Immunohistochemistry for the detection of neural and inflammatory cells in equine brain tissue

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Phenotypic characterization of cellular responses in equine infectious encephalitides has had limited description of both peripheral and resident cell populations in central nervous system (CNS) tissues due to limited species-specific reagents that react with formalin-fixed, paraffin embedded tissue (FFPE). This study identified a cassette of antibodies for investigating the immunopathology of infectious CNS diseases in horses. Multiple commercially available staining reagents and antibodies derived from antigens of various species for manual immunohistochemistry (IHC) were screened. Several techniques and reagents for heat-induced antigen retrieval, non-specific protein blocking, endogenous peroxidase blocking, and visualization-detection systems were tested during IHC protocol development. Boiling of slides in a low pH, citrate-based buffer solution in a double-boiler system was most consistent for epitope retrieval. Pressure-cooking, microwaving, high pH buffers, and proteinase K solutions often resulted in tissue disruption or no reactivity. Optimal blocking reagents and concentrations of each working antibody were determined. Ultimately, a cassette of monoclonal (mAb) and polyclonal antibodies (pAb) were identified for CD3+ (pAb A0452, Dako) T-lymphocytes, CD79αcy+ B-lymphocytes (mAb HM57, Dako), macrophages (mAb MAC387, Leica), NF-H+ neurons (mAb NAP4, EnCor Biotechnology), microglia/macrophage (pAb Iba-1, Wako), and GFAP+ astrocytes (mAb 5C10, EnCor Biotechnology). In paraffin embedded tissues, mAbs and pAbs derived from human and swine antigens were very successful at binding equine tissue targets. Individual, optimized protocols are provided for each positively reactive antibody for analyzing equine neuroinflammatory disease histopathology.

1 **Immunohistochemistry for the Detection of Neural and Inflammatory Cells in Equine**

2 **Brain Tissue**

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18 **Abbreviations**

19   CNS   Central nervous system

20   EDTA   Ethylenediaminetetraacetic acid

21   FFPE   Formalin-fixed, paraffin embedded

22   GFAP   Glial fibrillary acid protein

23   H_2O_2 Hydrogen peroxide

24   HIER   Heat induced epitope retrieval

25   HRP   Horseradish peroxidase

26   IgG   Immunoglobulin

27   IHC   Immunohistochemistry

28   mAb   Monoclonal antibody

29   MHC   Major histocompatibility complex

30   NF-H   Neurofilament – heavy chain

31   pAb   Polyclonal antibody

32   PBS   Phosphate buffered saline

33   WNV   West Nile virus

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36

38 **Abstract**

39 Phenotypic characterization of cellular responses in equine infectious encephalitides has had

40 limited description of both peripheral and resident cell populations in central nervous system

41 (CNS) tissues due to limited species-specific reagents that react with formalin-fixed, paraffin

42 embedded tissue. This study identified a set of antibodies for investigating the

43 immunopathology of infectious CNS diseases in horses. Multiple commercially available

44 staining reagents and antibodies derived from antigens of various species for manual

45 immunohistochemistry (IHC) were screened. Several techniques and reagents for heat-induced

46 antigen retrieval, non-specific protein blocking, endogenous peroxidase blocking, and

47 visualization-detection systems were tested during IHC protocol development. Boiling of slides

48 in a low pH, citrate-based buffer solution in a double-boiler system was most consistent for

49 epitope retrieval. Pressure-cooking, microwaving, high pH buffers, and proteinase K solutions

50 often resulted in tissue disruption or no reactivity. Optimal blocking reagents and concentrations

51 of each working antibody were determined. Ultimately, a set of monoclonal (mAb) and

52 polyclonal antibodies (pAb) were identified for CD3$^+$ (pAb A0452, Dako) T-lymphocytes,

53 CD79αcy$^+$ B-lymphocytes (mAb HM57, Dako), macrophages (mAb MAC387, Leica), NF-H$^+$

54 neurons (mAb NAP4, EnCor Biotechnology), microglia/macrophage (pAb Iba-1, Wako), and

55 GFAP$^+$ astrocytes (mAb 5C10, EnCor Biotechnology). In paraffin embedded tissues, mAbs and

56 pAbs derived from human and swine antigens were very successful at binding equine tissue

57 targets. Individual, optimized protocols are provided for each positively reactive antibody for

58 analyzing equine neuroinflammatory disease histopathology.

Introduction

59

60   Comparative pathology of many diseases that affect multiple species is hindered by the lack of

61   species-specific reagents. Although many antibodies that react with cellular antigens and

62   inflammatory molecules are available, there still are gaps in how these work in formalin-fixed

63   paraffin embedded (FFPE) tissues. This limits our ability to generate new knowledge about

64   disease processes that can benefit human and animal health as a whole. Horses develop many of

65   the same or similar central nervous system (CNS) infections as humans (Ritchey *et al.*, 2006,

66   Bourgeois *et al.*, 2011, Rushton *et al.*, 2013, and Yu *et al.*, 2015). Cell marker panels are often

67   composed of both equine and non-equine specific antibodies, of which most are used in flow

68   cytometry. Since manufacturers may not have supporting technical documentation on whether

69   their products will cross-react with equine antigens (Beckstead, 1994 and Ramos-Vara, 2005),

70   development of antibody panels to accomplish *in situ* disease characterization in formalin-fixed

71   tissue is a formidable task because cross-linking of antigens often renders epitopes non-reactive.

72   Additional investigative steps are required to determine if antigens can be retrieved and what

73   retrieval method works best. Because of this and other hurdles, there is limited information on

74   what antibodies react with FFPE tissue.

75   The goal of this study was to identify a panel of cell markers for studying cellular pathogenesis

76   in equine infectious brain diseases. In particular, protocols for phenotyping reactive glia,

77   neurons, and infiltrating peripheral blood mononuclear and polymorphonuclear cells in the

78   equine brain were developed. Commercially available antibodies for $CD3^+$ T-lymphocytes,

79    $CD8^+$ and $CD4^+$ T cell subpopulations, B lymphocytes, macrophages, microglia, astrocytes, and

80   neurons were investigated for reactivity in normal and diseased FFPE tissue. Optimization of

81 manual immunohistochemistry (IHC) protocols was performed using various IHC staining

82 reagents and methods.

