to even more cross-linking and subsequent antigen 'masking'. Formalin fixation is often followed by embedding the tissue in paraffin, enabling it to be easily stored and keeping the resolution and morphological detail for prolonged periods of time³⁴. Though paraffination also has adverse effects on antigen-antibody

binding, these are easily overcome by antigen retrieval techniques. During paraffination, the water in the specimen is replaced, first by alcohol or acetone, then by a paraffin solvent such as xylene or toluene. Finally, the tissue is impregnated with paraffin wax.

Other chemical fixatives are acetone and alcohols (methanol and ethanol). These fixatives are also called coagulative fixatives, as they precipitate proteins by breaking hydrogen bonds. However, these fixatives can cause denaturation of proteins as they remove water. In absence of water, hydrophobic and hydrophilic groups of proteins can dissociate, causing them to denature. Another disadvantage of these fixatives is their low tissue penetration. Especially since the introduction of methods to counter the adverse effects of formaldehyde, immunohistochemical studies are therefore generally fixated in formalin.

Tissue washing

The type of buffer used for washing the tissue slides and the dilution of antibodies has a significant impact on the quality of the immunohistochemical staining.

Thorough and effective rinsing between the various steps of the procedure is of utmost importance. PBS (phosphate buffered saline), TBS (tris buffered saline), distilled water and Tris are the most frequently used washing buffers in immunohistochemistry, all with varying contents. The pH and ions in buffers can greatly affect the quality of staining. For instance, PBS contains sodium ions, which can shield negatively charged epitopes³⁶. When using alkaline phosphatase-conjugated antibodies, PBS can not be used, as it contains high concentrations of inorganic phosphate. Another example is the pH. As said, this can cause conformational changes in the tissue. Especially when using monoclonal antibodies, this can have serious consequences for antigen-antibody binding.

Additions can be made to the washing buffer to improve its action. An example is the addition of a small amount of detergent, such as Tween 20. This assists in the uniform spread of the reagents, as a result of the reduction in surface tension.

Antigen retrieval techniques

As said, routine formaldehyde fixation and the rest of the paraffin embedding procedure can alter the conformation of proteins and subsequent a possible loss of antigenicity, eventually resulting in a decrease of staining quality. To recover the antigenicity, antigen retrieval techniques are at the disposal of the researcher. The goal of this technique is the renaturation or at least a partial restoration of the protein of interest³⁷.

There are two basic techniques for antigen retrieval, namely the heat-induced and the enzymatic technique. Currently, antigen retrieval is predominantly defined as the heat-induced technique, however the enzymatic technique is also a fully functional and potentially very useful technique.

Heat-induced epitope retrieval (HIER) has greatly improved the possibilities of immunohistochemical detection³⁶. The exact mechanisms involved are not yet fully known, however one of them is the hydrolysis of the methylene cross-links formed during fixation. That this is only one of the mechanisms is proven by the fact that it also enhances the staining of tissue fixated in for instance ethanol, which does not induce cross-linking. Other hypothesized actions are the extraction of diffusible blocking proteins, precipitation of proteins and rehydration of the tissue sections. Heating can be performed in a steamer or water bath, and also in a microwave. Temperatures and heating duration can be varied, as well as the solution in which

the sections are heated. Ready-to-use antigen retrieval solutions are commercially available, however solutions such as citrate buffer, EDTA and tris base buffers can easily be made and can provide good results as well.

Protease induced epitope retrieval (PIER) is another available antigen retrieval technique, and was the most commonly used technique until the introduction of HIER. The mechanism of PIER is probably the digestion of proteins by enzymes such as trypsin, proteinase K, pronase and pepsin³⁸. The cleavage of these enzymes is nonspecific, and therefore it might have adverse effects on some antigens. The effect of this technique depends on the fixation duration, the type of enzyme, concentration, and incubation time, temperature and pH. Some disadvantages of PIER are the rather small number of antigens for which it is the optimal antigen retrieval method and the possible alteration of tissue morphology³⁷.

Finally, several miscellaneous antigen retrieval methods exist. Treatment of tissue sections with formic acid, strong alkaline solutions, urea, acid solutions, borohydride and sucrose solutions can also be used³⁹.

Visualization of antigens

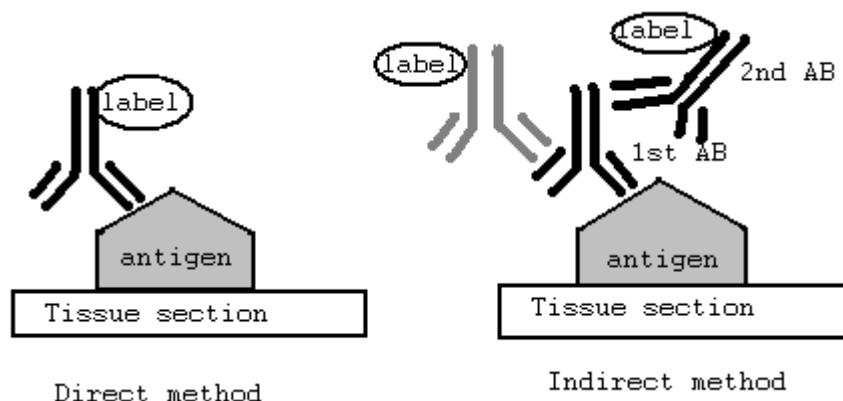
After the binding of antigen and antibody, this complex needs to be made visual under the microscope. This visualization can be accomplished by labelling either the primary antibody during direct immunostaining, or labelling the secondary or tertiary antibody in indirect immunostaining.

The direct method is the simplest way of labelling the antibody. This reaction is a one-step process, in which the primary antibody is conjugated with a label molecule. Numerous substances can act as a label molecule, including enzymes, fluorescent dyes, colloidal gold and biotin³⁴. Fluorescent dyes and colloidal gold can be visualized directly using fluorescence microscopy, for biotin and enzymes a chromogenic (colour producing) system needs to be added. In case of biotin, streptavidin conjugated with an enzyme can bind to biotin. The complex can be visualized after the addition of a chromogenic substrate for the enzyme conjugated to the streptavidin. Enzyme labels commonly used are horseradish peroxidase (HRP) and calf intestinal alkaline phosphatase (AP). In case of an enzyme functioning as a label, only the chromogenic substrate needs to be added. The enzyme converts the substrate from soluble and colourless to insoluble and coloured, which can be seen during microscopy. Common substrates are diaminobenzidine (DAB) and 3-amino-9-ethyl carbazole (AEC).

The direct method is useful for the detection of antigens with a high density or when working with antibodies with a high avidity. An advantage of the direct method is that it is fast to use, however when higher sensitivity is required, for instance when antigens are sparsely spread through the tissue, use of the indirect method is more appropriate³⁷.

In indirect immunostaining, the bound antibody is unlabeled, and is visualized with a secondary antibody which is conjugated with a label. The indirect method is more sensitive as a result of signal amplification, which occurs when several secondary antibodies bind to different antigenic sites (Fc and Fab) on one primary antibody. (Fig. 4) The signal can be even more amplified when another step is added to the process. In this way, the label is conjugated with the tertiary antibody. Schematically it can be seen as another "branch" on the antibody tree, leading up to more "labelled" leaves. However, when the density of antigens is very low, further amplification of the signal can be necessary.

Figure 4: Schematic overview of direct and indirect immunostaining.



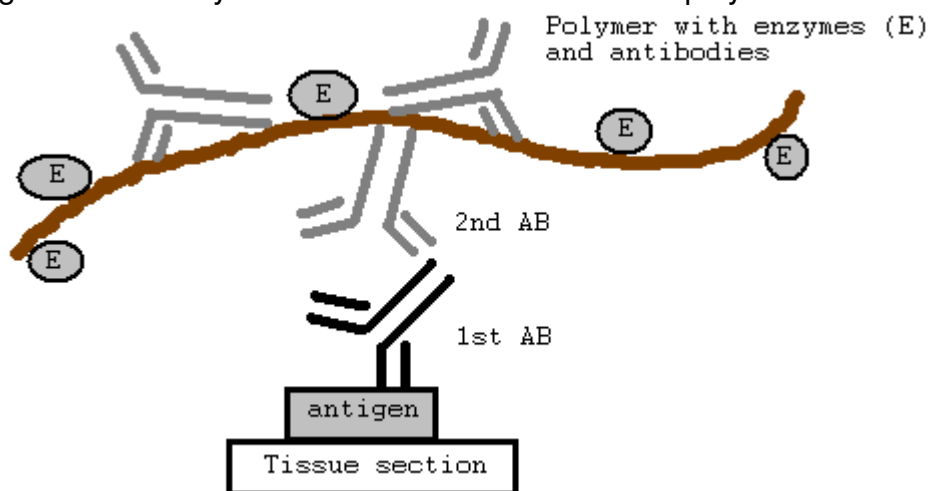
Further signal amplification options

Over the years several techniques have been developed to visualize antigens with a low density. One of them is the aforementioned avidin-biotin associated complex. It is based on the high affinity that avidin has for biotin³⁴. The bond formation is rapid and it is not affected by pH changes. Because avidin also tends to non-specifically bind other tissue components, nowadays it is largely replaced with streptavidin, which is produced by *Streptomyces avidinii*. The most common sequence of ABC application consists of three steps. First, primary antibody directed to a specific antigen, secondly a biotinylated secondary antibody and finally a labelled streptavidin. Recently, the reagents are also available in a preformed complex between streptavidin and a biotinylated enzyme, which increases the sensitivity and reduces staining time⁴⁰.

