

Use of immunohistochemistry and molecular assays such as RT-PCR for precise *post mortem* diagnosis of distemper-related encephalitis

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The wide spectrum of neurological presentations of nervous distemper, granulomatous meningoencephalitis (GME), and necrotizing encephalitis (NE) may complicate their clinical diagnosis. In addition, although there are some differences, distemper encephalitis, NE, and GME share similar histological changes such non-suppurative inflammation. Further, necrotic lesions may be present in both distemper and NE. As nervous distemper may mimic other inflammatory conditions of the brain, CDV infection should be always excluded before making the definitive diagnosis of a neuropathological entity such as GME, NE, or other non-suppurative encephalitis of unknown etiology. However, immunohistochemical (IHC) assays routinely used for *post mortem* CDV antigen detection may cross-react with auto-antigens, resulting in non-specific immunolabeling. Accordingly, in this mini-review a critical approach to the usefulness of IHC for definitive diagnosis of nervous distemper will be discussed. Additionally, the use of CDV gene detection by molecular assays such as RT-PCR for *post mortem* diagnosis of nervous distemper will be commented on.

Keywords dog; canine distemper virus; central nervous system; encephalitis; immunohistochemistry; RT-PCR

1. Differential diagnosis of encephalitis in dogs

In the practice of veterinary neurology, it is known that different causes may lead to neurological disturbances with degenerative, metabolic, autoimmune, nutritional, inflammatory, toxic, and vascular etiology [1-3]. Among all the possible etiologies, inflammatory conditions of the central nervous system (CNS) are especially important and may contribute to almost 1/3 of the neurological diseases of the brain in small animals [1, 4].

Two distinct causes of brain inflammation have been reported: non-infectious idiopathic and infectious encephalitis [5-7]. *Ante mortem* diagnosis of infectious and non-infectious encephalitis occasionally remains very difficult if not impossible, even when extensive diagnostic testing (ancillary diagnostic aids) is performed [8, 9]. The precise diagnosis of the brain's inflammatory condition is often challenging, even with the use of techniques for the detection of infectious agents such as PCR and immunohistochemical assays [2-7].

Idiopathic inflammatory diseases or inflammatory disturbances of unknown etiology are often involved in brain disease in dogs [6, 7, 13]. Breed-specific types of necrotizing encephalitis (NE) such as necrotizing meningoencephalitis (Pug-type NE) and necrotizing leukoencephalitis (Yorkshire-type NE) have been recognized as important diseases in different small-sized breeds [14-19]. Granulomatous meningoencephalomyelitis (GME) is also a type of encephalitis that has been commonly identified in small-sized dog breeds [14, 15, 20, 21].

Important causes of infectious diseases with neuroparenchymal inflammation include viral, fungal, protozoal, and bacterial meningoencephalitis [5-9]. Canine distemper virus (CDV) is the most important pathogen for meningoencephalomyelitis in dogs [9, 22, 23]. Despite the extensive practice of vaccination worldwide, CDV is an important re-emerging agent of nervous disease in dogs [24-27]. Distemper remains endemic in many regions [28-30] and has also even been recorded in vaccinated dogs [28, 31]. In addition, some special forms of chronic nervous distemper may be observed in vaccinated dogs, such as *multifocal chronic distemper encephalomyelitis in mature dogs* [32] and the extremely rare chronic variant form of distemper in mature/old animals known as *old dog encephalitis* [33, 34].

Neurological examination may be not useful to differentiate nervous distemper from other encephalitis [35, 36]. The wide spectrum of neurological signs of distemper, NE, and GME [14-17, 20, 23, 29, 37-41] may complicate the clinical diagnosis of such encephalitis, and the methods available for *ante mortem* diagnosis of such brain conditions to date are of limited value [4, 10, 12, 42]. Thus, in many cases the definitive diagnosis is only possible *post mortem*, and histological examination of the CNS is essential for the specific *post mortem* diagnosis.

Distemper-related encephalitis, NE, and GME have been classified as non-suppurative encephalitis on histopathological examination [5]. Although there are some differences, these inflammatory brain conditions in dogs may share similar histological changes as determined by routine methods (H&E staining), including meningitis, perivascular cuffing, and reactive and inflammatory changes within the nervous parenchyma characterized, respectively, by glial reaction and mononuclear cell infiltration, mainly by lymphocytes and monocyte/histiocyte-lineages [15, 18, 23, 34, 43, 44]. Necrotic and malatic lesions may also be present in distemper encephalitis [23, 29, 45-47] and NE [14, 15, 17, 18]. Additionally, such necrotic lesions in distemper may occasionally be recognized in both

the gray and white matter of the forebrain and may mimic the microscopic and eventually even the gross findings of NE [42].