Materials and methods

84 *Tissue samples*

85 Immunohistochemistry protocols were developed on diseased and normal horse tissues.

86 Diseased horses consisted of clinically affected West Nile virus (WNV), both naturally and

87 experimentally infected, and *Sarcocystis neurona* infections (Beckstead, 1994, Gutierrez *et al.*,

88 1999, Porter *et al.*, 2003, and Seino *et al.*, 2007). Neurologically, normal horses were obtained

89 by owner surrender for humane euthanasia due to loss of use. Brain, spinal cord, lymph node,

90 spleen, thymus, and liver were collected and archived from these animals under University of

91 Florida (Gainesville, FL) Institutional Animal Care and Use Committee protocols #F077, #F093,

92 #D163, and #4109. Tissues were fixed in 10% neutral buffered formalin and processed into

93 paraffin-embedded blocks approximately one-week after fixation. Initial evaluation of antibody

94 binding was tested on non-infected equine lymph node and spleen for lymphocytic targets, liver

95 and thymus for tissue macrophage targets, and brain for microglia, astrocytes, and neurons.

96 *Tissue processing*

97 The invariable IHC procedures for all protocols included sectioning FFPE tissues at 5 µm and

98 placing them on positively charged glass slides. The slides were soaked in xylene (Fischer

99 Scientific, Pittsburg, PA, USA) three times for 5 min to remove paraffin. These sections were

100 then rehydrated through a gradient of ethanol (Fischer Scientific) for 5 min in each

101 concentration, 100%, 100%, 95%, and 70% ethanol, followed by de-ionized water. In order to

102 reduce the volume of the reagents tested and liquid loss, tissues were encircled with a

103 hydrophobic barrier pen (ImmEdge™ Pen, Ted Pella Inc., Redding, CA, USA).

104 *Antigen Unmasking*

105 Three methods of heating slides for investigating heat induced epitope retrieval (HIER)

106 effectiveness included using a pressure cooker, microwave, and a double boiler. Pressure

107 cooking was performed at 125°C for 30 s followed by 90°C for 10 s (Matyjaszek *et al.*, 2009 and

108 Grosche *et al.*, 2012), or microwaving was performed for 10 min (DAKO, 2009) using a

109 commercial countertop GE 1000W oven. For double boiling, two tissue-slides were floated

110 back-to-back in 25 ml of retrieval solution in a 50 ml plastic conical tube. Conical tubes were

111 then placed in pre-warmed water of a 250 ml glass beaker on a hotplate. Water temperature was

112 maintained at 90°C. After approximately 5 min of warming the retrieval solution, HIER was

113 timed for 10 min. Conical tubes were then removed from the double boiler and allowed to cool

114 for 15 min at 27°C. Tissues were rinsed in deionized water three times for 2 min.

115 Heat induced epitope retrieval buffers were tested primarily using the double boiler system.

116 Regents included two commercial citrate buffers, Epitope Retrieval Solution pH 6 (Novacastra,

117 Leica, Newcastle Upon Tyne, UK) and Target Retrieval Solution pH 6 (Dako, Glostrup,

118 Denmark), and an ethylenediaminetetraacetic acid (EDTA) solution buffered at pH 9 (10 mM

119 Tris Base, 1 mM EDTA solution, 0.05% Tween 20, and NaOH to titrate to pH 9). A 1 ×

120 concentration of each solution was freshly made by diluting stock solutions with deionized

121 water. For proteolytic epitope retrieval, tissues were treated with 200 ug/ml proteinase K

122 solution [Tris HCL 100 mM pH 8.2, Tween 20, and Proteinase K (Ambion, Foster City, CA,

123 USA)] for 10 min at 37°C.

124 *Endogenous Peroxidase Blocking*

125 Peroxidase neutralizing solutions that were tested included two prepared solutions of hydrogen

126 peroxide (H_2O_2), 3% and 0.3% H_2O_2, and a ready-to-use commercial reagent, Peroxidase Block

127   (Novolink™ Polymer Detection System, Leica). Solutions containing 3% and 0.3% H_2O_2 were

128   made fresh for each staining attempt by diluting 30% H_2O_2 (Fischer Scientific) in $1 \times$ phosphate

129   buffered saline (PBS) ($10 \times$ PBS, Fischer Scientific). Tissues were immersed in peroxidase

130   blocking solution for 5 min followed by two, 5 min rinses in PBS.

131   *Non-specific Protein Blocking*

132   Non-specific blocking techniques included four commercial reagents and one lab prepared

133   solution. Commercial reagents included 10% Normal Goat Serum (Invitrogen, Frederick, MD,

134   USA), Protein Block (Novolink™ Polymer Detection Systems, Leica), Novocastra™ IHC/ISH

135   Super Blocking Solution (Leica), and Novocastra™ Liquid Serum, Normal Goat Serum

136   Blocking Reagent (Leica). Additionally, a 5% goat serum solution was prepared by diluting

137   Immunopure® Goat Serum (ThermoFischer Scientific, Waltham, MA, USA) in $1 \times$ PBS.

138   Protein blocking reagents were applied for 20 min with the exception of Novolink™ Protein

139   block for only 5 min. Blocking reagents were removed without rinsing before adding primary

140   antibody.

141   *Primary Antibodies*

142   Primary antibodies tested targeted cell populations of astrocytes, microglia, neurons, T

143   lymphocytes, B lymphocytes, and macrophages (Table 1). All primary antibodies were diluted

144   in one of two commercial diluents [IHC Diluent (Novacastra, Leica); Dako Antibody Diluent

145   (Dako)]. Two-fold serial dilutions of each antibody were tested to determine an optimal staining

146   range. If the signal was weak to absent or background staining was present, additional dilution

147   tests were performed until optimal staining was achieved. Antibodies were applied in a 37°C,

148   humidified incubator for 60-120 min or overnight (~16 hrs) at 4°C in a humidified, covered dish.

149 After primary antibody incubation, slides were washed three times for 5 min each in 1 × PBS

150 before secondary antibody application.

151 Negative controls for each primary antibody consisted of either an isotype-matched negative

152 primary control (MCA928, AbD Serotec, Kidlington, UK) for monoclonal antibodies or rabbit

153 serum for polyclonal antibodies.