The ABC technique itself has been further fine-tuned with the development of catalyzed signal amplification (CSA). CSA uses an amplification reagent subsequent to the use of the streptavidin-biotin-peroxidase complex. It contains a soluble substrate (biotinyl-tyramide) that is catalyzed by the bound peroxidases, to form insoluble biotinyl-phenols. The deposited biotinyls then react with a streptavidin-peroxidase complex. This results in an increase of the number of enzymes at the target location, and subsequent amplification of the signal when the substrate for the peroxidase is added³⁶.

An even higher antigen detectability can be achieved with the chain polymer-

Figure 5: Schematic overview of the chain polymer-conjugated technology.



conjugated technology. This technology uses an inert 'backbone' molecule of dextran as a peg for several enzymes. About 70 molecules of enzyme, along with ten molecules of antibody can be attached to the dextran polymer. Since it is not

necessary to make use of biotin and avidin in this technique, nonspecific staining caused by endogenous biotin is prevented.

Background staining and blocking

Background staining is one of the most encountered problems during immunohistochemistry. It can severely impede a correct interpretation of the results and is often caused by more than one factor.

Fc receptors are present on the cell membrane of several cell types, including macrophages, monocytes, granulocytes and lymphocytes. These receptors can non-specifically bind the Fc part of antibodies that are used during immunohistochemistry³⁴. The action of Fc receptors can be easily blocked by adding normal serum, derived from the same species that is used to produce the secondary antibody.

The enzymes that are used as a label in immunohistochemistry, most commonly peroxidase and alkaline phosphatase, can also occur endogenously in some cell types. When substrate for the enzyme labels is added, their action can lead to nonspecific background staining. Though the majority of the enzymes are destroyed during fixation, especially in tissues such as liver, bone and intestines it may still cause problems³⁶. Therefore it may be necessary to treat the tissue sections with substances, preferably levamisole for alkaline phosphatase and hydrogen peroxide for peroxidase, to block their action.

Another common cause of 'background' is the hydrophobic interaction that can occur between molecules. Fixation with aldehydes and the subsequent molecular cross-linking leads up to an increase in hydrophobicity⁴¹. Other contributors are immunoglobulins (antibodies), which are particularly hydrophobic, and dependent of their isoelectric point, can be charged negatively or positively. Two hydrophobic molecules can interact, when their surface tension are lower than that of water, resulting in possible nonspecific staining. Like in the case of the Fc receptors, hydrophobic reactions can be prevented by adding protein in the form of normal serum. Other solutions can be the addition of detergents to lower the surface tension and raising the ionic strength of the diluent solution with ions.

Finally, nonspecific staining often occurs when tissues are physically injured. This physical injury can be due to drying out, an insufficiently penetrating fixative and necrosis or autolysis of tissues. These tissues often stain diffusely. Obviously, the correct handling of tissues is essential to prevent this from happening.

Controls for immunohistochemistry

Controls are necessary for the validation of immunohistochemical staining results. Without it, interpretation would be more or less random, and make the value of the results doubtful. It also helps the researcher in assessing his or her correct following of staining protocols⁴². Commonly used controls for immunohistochemistry are positive and negative tissue controls, with known presence or absence of the antigen.

Materials and Methods

In order to establish a successful immunohistochemical method to detect West Nile virus, a number of experiments were undertaken, investigating possibilities to enhance the quality of staining. The protocol introduced by Erdelyi and his associates¹¹ was used as a basic protocol, and was subsequently adapted as results of the experiments were satisfactory or not. After determining which protocol gave the best results, several tissue sections from animals with a possible West Nile virus infection were tested. These tissue sections were obtained from the archives of the Veterinary Pathologic Diagnostic Centre of the Faculty of Veterinary Medicine, at the Utrecht University.

Materials and methods

The staining experiments were performed on positive control tissues obtained from crows which were experimentally infected with West Nile virus. All procedures were carried out at room temperature, except if mentioned differently. The tissues were fixed in formalin and thereafter embedded in paraffin. Before staining, the tissues were cut into 4 µm thick slides using a microtome and subsequently mounted on tissue-adhesive-coated slides. Dewaxing was performed by consecutively immersing the slides in xylene and 100, 96 and 70% alcohol solutions. The xylene immersion was carried out twice and lasted 5 minutes each. The alcohol immersions were performed twofold as well, but lasted 3 minutes. Rehydration was performed by immersing the slides in distilled water twice for 3 minutes. Antigen retrieval was carried out with protease type XIV from *Streptomyces griseus* (Sigma Aldrich co., Saint Louis MO. prod. No. p5147), 0,1% in either distilled water or PBS at either room temperature or 37°C. Blocking of endogenous peroxidase activity was performed by immersing the slides in a 1% hydrogen peroxide in methanol solution, lasting for 30 minutes. Secondary blocking of endogenously acquired background staining was performed, depending the following treatments, with normal horse or normal goat serum at a 1:10 dilution in PBS, for a time span of 15 minutes. Rinsing of the slides in between the treatments was performed with PBS (0.01 M Na₂HPO₄·2H₂O; 9% NaCl; pH 7,2 - 7,4; Merck chemicals, Germany) washing solution, or PBS combined with Tween (0,1% Tween 20 detergent, Merck, Darmstadt, Germany). The primary antibody used was a mouse anti-West Nile/Kunjin virus (mAB 8150, Millipore, Temecula CA). During the research, several detection methods were tested, including avidin-biotin complexes (Vectastain®, ABC standard and elite, Vector Laboratories, Burlingame CA), the polymers Histofine®, Simple Stain MAX PO (Nichirei Biosciences, Tokyo) and BrightVision Poly-HRP-Anti Ms (ImmunoLogic, Duiven, The Netherlands). Diaminobenzidine (DAB) and 3-amino-9-ethyl carbazole (AEC) were used as a substrate for the enzymes used during the staining. Counterstaining of the tissue sections was accomplished with Mayer's hemalaun (Merck, Darmstadt, Germany). Finally, the tissue sections were mounted under the coverslip using either Eukitt (Kindler GmbH, Freiburg Germany) in case of DAB staining or Aquatex (Merck, Darmstadt Germany) in case of AEC staining

The tissues were selected from the archives based on several requirements. First of all, the search we executed in the database was based on two criteria, namely the presence of encephalitis, and in birds for the combination of encephalitis and additionally myocarditis, based on findings from literature^{24, 25, 26}. The hereby selected cases were further studied, paying attention to the case history. Cases that were clearly not indicative of an infection with West Nile virus, for instance

encephalitis cases with purulent exudate, were excluded. Haematoxylin and eosin tissue slides of the cases with a possible West Nile virus, were subsequently examined histologically, by light microscopy. This examination was performed by a qualified pathologist, with expertise with West Nile virus cases. Again, when the observed lesions were clearly not indicative of West Nile virus, these cases were excluded. Thereafter the remaining cases were tested with the developed immunohistochemical procedure for absence or presence of West Nile virus.

Results

Experiment one

The first experiment that was carried out was primarily based on the protocol introduced by Erdelyi (2007). Since a different antibody is being used, the goals of this first experiment were mainly to determine proper antibody dilutions. The dilutions tested were 1:25, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600, and were diluted in PBS. Antigen retrieval took place at 37°C with 0,1% protease XIV in PBS, and lasted for 10 minutes. The ABC standard kit was used for detection and DAB as chromogenic substrate.

The results of the experiment were fairly satisfactory. A good signal was seen at an 1:25 dilution, especially in the vicinity of vessels and in infiltrates (Appendix figure 1). Background staining was very low, if observed at all. A remarkable fact was that at 1:50 and higher dilutions, a signal was no longer observed (Appendix figures 2 and 3). Finally, we may note that the negative control (treated with PBS instead of the antibody) showed no positivity at all (Appendix figure 4).

Experiment two

After reconsidering the results of the first experiment, we decided to keep using the antibody at a 1:25 dilution, and test the effects of two parts of the protocol: the length of antigen retrieval and the use of ABC standard and ABC elite kits. We tested the antigen retrieval time by prolonging the time of exposure to the enzyme to 15 and 20 minutes (37°C). We hypothesized that a longer exposure time to the enzyme might lead up to a higher grade of unmasking of the antigen. Furthermore we wanted to test the ABC elite kit, since the sensitivity is 5 to 10 times higher than that of the standard ABC kit. If this procedure would give fair results, we might be able to use a higher dilution of the costly antibody in subsequent experiments.

The main thing we noted during the second experiment was the degree of tissue damage. Tissue washed of the glass slides very easily, especially after application of antigen retrieval. Both macro- and microscopically, artifacts and tissue damage were present. The observed tissue damage seemed to be worse in the slides which were treated for 15 and 20 minutes during antigen retrieval. A possible cause is a loss of integrity of the tissues, during the enzymatic treatment of the tissues. In the unaffected parts of the tissues, the effect of standard versus elite ABC kits could still be observed (Appendix figures 5 and 6). However, a clear superiority of one kit over the other could not be established.