In the conventional neuropathological presentation of distemper encephalitis (CDV demyelinating encephalomyelitis), white matter vacuolation (spongy degeneration) is a typical and often neuropathological feature. However, white matter vacuolation is not synonymous with CDV infection; other conditions such as inflammatory/infectious [5, 7], degenerative [48], metabolic [49], and toxic [50] diseases of the nervous system may also present with spongy degeneration of the white matter. Accordingly, vacuolar change of the white matter should be not used as a histological hallmark of CDV demyelinating encephalomyelitis.

The pathogenesis of CDV neuropathology is multifactorial and not fully elucidated, and non-conventional neuropathological features of CDV infection have also a broad spectrum [42]. CDV encephalitis has been diagnosed in the absence of typical neuropathological features expected in nervous distemper, even in an instance where severe infiltration of lymphocytes and monocyte/histiocyte-lineages concentrically through the white matter of the hindbrain mimicked GME [42]. With the use of advanced molecular techniques, CDV was also demonstrated in non-suppurative meningoencephalitis of unknown etiology, which was never previously associated with CDV [51].

Given the above considerations, nervous distemper should always be excluded before the definitive diagnosis of NE, GME, or other neuropathological entity, as CDV neuropathology is diverse [42], and distemper-related encephalomyelitis may mimic other diseases of the CNS [20, 42, 43]. Accordingly, to confirm or even exclude distemper encephalomyelitis, *post mortem* immunohistochemical (IHC) detection of CDV antigens has been widely performed, although the sensitivity and specificity of the technique are not available for comparison.

2. CDV and viral antigens

CDV belongs to the *Paramyxoviridae* family and has been classified in the *Morbillivirus* genus together with the measles virus. The virion is relatively large (150-240 nm) and surrounded by a lipoprotein envelope composed of viral glycoproteins that incorporate into the cell membrane [22, 52].

CDV is a non-segmented single-stranded negative-sense RNA virus, and its genome has approximately 16,000 nucleotides that comprise 6 genes. The sequence of the genes, from 3' to 5', is nucleocapsid (N) protein, phosphor (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin (H), and large (L) protein (Lamb and Kolakofsky, 1996). The virus possesses six structural proteins: three internal or core proteins and three envelope proteins. CDV also possesses two non-structural polypeptides (V and C proteins). The core proteins are the L, N, and P proteins, and the envelope polypeptides consist of the M, H, and F proteins [53].

3. Development of chronic CDV encephalitis and the expression of viral target antigens for IHC assay

Chronic CDV encephalomyelitis coincides with recovery from severe CDV-induced immunosuppression [44], and in this stage of the nervous disease there is an obvious intrathecal antiviral immune response [54]. Antiviral antibodies have been found in both the cerebrospinal fluid (CSF) and the nervous system parenchyma of dogs with chronic CDV encephalomyelitis [54-56]. Anti-CDV antibodies are highly effective in neutralizing extracellular CDV and in preventing intercellular spread of the virus.

Prolonged exposure of infected target cells to high concentrations of antibodies leads to redistribution of viral surface antigens and their subsequent disappearance [57]. Therefore, the presence of intrathecal antiviral antibodies may cause a restricted synthesis of CDV envelope proteins within the CNS of dogs with chronic distemper encephalomyelitis. Alldinger et al. [46] observed that restricted expression of viral surface proteins (M, F, and H) was correlated with lesion-associated inflammation. The escalation of the intrathecal antiviral immune response coincides with the beginning of the inflammatory changes within the nervous system parenchyma, and both may decrease the expression of CDV envelope proteins in the infected cells. Müller et al. [58] suggested that technical reasons associated with the particular mode of transcription of morbilliviruses might also play a role in the early disappearance of viral surface proteins in inflammatory CDV lesions.

All of the above considerations make the envelope proteins non-recommended for usage as target antigens for IHC detection of CDV, especially in cases of chronic inflammatory distemper encephalitis. In addition, surface proteins are more adversely affected by formalin fixation than the core proteins [45]. Consequently, envelope CDV proteins should be avoided as target CDV antigens for IHC detection, and core proteins should be preferred. Due to the transcriptional polarity of the N gene in the genomic RNA of CDV, the N protein is the most transcribed core antigen within the infected cell and seems to be a good indicator of infection [59]. Demonstration of N protein expression by IHC assay is suitable for CDV detection [45, 46], and is currently used for the diagnosis of nervous distemper [34, 60].