154 *Detection Systems*

155 Three commercial, horseradish peroxidase (HRP)-linked conjugate detection kits were utilized

156 by following manufacturer's instructions. These kits included the Vectastain® ABC Kit – Mouse

157 IgG (Vector Laboratories, Burlingame, CA, USA), the Vectastain® ABC Kit – Rabbit IgG, and

158 the Novolink™ Polymer Detection System (Leica).

159 Lastly, a substrate-chromogen (Vector NovaRED Peroxidase Substrate, Vector Laboratories)

160 was applied for 10 min, rinsed with de-ionized water for 5 min, counterstained with Lab

161 Vision™ Mayer's Hematoxylin (ThermoFisher Scientific) for 1 min, and rinsed in running tap

162 water for 2 min. All sections were then dehydrated through an increasing gradient of ethanol,

163 50%, 70%, 95%, and 100%, for 2 min each. Slides were cleared in xylene three times for 3 min

164 each before coverslipping with Permount™ mounting medium (Fischer Scientific).

165 **Results**

166 *Antigen Unmasking*

167 Of the reagents tested, low pH citrate based solutions resulted in superior staining (Table 2).

168 Target Retrieval Solution pH 6 by Dako was sufficient for most antibodies; however, use of

169 Epitope Retrieval Solution pH 6 by Leica resulted in positive staining when the Dako product

170   failed to produce staining with the CD79αy$^+$ antibody. Proteinase K solution was tested with

171   macrophage antibodies and resulted in increased staining intensity of MAC387$^+$ antibody.

172   Various issues were encountered with the method of applying heat. Utilizing, one antibody,

173   CD79αy$^+$, only the double boiling method resulted in consistent positive staining of lymph

174   nodes. Pressure cooking and microwaving resulted in limited to no staining or uneven staining,

175   respectively. In addition, tissue disruption was minimal with the double boiling method. One

176   antibody, Iba-1$^+$, did not require heat retrieval and any HIER resulted in non-specific background

177   staining.

178   *Endogenous Peroxidase Blocking*

179   In FFPE tissues, 3% H_2O_2 solution was reliable for reducing background staining without

180   disruption of the tissue. There was no difference between commercial and lab diluted reagents.

181   *Non-specific Protein Blocking*

182   Multiple combinations of IHC antibody diluents and non-specific protein blocking reagents were

183   tested. No difference in staining intensity was noted between of either diluent used. Among

184   protein blocking solutions, 10% Normal Goat Serum, Protein Block from the Novolink™

185   Polymer Detection System, and 5% Immunopure® Goat Serum in PBS solution were all equal in

186   blocking non-specific staining.

187   *Specificity and Sensitivity of Monoclonal and Polyclonal Antibodies*

188   Several antibodies were tested for lymphocyte specific CD antigens including CD3$^+$, CD4$^+$,

189   CD5$^+$, CD8$^+$, CD20$^+$, CD21$^+$, and CD79αy$^+$ (Table 1). Of the two CD3$^+$, pan-T cell markers

190   examined, rabbit anti-human polyclonal antibody (A0452, Dako) appropriately stained lymph

191   node cortex (Fig. 1A), periarterial lymphatic sheaths of spleen, and perivascular cuffs in WNV

192 infected brain (Fig. 1B). Four CD4$^+$ T-helper cell antibodies were tested. Only mouse,

193 monoclonal anti-equine antibody (HB61A, VMRD, Pullman, WA, USA) at 1:25 dilution

194 positively stained lymph node cortex; however, background staining was high when applied to

195 brain tissues. Two CD8$^+$ cytotoxic T cell markers were investigated, but neither marker had

196 reactivity in FFPE tissue.

197 Antibodies against CD5$^+$, CD20$^+$, CD21$^+$, CD79αy$^+$, and IgG (H+L) (Table 1) were tested for

198 identification of B cell populations. A putative lymphocyte marker, CD5$^+$ (B29A, VMRD),

199 reportedly selects for B cells in equine tissues. Although this antibody intensely stained the

200 germinal centers of FFPE lymph nodes, cortical staining was also noted. Because of this, CD5$^+$

201 (B29A) was unreliable for distinction between B cell and T cell populations. Additionally in

202 brain tissue, this antibody resulted in non-specific background staining, which could not be

203 resolved. No staining was achieved with CD20$^+$, CD21$^+$, or any IgG (H+L) antibodies. Anti-

204 human CD79αcy$^+$ (HM57, Dako) monoclonal antibody at 1:100 successfully stained lymph node

205 germinal centers with no background staining in equine brain (Fig. 1C, D).

206 Multiple macrophage-targeting antibodies were investigated. RAM 11 (Dako), AM-3K

207 (TransGenic Inc., Kobe, Japan), and CD68$^+$ (KP1, Leica) antibodies had no reactivity with

208 control tissues; however, MAC387$^+$ (Leica) was reactive. This marker positively stained control

209 hepatic and thymic macrophages (Fig. 2A) with limited staining noted in lymph node sections.

210 Based on cell morphology, polymorphonuclear and mononuclear cells also stained positively due

211 to lack macrophage-specificity of the antibody. No reactivity was noted in normal equine brain.

212 The MAC387$^+$ cell population was distinct from the distribution of CD3$^+$ lymphocytes in brain

213 sections with inflammation due to WNV infection (Fig. 2B). Additionally, this macrophage

214   antibody had little to no cross reactivity with brain microglia since few MAC387⁺ cells were

215   visualized within glial nodules of WNV⁺ brain.

216   To characterize reactive gliosis, antibodies against both microglial and astrocytic markers were

217   tested. In non-infected brain tissue, Iba-1⁺ antibody stained scattered resident microglia with low

218   intensity, yet microglial processes were visualized (Fig. 2C). Staining intensity was higher in

219   WNV- infected brains (Fig. 2D). Astrocyte populations were identified with antibody against an

220   intermediate filament, glial fibrillary acid protein (GFAP), specific to astrocytes in CNS tissues.

221   This antibody reacted strongly with astrocytes in both normal and infected brains with distinct

222   astrocytic processes notable. GFAP⁺ astrocytes were distributed within the brain parenchyma, at

223   the glia limitans, along brain vasculature, and in areas of gliosis of WNV⁺ brains (Fig. 3).