Generally, in the tissue sections that could still be assessed, a fairly well signal was observed. Again, the majority was observed in cells surrounding the vessels and in inflammatory infiltrates in the neuropil.

Experiment three

The results of the second experiment lead us to think that a part of the tissue damage may have been caused by the length of the antigen retrieval. Therefore we decided to limit the time of this procedure to 10 minutes at 37°C. Moreover, we performed antigen retrieval with the enzyme dissolved in both distilled water and PBS, to see if the diluents had any effects on antigen retrieval. We also tested a polymer (Histofine) as a second antibody, to see if it would lead to better results than the ABC standard kit. Finally, we changed the chromogenic substrate from DAB (brown) to AEC (red), for better contrasting with the hemalaun counterstain.

After microscopical examination of the slides, we concluded that the tissues were again damaged quite seriously. The detachment already started at deparaffination,

but was worse after antigen retrieval. In the unaffected parts of the tissues, the difference between a treatment with protease XIV in PBS and distilled water could be determined. Although the differences were not very distinct, in slides treated with protease in PBS there was less background staining, and overall staining looked better. The polymer showed no better results than the standard ABC kit and was therefore no longer used in further experiments (Appendix figures 7 and 8).

Experiment four

Due to the tissue damage we experienced on silan coated glass slides, we decided to proceed testing on KP+ glass slides. These slides are more suitable for adhesion of soft, vulnerable tissues, such as brain tissue. Because we bore in mind that the tissue damage might have influenced the results of our previous experiment, we also decided to once more perform experiment three, though now we used the BrightVision polymer instead of the Histofine polymer. An advantage of the BrightVision polymer is that it is faster to use. Since the BrightVision is an anti-mouse antibody generated in goats, we now used normal goat serum during blocking of endogenous alkaline phosphatase activity. The remainder of the protocol was, as said, identical to the one used in experiment three.

The results of our fourth experiment were somewhat disappointing, since the tissues were still damaged. The degree however was less stern in all sections, and in the sections in which antigen retrieval took place in PBS damage was even more reduced. This lead us to believe that the damage may be partially attributed to how antigen retrieval is performed, and we therefore decided not to use protease XIV in distilled water anymore. Concerning the BrightVision polymer, a distinct improvement of the staining quality was observed in the tissue sections treated with this polymer, compared to those treated with the ABC standard kit (Appendix figure 9). The polymer-treated slides showed significant positivity, and as in the previous experiments the majority was localized in the perivascular cuffs and in inflammatory infiltrates (Appendix figure 10).

Experiment five

In the fifth experiment we tested three new variables: we compared a lower grade of dilution (1:10) to the one we used before (1:25) and the temperature whereby antigen retrieval was performed. Besides that, we also tested the influence of the order in which antigen retrieval and the blocking of endogenous peroxidase are performed. We tested this, because the blocking of endogenous peroxidase, which is carried out by treating the slides with hydrogen peroxide in methanol, may by itself cause an increase in antigen masking, and therefore, if blocking is performed after antigen retrieval, it may decrease the efficiency of antigen retrieval.

Generally, the results of this experiment were very promising. Especially the 1:10 dilution treated sections resulted in a very complete representation of the antigen, though with low background staining (Appendix figure 11). Distinct granules, localized near the vessels, were visible. As concerns the order of antigen retrieval and blocking, no clear advantage of any one was visible. Slides upon which antigen retrieval took place firstly, showed results that could be considered better, however the other way around also lead to good results. This also applies to the temperatures whereby antigen retrieval takes place. No clear differences were observed (Appendix figure 12). All in all, we supposed that the protocol that produced the best result in our fifth experiment should become the backbone of our final protocol.

The subsequent experiments that we performed can be considered as “fine-tuning” of this protocol.

Experiment six

In this experiment we wanted to investigate if it was possible to achieve results with a 1:25 antibody dilution that were similar to the ones obtained with a 1:10 dilution. Therefore we tested our ‘backbone protocol, with a 1:25 antibody dilution, and then increased the incubation time of the chromogenic substrate AEC. We hypothesized that, since the actual detection of the antigen was successful in the preceding tests, it should be possible to use the antibody at a lower dilution.

The results of this experiment were, as expected, satisfactory. The positive signals were seen diffusely throughout the tissue, with the majority around the perivascular cuffs and in infiltrates. Background staining was more intense than when they were stained for 15 minutes, but did not interfere with the interpretation of the results. Combined with the fact that working with a 1:25 antibody is also less costly, we decided to keep the 20 minute staining procedure in our protocol.

Experiment seven

Finally, we tested one more adaption to our protocol. This time we tested a 1:15 antibody dilution, combined with a twenty and fifteen minute’s incubation with AEC. As may be expected, the results were good. Positivity was also visible at lower magnifications and diffusely spread throughout the tissue. However, when comparing to the 1:25 treated tissues, it did not lead up to results significantly better than the 1:25 treated tissues. Therefore, in the final protocol we will use a 1:25 diluted antibody. The establishment of the immunohistochemical method to detect West Nile virus was now finished. The protocol we established is the following.

Protocol for immunohistochemical detection of West Nile virus in formalin-fixed paraffin-embedded tissues.

1. Mount 4 micrometer paraffin section on KP+ coated microscope slide.
2. Dry overnight at 55 °C.
3. Deparaffinize and rehydrate in 2x xylene (2x5 min), 2x alc100%, 2x alc96%, 2x alc70%, 2x AD (2x3 min).
4. Pretreat the slides with 0,1% protease type XIV in PBS. 10 min 37 °C.
5. Rinse in PBS 3x5 min
6. Block endogenous peroxidase activity in 1% H₂O₂ in methanol. 30 min
7. Rinse in PBS/Tween. 3x5 min
8. Treat with Normal Goat Serum 1:10 in PBS. 15 min
9. Incubate with Mouse anti-WNV/Kunjil 1:25 in PBS. 4 °C ON
10. Rinse in PBS/Tween 3x5 min
11. Treat with BrightVision poly-HRP goat anti-mouse. 30 min
12. Rinse in PBS. 3x5 min
13. Incubate with AEC staining solution. 20 min
14. Rinse once in tapwater in fumehood, discard in jerrycan IV.
Rinse in running tapwater 5 min
15. Stain nuclei with haematoxylin. 30 sec
16. Rinse in running tapwater 10 min
17. Cover slides with Aquatex and coverslip .

Experiment eight

After the final establishment of the protocol, the specimens from the database selected for immunohistochemical testing were tested. These specimens contained both equine, bird and wildlife cases. Below the pathological case descriptions are mentioned shortly.

Equine case 1: P0113318, female

Lymphocytic meningo-encephalitis with vasculitis and malacia, possible viral inflammation.

Equine case 2: P9401040, female

Meningo-encephalitis of unknown origin.

Equine case 3: P9406061, female

Milde meningo-encephalitis, predominantly round cell infiltrates.

Avian case 1: B0002437 *Gallus gallus*, gender unknown.

Encephalitis with round cell infiltrates. Suspected of a viral infection.

Avian case 2: B0102220 *Gallus gallus*, male.

Hepatitis, nephritis, enteritis and reactive spleen, severe encephalitis of round cell origin.

Avian case 3: B 0302229 *Rollulus roulroul*, gender unknown.

Lymphoplasmacytic panophthalmia and encephalitis. A viral cause could not be excluded.

Avian case 4: B0401368 *Serinus canaria*, gender unknown.

Encephalitis with perivascular cuffing with round cells. An underlying viral infection could not be excluded nor be demonstrated.

Avian case 5: B0501733 *Gallus gallus*, gender unknown.

Meningo-encephalitis, possibly on a viral basis.

Avian case 6: B0601225 *Rollulus roulroul*, male.

Myocarditis, encephalitis and vasculitis, fitting with a viral background.

Avian case 7: B9701589 *Chloebia gouldiae*, gender unknown.

Widespread round cell encephalitis. Suspected of a paramyxovirus infection.

Avian case 8: B9901418 *Gallus gallus domesticus brisson*, female.

Meningitis, encephalitis.

Avian case 9: B0300227 *Gallus gallus*, male.

Encephalitis with perivascular cuffing. Suspected to have paramyxovirus.

Avian case 10: B0200071 *Somateria mollissima*, male.

Diffuse round cell encephalitis, expanded to the autonomous system.

Avian case 11: B9802256 *Perdix perdix*, gender unknown.

Severely extended lymphocytic meningo-encephalitis. Hepatitis.

Wildlife case 1: B3091117024 *Capreolus capreolus*, female.

Animal found with kyphosis, no longer afraid of people. Still in summer coat, whilst it was already November. Euthanised.

Alongside the selected cases with a possible West Nile virus, two cases were selected which were definitely negative for West Nile virus. These two cases were selected after microscopical examination by a qualified pathologist at the Veterinary Pathologic Diagnostic Center of the Faculty of Veterinary Medicine, at the Utrecht University, with expertise in West Nile virus cases. These cases functioned as negative control tissues in our experiment. Below the two case description are shortly mentioned.

Equine case 1: P0302828, female

No obvious cause found, mild encephalitis, herpesvirus improbable.

Avian case 1: B0402168 *Neophema pulchella*, gender unknown.

Mild lymphocytic to histiocytic meningo-encephalitis. Mild pancreatitis.

A positive signal was not found in anyone of these cases.