4. IHC assay for CDV antigen detection

Astrocytes are the main cell target for CDV, comprising nearly 95% of cells infected in the CNS [61]; however, CDV antigens may also be found in virtually all cell types of the CNS (neurons, glial cells, ependymal cells, choroid plexus epithelium, endothelial cells, and meningeal cells) in conventional cases of distemper encephalomyelitis [45, 62, 63].

CDV proteins are found mainly within the cytoplasm of infected cells; however, viral antigens may also be observed within the intranuclear compartment, as by a paradoxical mechanism the intracytoplasmic replication of CDV may lead to aggregation of viral proteins in the nuclear region [5].

Although CDV antigens may be found in nearly all cell types of the CNS [45, 62, 63], the main cell affected is the astrocyte [61]. However, in some special cases CDV antigens may only be found within neurons [33, 34, 37, 64]. In the chronic form of distemper known as old dog encephalitis, CDV antigens may be found inside isolated neurons in the gray matter of the forebrain [33, 64]. Cantile et al. [37] described a case of non-conventional distemper encephalitis in which CDV antigens were only detected in the cytoplasm of neurons of the cerebral cortex and pons. Nessler et al. [65] found weak neuronal CDV immunolabeling by IHC in CDV inclusion body polioencephalitis, a non-conventional form of CDV-induced encephalitis. In addition, Alldinger et al. [46] predominantly observed CDV antigens in neurons of the forebrain gray matter even in some cases of conventional distemper encephalomyelitis.

Studies on nervous distemper have been shown that in contrast to acute non-inflammatory CDV lesions, where abundant CDV antigens (N protein) are found in the center of the lesions, viral proteins may be cleared from chronic inflammatory lesions due to an effective inflammatory/immune response [45, 54, 62, 58]. Therefore, in such chronic lesions, CDV antigens may be cleared from the lesion, and IHC might result in negative CDV immunolabeling.

4.1 Non-specific immunoreactions in IHC assay for CDV antigen detection

Monoclonal antibodies routinely used in the IHC assay for CDV N protein detection may occasionally and nonspecifically cross-react with dog self-antigens in the CNS, resulting in non-specific immunolabeling [45]. An IHC assay for CDV N protein detection revealed non-specific immunolabeling characterized by fine granules in the cytoplasm of most forebrain neurons (cortical neurons and in some nuclei of the cranial brainstem) closely associated with lesions of NE in a Yorkshire Terrier dog and in some neurons of a dog with typical GME [17]. Suzuki et al. [15] also found non-specific immunolabeling for CDV antigen in neurons that could be misdiagnosed as intra-neuronal CDV antigens in both GME and NE cases (CDV-free encephalitis).

The non-specific pattern of immunolabeling characterized by fine granules in the cytoplasm of neurons in CDV-free encephalitis [15, 17] may represent a challenge for the pathological diagnosis, as such non-specific immunolabeling resembles the CDV immunolabeling observed in some special cases of nervous distemper. CDV antigens may be found only within neurons in some non-conventional cases of distemper encephalitis [33, 37, 64].

5. The role of viral persistence in CDV antigen detection by IHC

Chronic inflammatory nervous lesions in distemper-related encephalomyelitis coincide with recovery from severe CDV-induced immunosuppression and are characterized by influx of inflammatory cells, mostly mononuclear, in both perivascular and nervous parenchyma [5, 44, 66]. It is already known that chronic nervous distemper is associated with CDV persistence within the CNS [62, 66, 67]. *In vitro* studies suggest that viral persistence may be linked to spread of virulent CDV from cell to cell via cell processes with very little release of infectious particles into the extracellular space and without cytolysis [68-70]. This mechanism of viral spread in chronic inflammatory encephalomyelitis might allow CDV to persist in the CNS even in the presence of an effective antiviral inflammatory response. Therefore, CDV escapes from virus-hostile inflamed sites to other sites where inflammation has not yet occurred [45, 58, 62, 54].

Experimentally induced and naturally occurring nervous system diseases have been used for additional *in vivo* studies on CDV persistence within nervous tissues [58, 65, 71, 72]; such studies showed that although the virus may be cleared from chronic inflammatory lesions [54, 58], persistence of CDV within CNS is favored by restricted infection of certain cells (neurons and oligodendrocytes) with reduced (neurons) to absent (oligodendrocytes) viral protein expression [58, 65, 71, 72]. The reduced viral protein expression in neurons may result in intraneuronal immunolabeling characterized by fine granules in the cytoplasm of neurons [58]. Thus, the mechanisms of CDV persistence within nervous tissue may lead to limited translation of CDV RNA in neurons with low expression of viral antigens on infected cells [58, 65], leading to fine and weak CDV immunolabeling in the cytoplasm of neurons by IHC.