224   Three antibodies that target neurofilament heavy-chain proteins (NF-H) of neuronal axons

225   successfully stained equine brain tissue. Swine derived, NAP4 (EnCor Biotechnology Inc.,

226   Gainesville, FL) antibody, was superior and stained neurons in both normal (Fig. 4) and infected

227   brains. Mouse monoclonal Neu-N antibody (A60, Millipore, Billerica, Massachusetts, USA),

228   which targets neuronal perikaryon, was also tested but without successful reactivity in equine

229   tissue.

230   Successful manual IHC protocols for these antibodies in equine neural tissue were identified

231   (Table 2). It should be noted that overnight, refrigeration with CD3⁺, CD79⁺, MAC387⁺,

232   GFAP⁺, and Iba-1⁺ primary antibodies will successfully stain tissues and may provide flexibility

233   in laboratory scheduling if single-day protocol completion is not possible.

234 **Discussion**

235 Formalin-fixed, paraffin embedded tissues are commonly archived for histological examination.

236 Formalin fixation and paraffin embedding samples retains tissue architecture and deactivates any

237 potential infectious agents (Cantile *et al.*, 2001, Ritchey *et al.*, 2006, and van Marle *et al.*, 2007);

238 however, this process can block or destroy sensitive epitopes (Beckstead, 1994, Mérant *et al.*,

239 2003, Terio *et al.*, 2003, and Ibrahim *et al.*, 2007). Despite reported reversal of these treatments,

240 the tested equine antigen derived CD4$^+$ and CD8$^+$ T cell were not detectable in FFPE. This

241 result has been described in other species (Beckstead, 1994, Bilzer *et al.*, 1995, Zeng *et al.*,

242 1996, Gutierrez *et al.*, 1999, and Härtig *et al.*, 2009). Anti-CD4$^+$ (mAb HB61A, VMRD) and

243 CD8$^+$ (mAb HT14A, VMRD) monoclonal antibodies, however, were reactive to fresh, frozen

244 equine lymph node and infectious CNS tissue. Archiving tissues in both FFPE and fresh, frozen

245 tissue formats is recommended when investigating epitopes that may be affected by fixation.

246 After successful staining of control tissues, normal and pathologic brain tissue samples were then

247 tested alongside positive and negative tissue controls. Pathologic tissue samples included WNV-

248 infected brain, which contains reactive gliosis and perivascular cuffing of inflammatory cells

249 (Cantile *et al.*, 2001, Ibrahim *et al.*, 2007, van Marle *et al.*, 2007, and Schnabel *et al.*, 2013). *S.*

250 *neurona* infected tissues were also used, which contained focal nonsuppurative inflammation,

251 mononuclear perivascular cuffing, and the occurrence giants cells and eosinophils (Boy *et al.*,

252 1990 and Dubey *et al.*, 2001). Following this workflow of tissue testing aided in the

253 identification of successful antibody reactivity.

254 Several hurdles must be overcome to optimize the interaction of antibodies with their intended

255 targets. Aldehyde cross-links that bind tissue proteins during formalin fixation process must be

256 removed by HIER or proteolytic epitope retrieval to allow epitopes to resume a more natural

257 confirmation and increase antibody-binding capacity (Shi et al., 1991, Ferrier et al., 1998, and

258 Krenacs et al., 2010). Peroxidases that naturally occur within tissues must be neutralized with

259 H_2O_2 in order to prevent non-specific staining during the application of peroxidase-based

260 substrate kits (Wendelboe and Bisgaard, 2013). Endogenous proteins may non-specifically

261 interact with antibodies and cause background staining that masks target antigen signal

262 (Daneshtalab et al., 2010 and Buchwalow et al., 2011). These unwanted binding sites must be

263 blocked. In this study a variety of reagents and methods were tested for each individual manual

264 IHC protocol. A base set of reliable solutions and techniques for testing antibodies was

265 identified in this study. These included 3% H_2O_2, low pH, citrate based HIER reagents in a

266 double-boiler, 5% Immunopure® Goat Serum in PBS, Leica's IHC Diluent, antibody incubation

267 for one hour at 37°C, and the Novolink™ Polymer Detection System. However, with meticulous

268 tailoring of each antibody protocol, our results contained a variety of reagents that were

269 ultimately selected for each staining procedure.

270 Identification of equine-reactive antibodies can be challenging due to limited knowledge of

271 commercial antibodies' reactivity with veterinary tissues and lack of development of multiple

272 species-specific reagents. Evaluation of cross-species reactivity of commercially available

273 antibodies, particularly CD antigens, has been largely performed in equine tissues prepared for

274 whole-cell analysis like flow-cytometry (Johne *et al.*, 1997, Mérant *et al.*, 2003, Terio *et al.*,

275 2003, Kunisch *et al.*, 2004, and Ibrahim *et al.*, 2007) or on fresh or frozen specimens (Bilzer *et

276 al.*, 1995, Zeng *et al.*, 1996, Lemos *et al.*, 2008, and Härtig *et al.*, 2009). Large screenings of

277 non-equine derived antibodies have often resulted in limited identification of equine reactive

278 reagents (Ibrahim *et al.*, 2007, Schnabel *et al.*, 2013, and Szabo and Gulya, 2013). Of the 26

279 antibodies in this study, six antibodies successfully reacted with FFPE equine tissues. All six

280   antibodies were derived from non-equine antigens. Their reactivity to equine tissue is likely due

281   to conserved epitope targets and the intracellular location of the antigen peptides (Jones *et al.*,

282   1993 and Ahmed *et al.*, 2007). If an antigen's peptide sequence is highly conserved across

283   multiple species, the production of antibodies from the immunized host may be inhibited due to

284   immune tolerance. However, because these antigens have intracellular origins, the host (i.e.

285   rabbit or mouse) is more likely to elicit an immune response upon antigen exposure. In this way,

286   these antibodies against human or swine antigen may successfully be produced and react with

287   equine or other species' tissues.