Discussion

West Nile virus is a re-emerging, possibly neuroinvasive viral disease. After a more periodical re-appearance in the past century, the 21st century is characterised by outbreaks on a larger scale. Hitting Northern America²⁴ and Eastern Europe¹² in the early 2000s and more recently in 2010 in Russia and Greece, West Nile virus has evolved into a pathogen that needs to be taken seriously.

Principal findings

In this study we aimed to determine a successful immunohistochemical protocol to detect West Nile virus in formalin-fixed and paraffin-embedded tissues. We did so by performing a series of experiments, in which we tested a large number of variables. After the establishment of this protocol we tested selected cases from patient databases for the presence or absence of the virus. West Nile virus was not detected in these tissues.

Strengths and weaknesses of our study

A weakness of the study is that we had to use the antibody in a 1:25 dilution. This dilution was sufficient for detection in experimentally infected tissues, in which the antigen was obviously abundantly present. However, to what extent West Nile virus causes pathologic changes and clinical illness, varies between species and individuals. The amount of antigen present in tissue may vary as well, and therefore cases which actually do have an infection with West Nile virus, may not be detected as such with this immunohistochemical method.

Another weakness of our study is the limited number of tests ran in the separate experiments during the protocol development. Since the results are dependent of many factors, a limited number of probes might more easily be affected by external factors. However, this did not prevent the establishment of a successful protocol. On the other hand, more testing might have directed us to different protocol.

A strength of our study is the fact that we had the opportunity to establish our protocol with the use of a positive control tissue. This gave us the chance to confidently assess the influence of certain factors on the results of our tests and determine if our results were reliable.

Meaning of the study and implications for clinicians

The protocol developed in this study offers a method to detect West Nile virus, a pathogen of growing importance in Western Europe. In our study the results obtained utilizing this protocol were satisfactory. Many infections with West Nile virus can be asymptomatic. Antigen distribution also varies between species and individuals. Especially neurovirulence is not always accomplished by the virus. Besides the fact that in order to perform immunohistochemistry tissues are required, this technique alone therefore can not be considered as the gold standard for detection of West Nile virus by clinicians. However, combined with other techniques such as PCR and virus isolation, one can confidently say if a patient is or is not infected with West Nile virus. A question that is likely to be asked more frequently in the future.

The protocol itself offers other researchers an opportunity to utilize the immunohistochemical method, without the burden of having to find out how to do this in a correct way.

Unanswered questions and suggestions for research

After this study, a number of questions still remain unanswered. One of them is if the use of another antibody may be possible in the future. The antibody we used in our study lacked somewhat sensitivity, visible in the fact that we had to use it in a 1:25 dilution. Other researchers¹¹ mentioned using their antibody in 1:1000 or even higher dilutions. A suggestion for future research might therefore be the use of and subsequent development of an immunohistochemical protocol for a more sensitive antibody.

Another question that still remains is if West Nile virus will reach Western Europe, or already has. Most recent articles mention a spread to as far as Austria, however in the United Kingdom, birds were found with antibodies against West Nile virus⁴³.

Whether West Nile virus would reach Western Europe depends on several factors. Theoretically, all that is needed for an outbreak are the vector, a reservoir, and hosts. Since these are all present, basically only the introduction of the virus is necessary. A very good suggestion for research therefore may be a serological survey amongst animals, as performed in the United Kingdom, and perhaps the more frequent testing of animals, with a case history remotely resembling that commonly observed in West Nile virus infections.

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Appendix

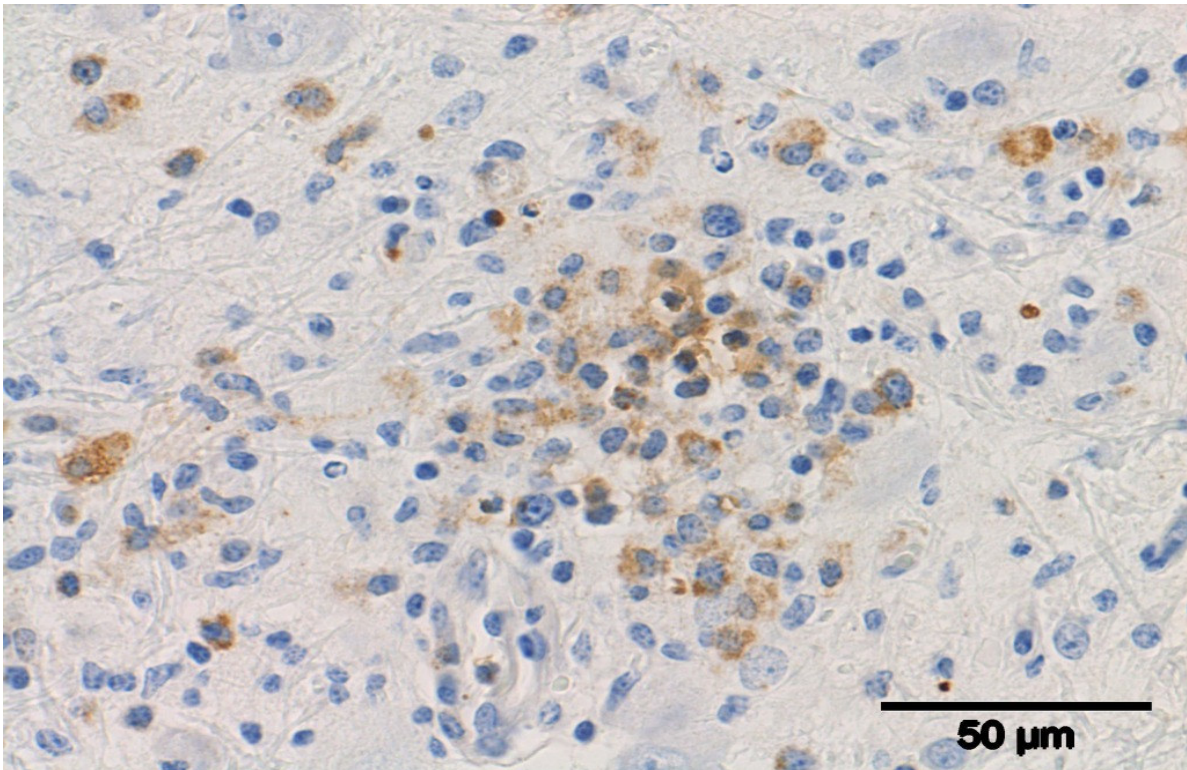


Figure 1: (40x magnification)

Tissue section treated with a 1:25 primary antibody dilution. A positive signal can be observed in the cytoplasm of some cells in this inflammatory infiltrate.

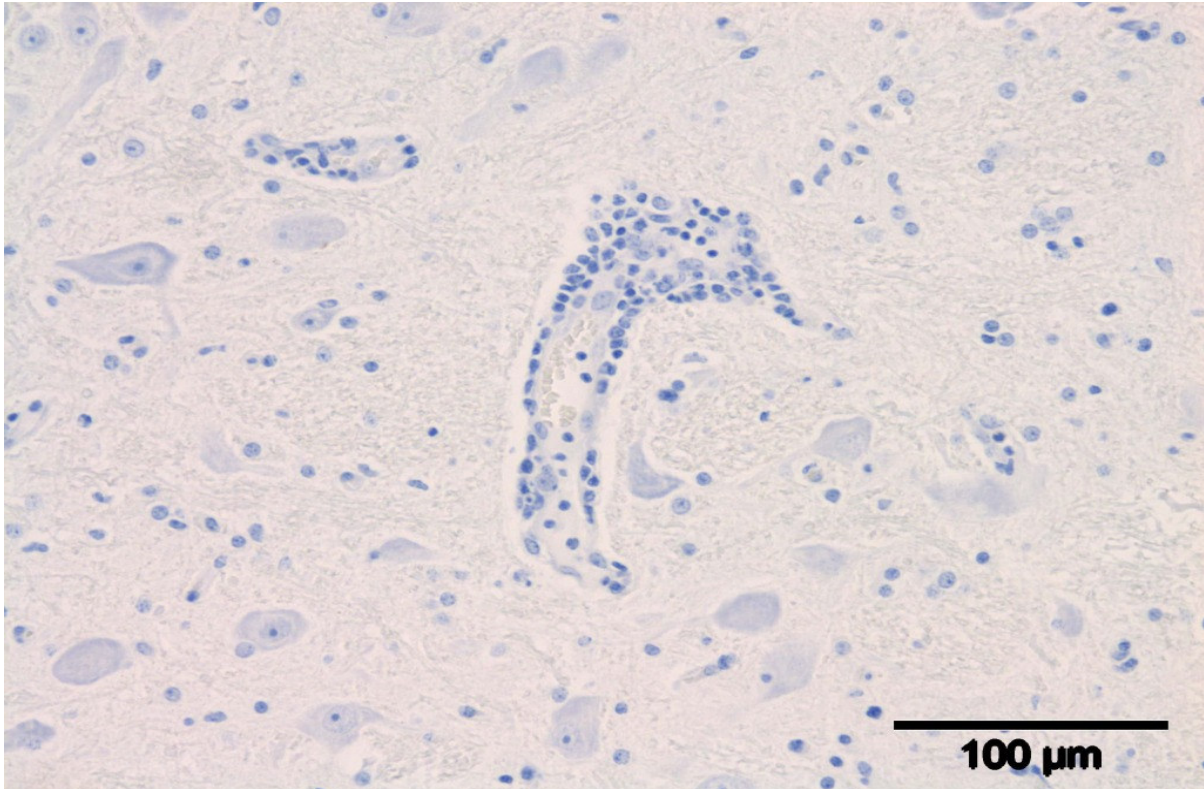


Figure 2: (20x magnification)

Tissue section treated with a 1:50 primary antibody dilution. Contrarily to the section treated with a 1:25 dilution, almost all positivity is lost.