The fine and weak CDV immunolabeling in cytoplasm of neurons due to CDV persistence [58, 65] might mimic the non-specific immunolabeling reported in CDV-free encephalitis [15, 17]. In brief, the interpretation of IHC assays for CDV detection may be difficult when the immunolabeling is characterized by fine granules present only within the cytoplasm of neurons, as such an immunoreactive pattern might be found in both naturally occurring nervous distemper and CDV-free encephalitis (non-specific immunolabeling).

6. CDV persistence and the molecular diagnosis of nervous distemper by RT-PCR

Studying CDV persistence in nervous tissues from different neuropathological presentations of nervous distemper, Müller et al. [58] and Nessler et al. [65, 72] observed that virus persistence is favored by abundant expression of all viral protein mRNA with reduced to absent protein translation, leading to an almost complete lack of viral antigen. Such reduced or absent translation of CDV RNA, in neurons and oligodendrocytes, respectively, provides a reservoir of immune-indifferent virus nucleic acid sequences [58].

CDV persistence may be used for the specific molecular diagnosis of CDV by detection of viral RNA by RT-PCR. Thus, RT-PCR should be carried out on formalin-fixed paraffin-embedded CNS sections for correct diagnosis of CDV in two situations: first, when CDV antigens are cleared from chronic nervous lesions due to an immune and inflammatory reaction [54] but virus persistence in nervous tissue is yet evident [58], and second, in special cases where the virus infection take place mainly in neurons and the molecular events of CDV persistence may result in IHC immunolabeling characterized by fine granules in cytoplasm of isolated neurons [33, 37, 64], where such labeling might be misdiagnosed as non-specific CDV immunoreactivity as previously reported for CDV-free encephalitis [15, 17].

Studies on a molecular level that employ CDV gene-specific primers for RT-PCR on formalin-fixed paraffin-embedded CNS sections might be useful for confirming the real presence of CDV; however, the choice of virus gene-specific primers requires caution and knowledge about paramyxovirus replication. As distal genes (L, H, and F) are the least transcribed during viral replication and core proteins genes (N, and P) are more frequently transcribed [53], the target CDV gene for RT-PCR amplification should preferentially be a core gene to avoid or minimize false-negative results. The authors optimized a RT-PCR assay for CDV gene detection from formalin-fixed paraffin-embedded tissue and observed that the CDV N gene could be easily detected from formalin-fixed paraffin-embedded nervous sections; however, when the target was the H gene, the number of false negative results was considerable [42].

7. Combination of IHC and RT-PCR assay

Combination of techniques such as IHC for CDV protein detection and molecular assays for CDV nucleic acid detection could establish a definite diagnosis of distemper-related encephalomyelitis. As pre-analytical, analytical, and post-analytical errors may generate false-negative results for protein and RNA detection [42] the use of the two techniques simultaneously might reduce the chances of misdiagnosis. In addition, IHC can be used to map the neuroanatomical distribution of CDV and identifies the nature of infected cells, while RT-PCR might be used to confirm that the IHC immunolabeling is CDV-specific.

The use of the RT-PCR assay may also be helpful because sequencing of RT-PCR amplicons could allow the classification of CDV strains [51, 73, 74]; moreover, sequencing of RT-PCR amplicons has been used to differentiate the wild-type CDV strain from vaccine CDV strains [34, 51].

8. Final Considerations

IHC for CDV protein detection is a good assay for *post mortem* diagnosis of nervous distemper. When immunoreactivity occurs in different cell types, especially astrocytes, CDV-induced nervous disease is the correct diagnosis, and an additional assay might not be necessary to confirm viral participation. In addition, the recognition of immunoreactions within the nuclear compartment may be used as a clue for distemper, as CDV infection can lead to aggregation of viral proteins in the nucleus of the infected cells. However, in instances where the immunoreactivity only occurs in the cytoplasm of neurons, the association of other techniques such as RT-PCR is necessary to confirm or exclude the real participation of CDV because such patterns of immunolabeling may occur in i) CDV-induced nervous disease or ii) CDV-free encephalitis (non-specific immunoreactivity).

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