288   Commercial macrophage antibodies are non-specific to macrophages and often cross react with

289   monocytes, granulocytes, dendritic cells, and fibroblasts (Johne *et al.*, 1997, Kunisch *et al.*,

290   2004, Shaw *et al.*, 2005, and Sellner *et al.*, 2014). Multiple macrophage-directed antibodies with

291   different target antigens were tested so that a distinction could be made between tissue

292   macrophage populations and cells that may cross-react with those antibodies. RAM11 (Dako)

293   recognizes an uncharacterized, cytoplasmic antigen specific to rabbit macrophages. AM-3K

294   (TransGenic) anti-macrophage antibody raised against human alveolar macrophage antigen

295   recognizes cytoplasmic membrane epitopes. Anti-CD68^{+} (KP1, Leica) recognizes primarily

296   lysosomal membrane proteins of macrophages, secondarily macrophage membranes, and is

297   found on monocytes, neutrophils, basophils, and large lymphocytes. None of these three

298   antibodies were successfully reactive in FFPE brain or lymphoid tissues. Although frequently

299   used as a macrophage-characterizing marker, MAC387^{+} (Leica) recognizes cytoplasmic, human

300   leukocyte antigen, L1 protein found on neutrophils, monocytes, and certain reactive

301   macrophages. Only MAC387^{+} antibody was reactive with FFPE equine tissues, so the positively

302   stained population must be considered as a mix of both macrophages and neutrophils. This cell

303   population had a distinct distribution in serial sections of WNV-infected brain each

304   immunolabeled for MAC387^{+}, CD3^{+} T lymphocytes, and microglia. In *S. neurona* infected

305   equine tissues, where multi-nucleated giant cells are observed, MAC387^{+} antibody did not react

306   with these cells even though there was reaction with single cells within each lesion.

307   Microglia, which are of a monocytic lineage, were identified with anti-Iba-1-antibody (Wako).

308   This antibody recognizes an intracellular, calcium binding protein basally expressed by both

309   microglia and macrophages, and upregulated in microglia following injury. Cell morphology,

310   specifically microglial processes, may be used to manually separate microglia and macrophage

311   populations. A limited number of equine studies have investigated microglial activation using

312   major histocompatibility complex (MHC) markers (Mullen *et al.*, 1992 and Lemos *et al.*, 2008).

313   MHC predominantly targets activated microglia populations, whereas anti-Iba-1^{+} antibody will

314   stain microglia in both their resting and activated form (Szabo and Gulya, 2013). Additionally,

315   using MHC as a marker for microglial cells in an inflammatory disease would also identify all

316   peripheral and CNS cells that have MHC upregulated due to this biological process.

317   Characterization of the total, both resting and activated, microglia population is therefore

318   improved with the use of Iba-1^{+} antibody (Ahmed *et al.*, 2007).

319   Regarding detection of neurons, it must be recognized that NF-H proteins are concentrated

320   within axons and are weakly present within cell bodies. Changes in staining intensity within cell

321   bodies and dendrites with NF-H antibodies, such as NAP4 that targets phosphorylated NF-H

322   proteins, are useful for identifying neuropathology (Shaw *et al.*, 2005 and Sellner *et al.*, 2014).

323   For quantification of neuronal populations, an antibody specific to neuronal nuclei should be

324   used. Neu-N antibodies, which target DNA-binding proteins of neural nuclei, are typically used

325   for the staining and counting of neuron perikaryons (Mullen *et al.*, 1992).

326   This study successfully identified a collection of protocols for the characterization of cell

327   populations in the histopathology of encephalitic diseases in equine CNS tissues. This suite

328   included IHC staining of CD3$^+$ T lymphocytes, CD79αcy$^+$ B lymphocytes, MAC387$^+$

329   macrophages and neutrophils, Iba-1$^+$ microglia, GFAP$^+$ astrocytes, and NF-H$^+$ neurons in FFPE

330   tissues. The suite was expanded with the inclusion of CD4$^+$ and CD8$^+$ T lymphocytes in fresh,

331   frozen tissue sections. Useful application of these antibodies was supported with the

332   characterization of neuropathology in diseased horses with clinical and experimental West Nile

333   encephalitis, clinical equine protozoal myeloencephalitis, and, preliminarily, clinical Eastern

334   equine encephalitis. Western blot analysis utilizing these antibodies should next be performed to

335   support the specificity of their reactivity in the horse.

336   **Acknowledgements**

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338   antibodies.

339

340

341   **Fig 1 –** For the detection of lymphocytes, (A, C) equine lymph node cortex and (B, D) WNV

342   infected equine brain incubated with (A, B) CD3$^+$ T lymphocyte primary antibody (pAb A0452,

343   Dako) for 60 minutes at 37°C and detected by Vectastain®ABC Kit, or incubated with (C, D)

344   CD79αcy$^+$ B lymphocyte primary antibody (mAb HM57, Dako) for 90 minutes at 37°C and

345   detected by Novolink™ Polymer Detection System. Vector NovaRED Peroxidase Substrate

346   chromogen and hematoxylin couterstain. Bar, 50 μm.

347   **Fig 2 –** For the detection of macrophage/microglial lineage cells, (A) equine thymus and (B)

348   WNV infected equine brain were incubated with MAC387$^+$ primary antibody (mAb MAC387,

349   Leica) for 60 minutes at 37°C and detected by Vectastain®ABC Kit. Additionally, (C) normal

350   (arrows) and (D) WNV infected horse brain were incubated with microglia/macrophage primary

351   antibody (pAb Iba-1, Wako) for 60 minutes at 37°C and detected by Novolink™ Polymer

352   Detection System. Vector NovaRED Peroxidase Substrate chromogen and hematoxylin

353   couterstain. Bar, 50 μm.

354   **Fig 3-** GFAP$^+$ astrocytes in WNV infected equine brain (A) near blood vessels and (B) at the

355   glial limitans. Tissues were incubated with GFAP+ astrocyte primary antibody (mAb 5C10,

356   EnCor Biotechnology) for 60 minutes at 37°C and detected by Vectastain®ABC Kit. Vector

357   NovaRED Peroxidase Substrate chromogen and hematoxylin couterstain. Bar, 50 μm.

358   **Fig 4 -** Normal equine brain incubated with NF-H$^+$ neuron primary antibody (mAb NAP4,

359   EnCor Biotechnology), for 60 minutes at 37°C and detected by Novolink™ Polymer Detection

360   System. Vector NovaRED Peroxidase Substrate chromogen and hematoxylin couterstain. Bar, 50

361   μm.

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466

Antibodies tested for immunohistochemical reactivity in equine tissues.