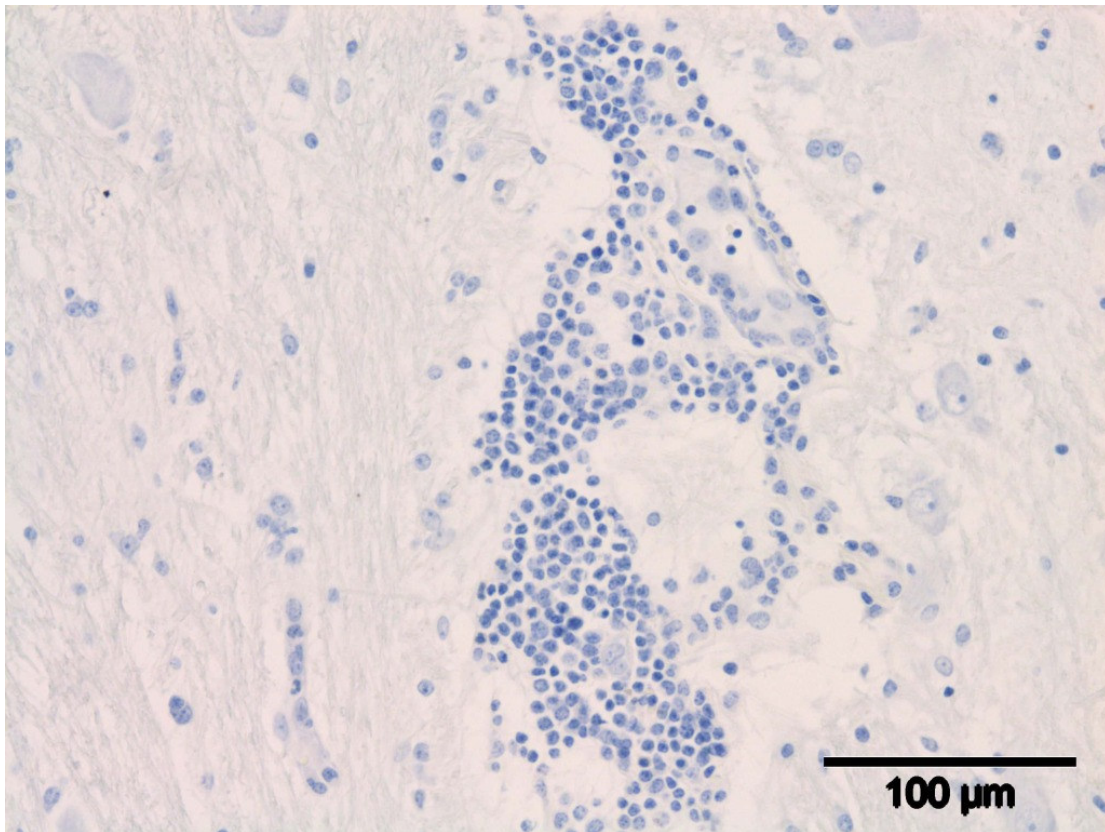


Figure 3: (20x magnification)
Tissue section treated with a 1:100 primary antibody dilution. In this section no positivity was observed.

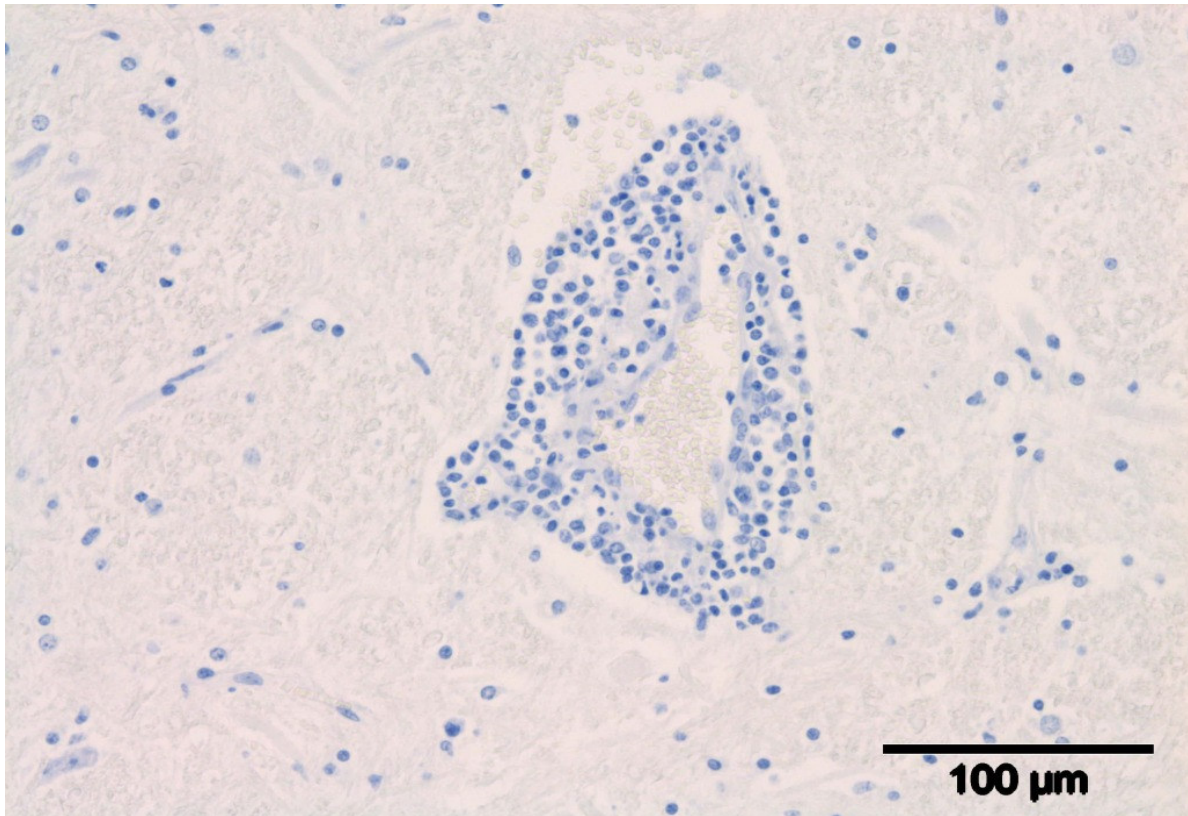


Figure 4: (20x magnification)

This image depicts the negative control tissue. As expected no positivity is observed. A perivascular cuff is visible, which is an accumulation of inflammatory cells surrounding the vessel walls. Occurrence of perivascular cuffs can be indicative of a viral infection.

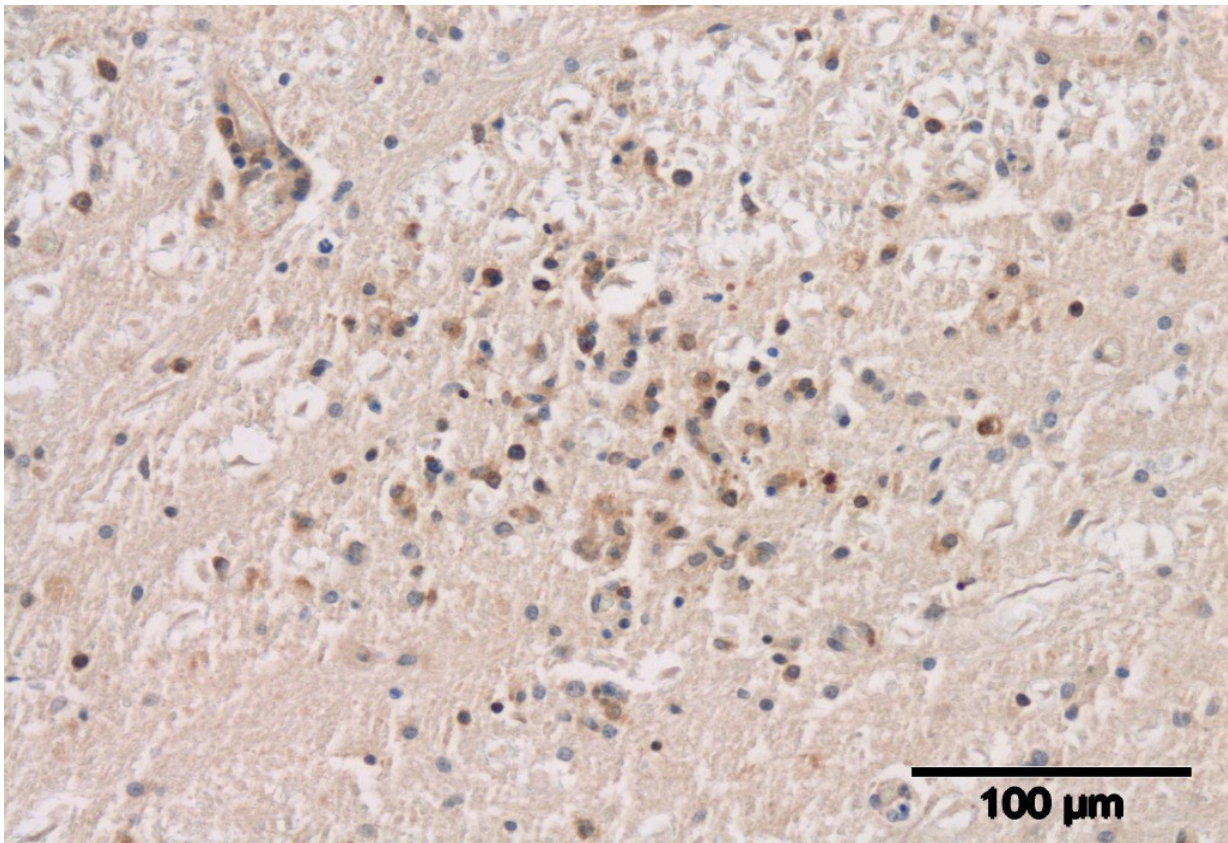


Figure 5: (20x magnification)

This slide was treated with the ABC Elite detection kit. A positive signal is visible, however the increase in background staining was a setback.

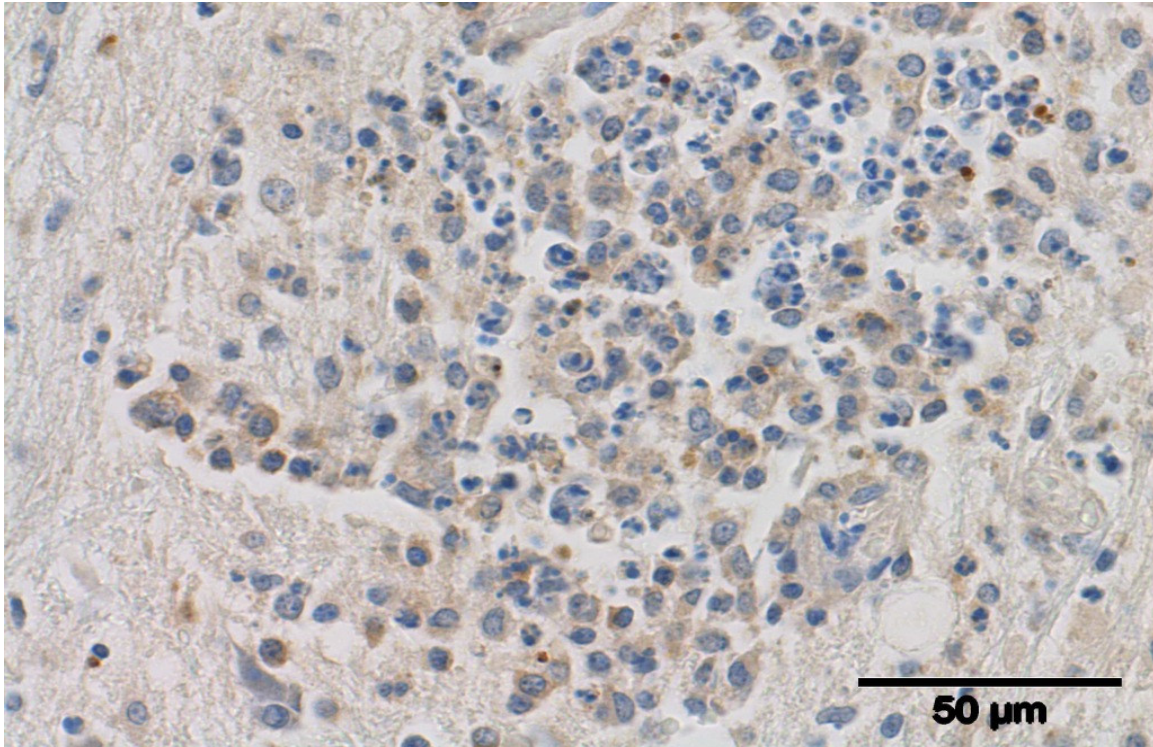


Figure 6: (40x magnification)

This tissue slide was treated with the ABC standard detection kit. As may be visible in both pictures, the difference in the quality of detection was not very significant.

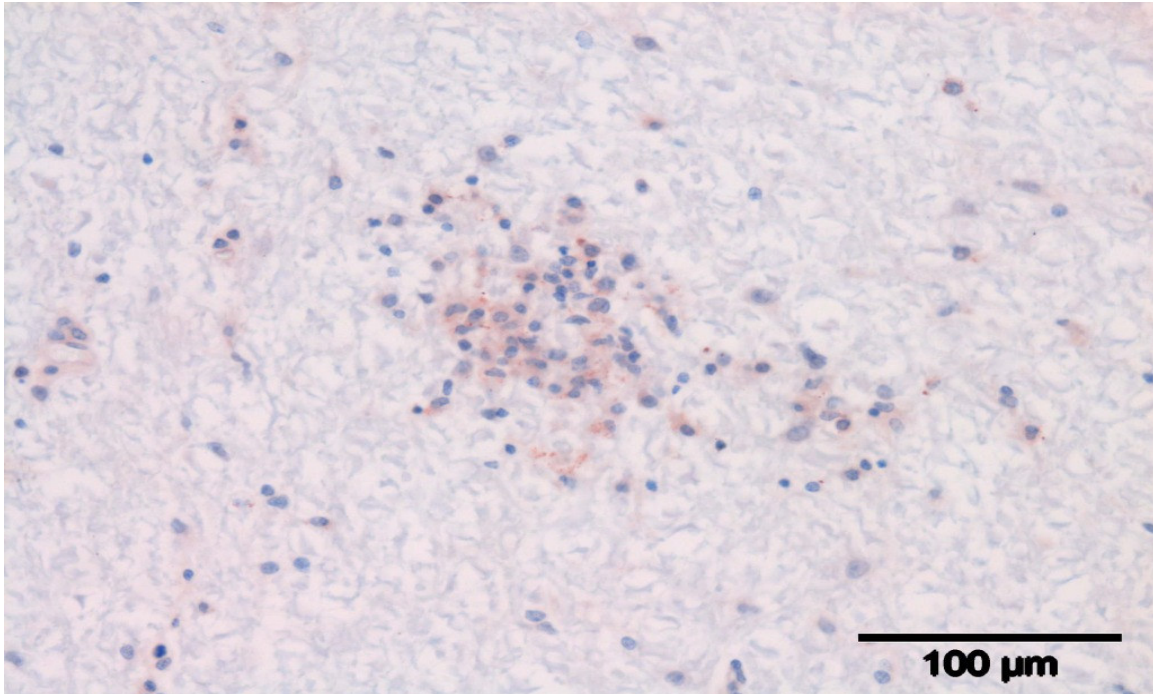


Figure 7: (20x magnification)

This tissue was treated with the Histofine polymer. Antigen retrieval took place in PBS. The signal is present, however amplification is necessary.

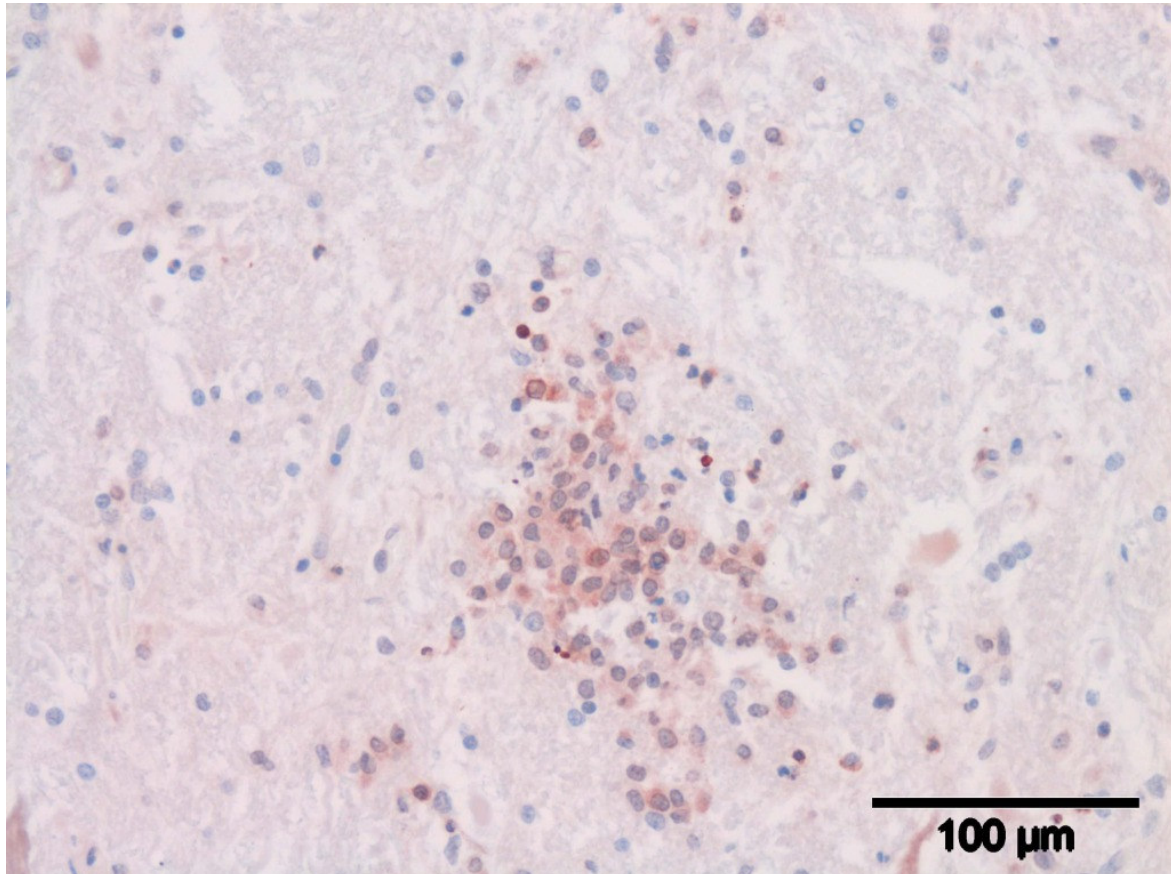


Figure 8: (20x magnification)

As the tissue in figure 7, treated with the Histofine polymer. In this slide, the antigen retrieval took place in distilled water.