1   **Table 1. Antibodies tested for immunohistochemical reactivity in equine tissues.**

Antibody	Antigen Species	Host Species	Isotype	Name or Clone	Protein Target	Source
CD3	Human	Mouse	IgG1	F7.2.38	Thymocytes, T lymphocytes, natural killer cells	Dako
CD3	Human	Rabbit	-	A0452	T lymphocytes	Dako
CD4	Equine	Mouse	IgG1	CVS4	T helpers	AbD
CD4	Human	Mouse	IgG1	4B12	Thymocytes, T helpers, mature peripheral T cells, TReg or cytotoxic T cell subsets	Dako
CD4	Human	Mouse	IgG1	1F6	T helpers	Leica
CD4	Equine	Mouse	IgG1	HB61A	Thymocytes, T helpers	VMRD
CD8	Equine	Mouse	IgG1	CVS8	Cytotoxic T lymphocytes	AbD
CD8	Equine	Mouse	IgG1	HT14A	Thymocytes, Cytotoxic T lymphocytes	VMRD
CD5	Equine/Bovine	Mouse	IgG2A	B29A	B lymphocytes only, in horses	VMRD
CD 20	Human	Mouse	IgG1	MJ1	B lymphocytes	Leica
CD21	Human	Mouse	IgG1	Bu33	B lymphocytes	AbD
CD21	Human	Mouse	IgG1	1F8	B lymphocytes	Dako
CD79αcy	Human	Mouse	IgG1	HM57	B lymphocytes	Dako
IgG (H&L)	Equine	Goat	IgG	A70-106	B lymphocytes	Bethyl
IgG (H&L)	Equine	Rabbit	IgG	A70-118	B lymphocytes	Bethyl
IgG (H&L)	Goat	Rabbit	IgG	A50-100	B lymphocytes	Zymed
Macrophage	Rabbit	Mouse	IgG1	RAM11	Macrophages	Dako
Macrophage	Human	Mouse	IgG1	MAC387	Macrophages	Leica
Macrophage	Human	Mouse	IgG1	AM-3K	Macrophages	TransGenic
CD68	Human	Mouse	IgG1	CD68-KP1	Macrophages	Leica
NF-H	Bovine	Mouse	IgG2b	9B12	Neurofilament-Heavy chain	EnCor-Biotechnology
NF-H	Bovine	Mouse	IgG1	AH1	Neurofilament-Heavy chain	EnCor-Biotechnology
NF-H	Swine	Mouse	IgG1	NAP4	Neurofilament-Heavy chain	EnCor-Biotechnology
GFAP	Swine	Mouse	IgG1	5C10	Astrocytes, Satellite cells, Schwann cells	EnCor-Biotechnology
Microglia	Human	Rabbit	-	Iba-1	Microglia, macrophages	Wako
Neu-N	Mouse	Mouse	IgG1	A60	Neurons	Millipore

Selected protocols for immunohistochemical visualization of resident and infiltrative cells in normal and WNV-diseased equine CNS tissues.

1 **Table 2. Selected protocols for immunohistochemical visualization of resident and**
2 **infiltrative cells in normal and WNV-diseased equine CNS tissues.**

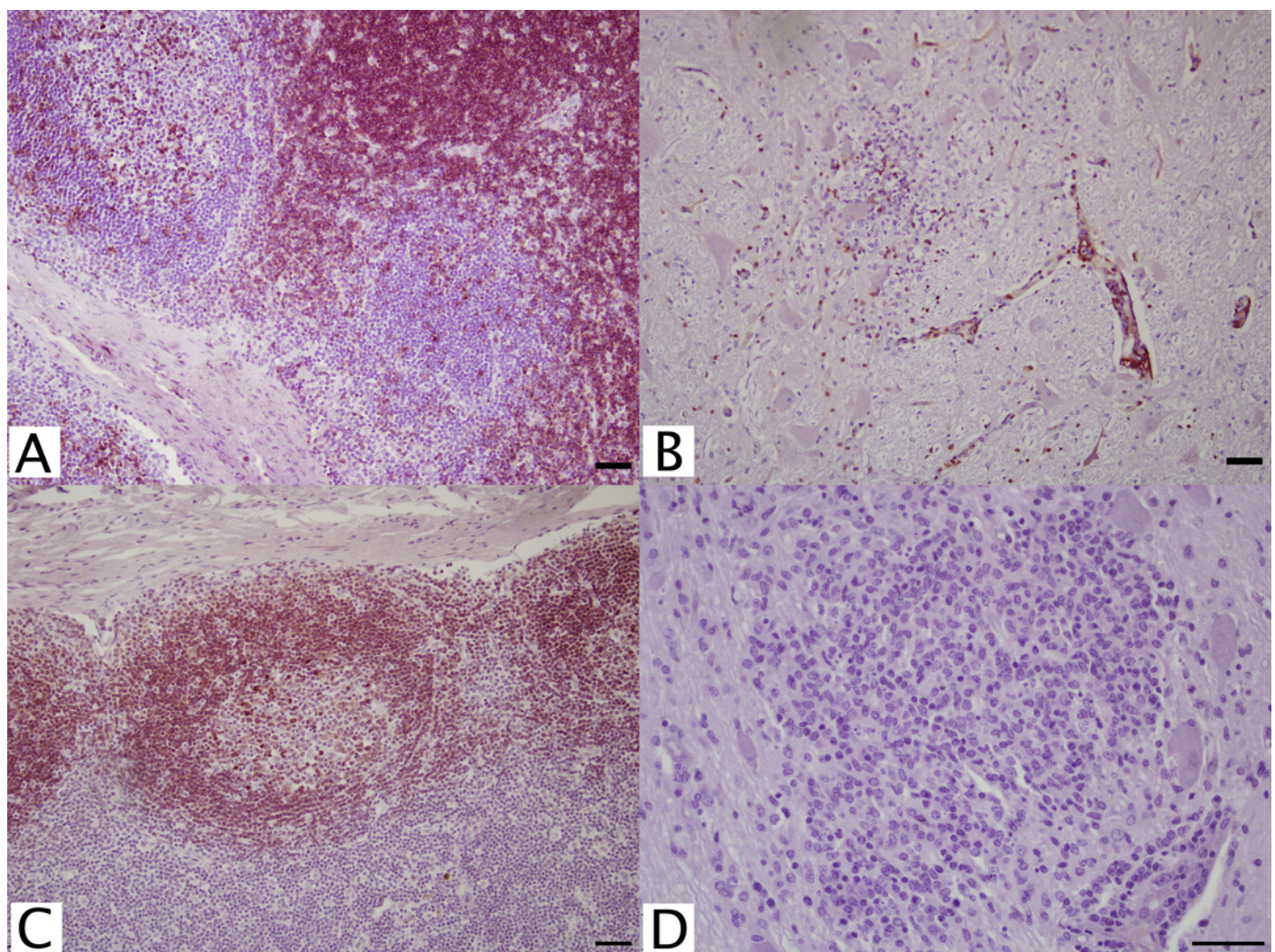
Antibody	ID/ Clone	Tissue format	Peroxidase Blocker	Antigen Retrieval	Blocker	Dilution	Incubation	Commercial Secondary Kit
CD3	A0452	FFPE	3% H_2O_2	Target Retrieval Solution pH6[5]	10% Normal Goat Serum[1]	1:100	60 min at 37°C	Vectastain®ABC Kit - Rabbit[3]
CD4	HB61A	FFT	0.3% H_2O_2	None	Protein Block[2]	1:800	120 min at 23°C	Post Primary Block & Novolink Polymer[2]
CD8	HT14A	FFT	0.3% H_2O_2	None	Protein Block[2]	1:800	120 min at 23°C	Post Primary Block & Novolink Polymer[2]
CD79αCY	HM57	FFPE	3% H_2O_2	Epitope Retrieval Solution pH6[2]	5% Immune Pure Goat Serum[4]	1:100	90 min 37°C	Post Primary Block & Novolink Polymer[2]
Macrophage	MAC387	FFPE	3% H_2O_2	Target Retrieval Solution pH6[5] Or Proteinase K	10% Normal Goat Serum[1]	1:100	60 min at 37°C	Vectastain®ABC Kit - Mouse[3]
NF-H	NAP4, AH1, 9B12	FFPE	3% H_2O_2	Epitope Retrieval Solution pH6[2]	Protein Block[2]	1:1,000	60 min at 37°C	Post Primary Block & Novolink Polymer[2]
GFAP	5C10	FFPE	3% H_2O_2	Target Retrieval Solution pH6[5]	5% Immune Pure Goat Serum[4]	1:8,000	60 min at 37°C	Vectastain®ABC Kit - Mouse[3]
Iba-1	-	FFPE	3% H_2O_2	None	Protein Block[2]	1:500	60 min at 37°C	Post Primary Block & Novolink Polymer[2]

3 FFPE - Formalin-fixed, paraffin embedded; FFT – Fresh, frozen tissue

4 [1]Invitrogen™ [2]Leica® – Novocastra™ Product Line [3]Vector Labs® [4]FischerScientific® [5]Dako®

1

IHC of CD3^{+} T lymphocytes and CD79^{+} B lymphocytes in euqine tissues.

Fig 1 – For the detection of lymphocytes, (A, C) equine lymph node cortex and (B, D) WNV infected equine brain incubated with (A, B) CD3^{+} T lymphocyte primary antibody (pAb A0452, Dako) for 60 minutes at 37°C and detected by Vectastain®ABC Kit, or incubated with (C, D) CD79αcy^{+} B lymphocyte primary antibody (mAb HM57, Dako) for 90 minutes at 37°C and detected by Novolink™ Polymer Detection System. Vector NovaRED Peroxidase Substrate chromogen and hematoxylin couterstain. Bar, 50 μm.

2

IHC of MAC387⁺ and Iba-1⁺ macrophages and microglia in equine tissues.

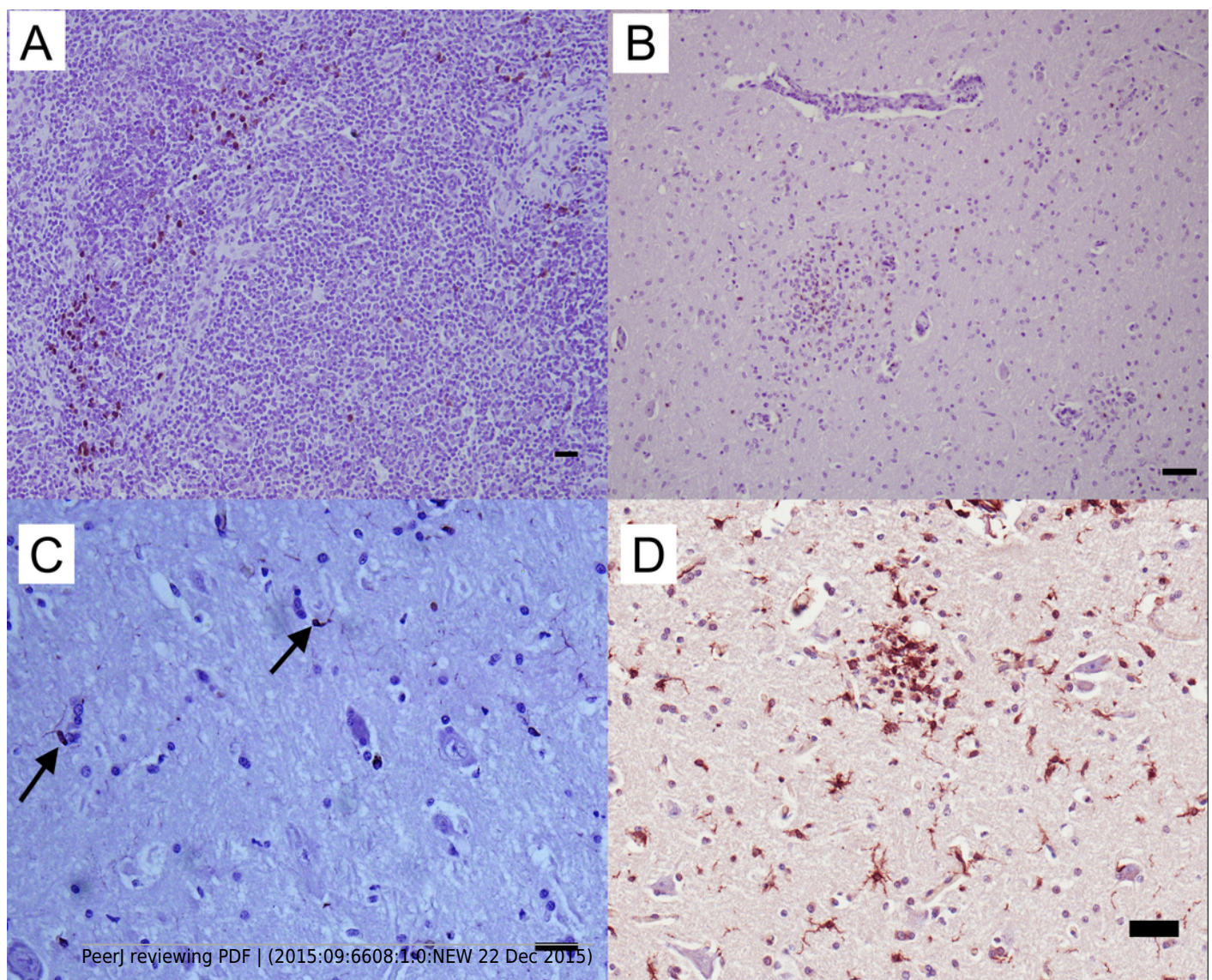
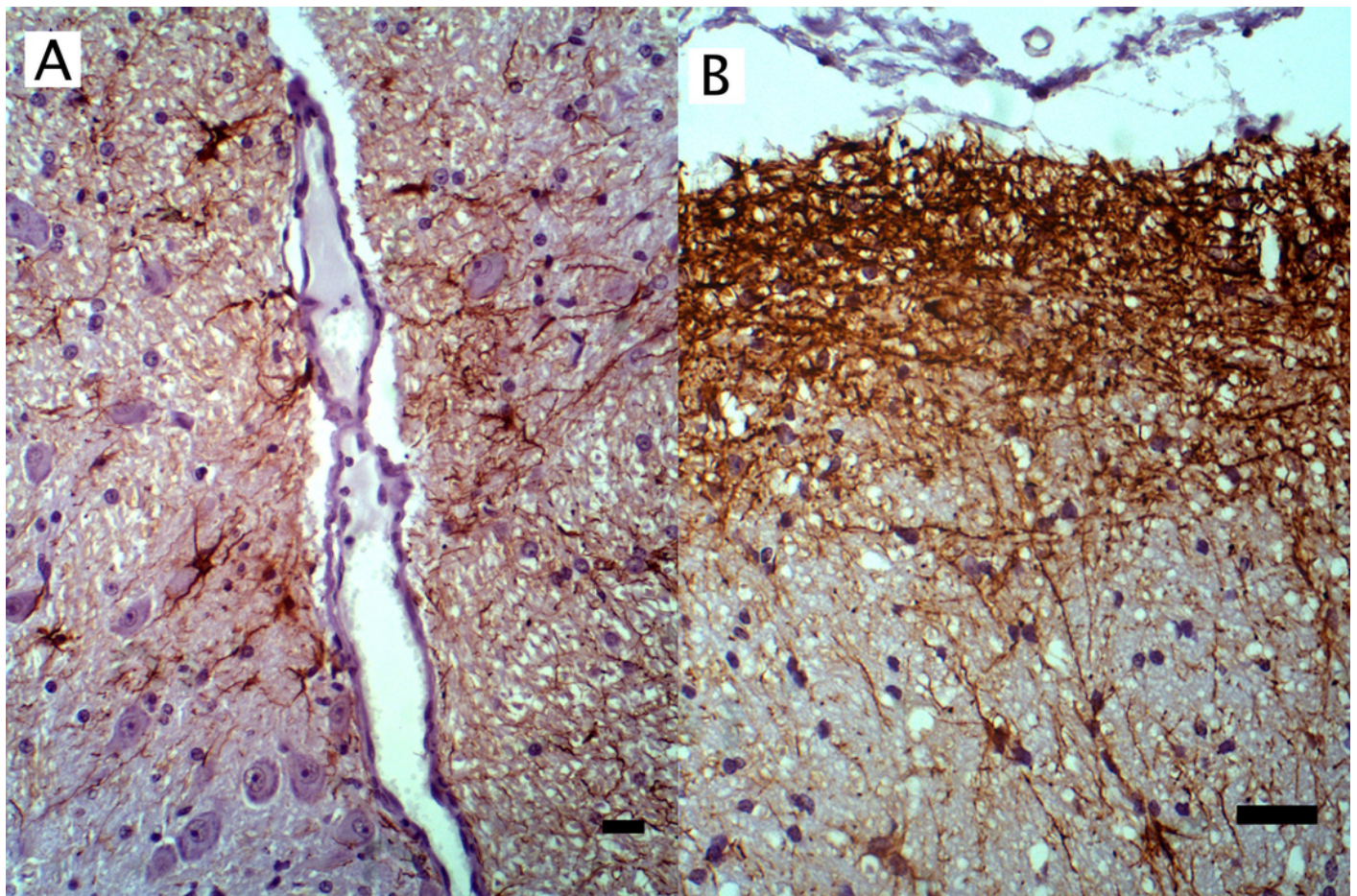
Fig 2 – For the detection of macrophage/microglial lineage cells, (A) equine thymus and (B) WNV infected equine brain were incubated with MAC387⁺ primary antibody (mAb MAC387, Leica) for 60 minutes at 37°C and detected by Vectastain®ABC Kit. Additionally, (C) normal (arrows) and (D) WNV infected horse brain were incubated with microglia/macrophage primary antibody (pAb Iba-1, Wako) for 60 minutes at 37°C and detected by Novolink™ Polymer Detection System. Vector NovaRED Peroxidase Substrate chromogen and hematoxylin couterstain. Bar, 50 µm.

IHC of GFAP$^+$ astrocytes in WNV infected equine brain.

Fig. 5. GFAP$^+$ astrocytes in WNV infected equine brain (A) near blood vessels and (B) at the glial limitans. IHC. Bar, 50 μm.

4

IHC of NF-H⁺ neurons in equine brain.

Fig 4 - Normal equine brain incubated with NF-H⁺ neuron primary antibody (mAb NAP4, EnCor Biotechnology), for 60 minutes at 37°C and detected by Novolink™ Polymer Detection System. Vector NovaRED Peroxidase Substrate chromogen and hematoxylin couterstain. Bar, 50 µm.