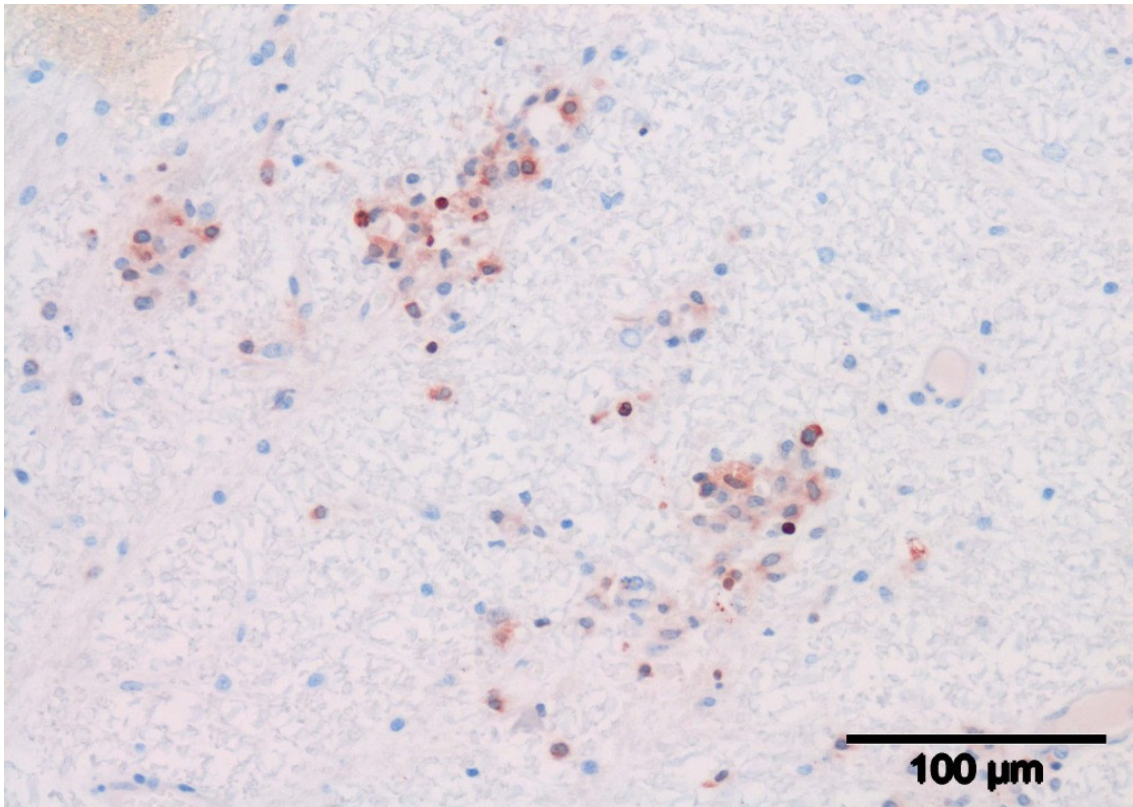


Figure 9: (20x magnification)

This tissue was treated with the BrightVision polymer. There is less background staining, leading up to more contrast and therefore a higher quality of detection.

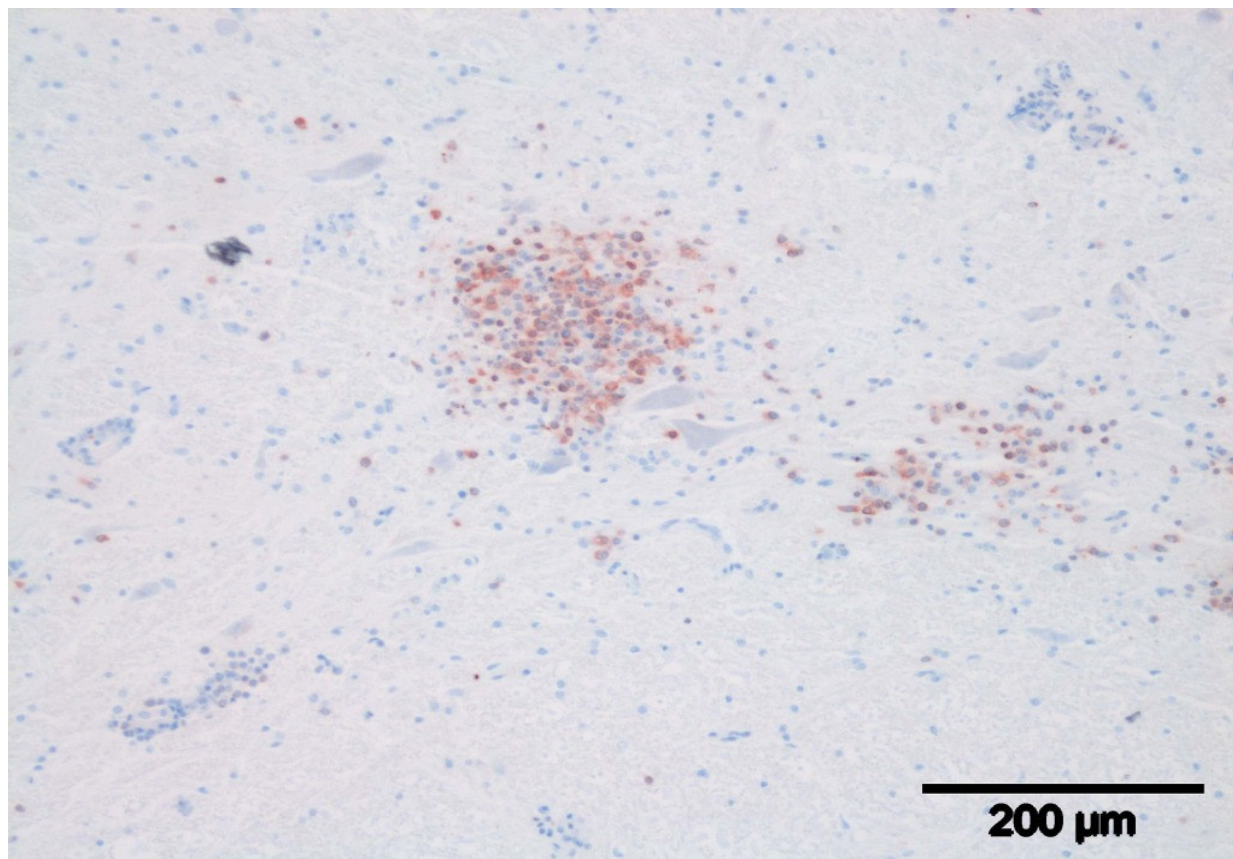


Figure 10: (10x magnification)

This tissue section was treated with the BrightVision polymer, and with a 1:25 primary antibody dilution. At a lower magnification (10x), a significantly positive signal can be observed.

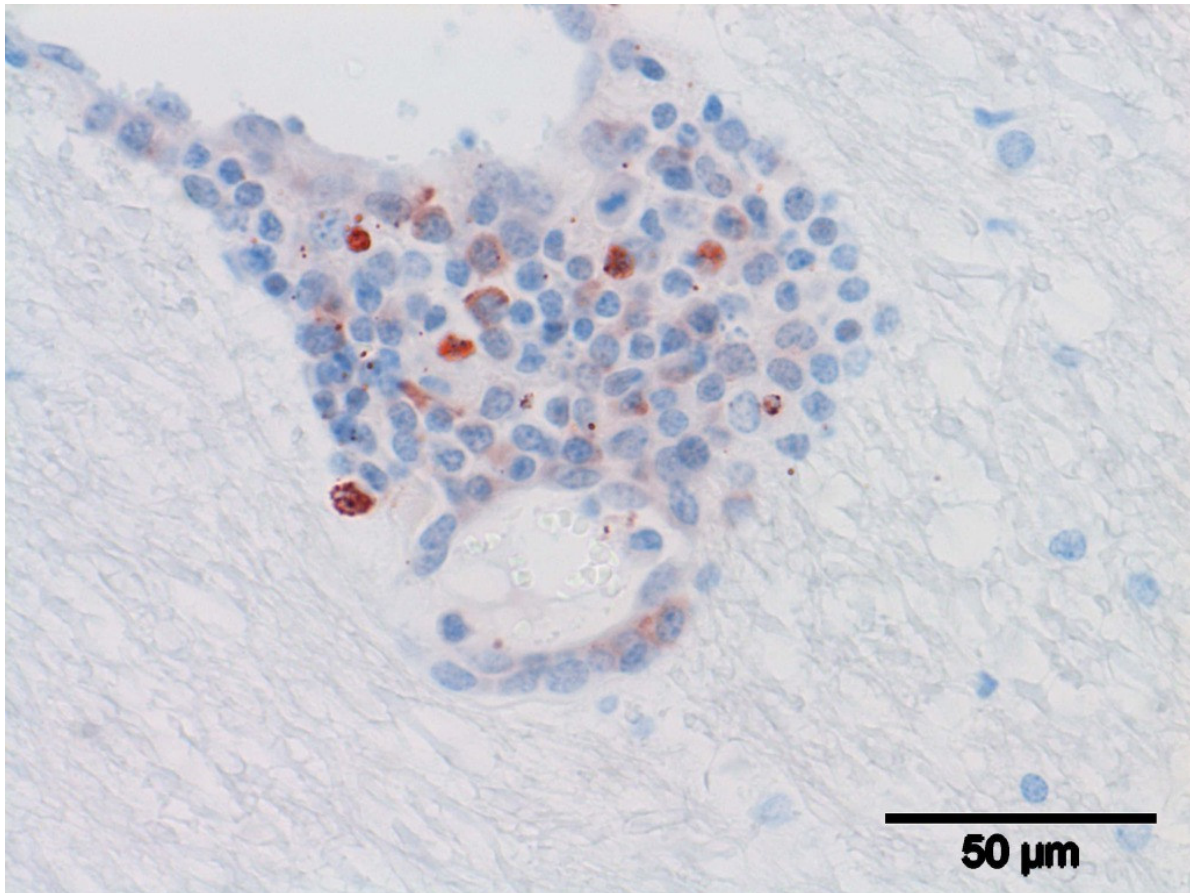


Figure 11: (40x magnification)

This tissue section was treated with a 1:10 primary antibody dilution. In the vascular cuff, distinct granules can be seen where the antigen is detected.

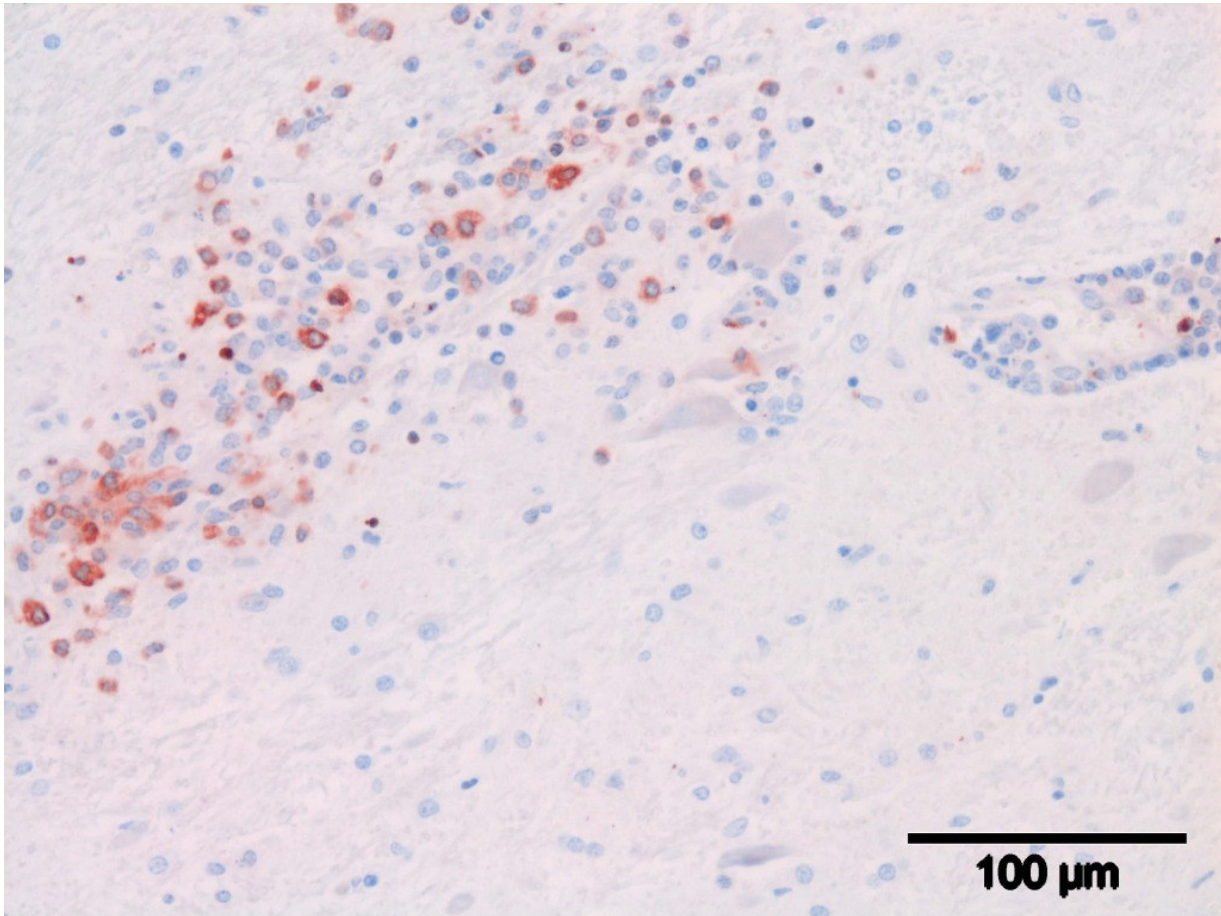


Figure 12: (20x magnification)

This tissue was treated with a 1:25 primary antibody dilution, and was incubated with AEC for 20 minutes. The results are comparable to those obtained with the 1:10 primary antibody dilution.

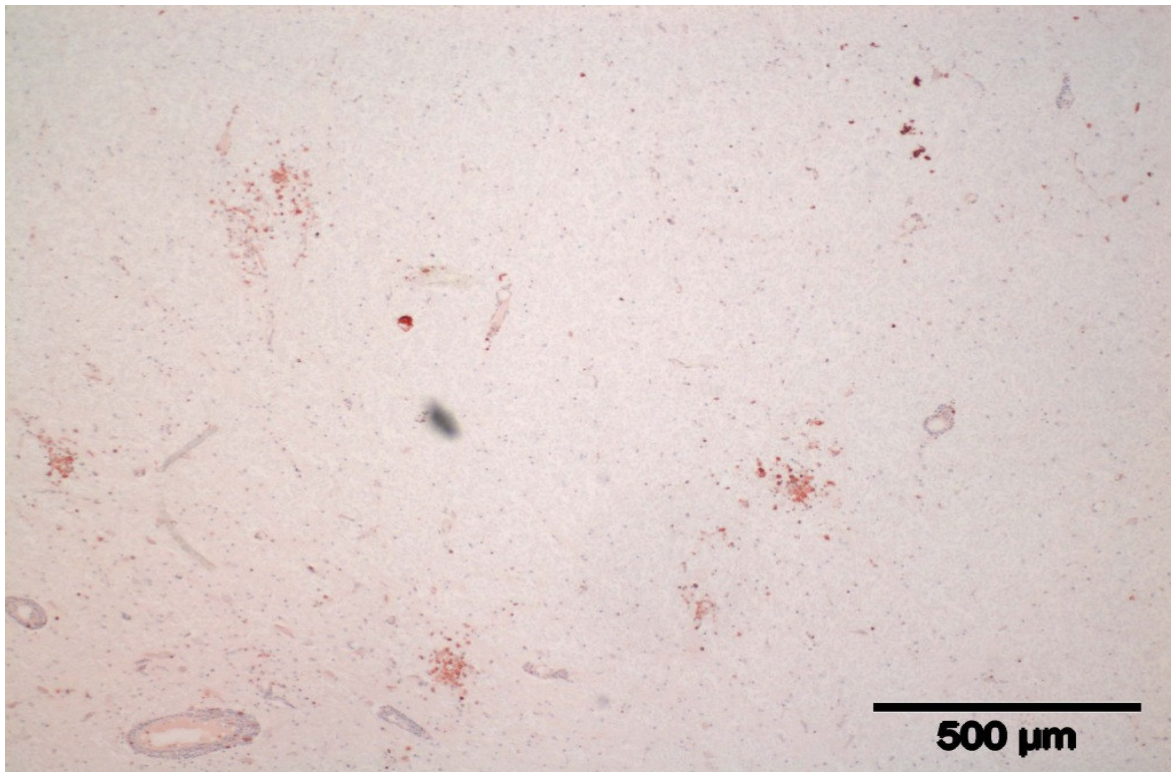


Figure 13: (4x magnification)
Tissue section treated with a 1:15 primary antibody dilution and incubated with AEC for 20 minutes. Antigen detection is of high quality and therefore visible at a low (4x) magnification.