Comparative Study on the Pathogenicity of the Generated 9a5b Newcastle Disease Virus Mutant Isolate between Chickens and Waterfowl

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ANIS ZAID

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Thesis submitted to the United Graduate School of Veterinary Science at Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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TABLE OF CONTENTS

ABSTRACT

The chicken and duck are important hosts of Newcastle disease virus (NDV) with distinctive responses to infection. NDV infection in ducks is often subclinical and chronic, while in chickens the infection is clinically apparent and transient. These differences may be due to in part to the host response to NDV infection. Lentogenic NDVs, circulating among waterfowl, have the potential to become highly pathogenic by replication in chickens. The pathological studies which compare between NDV infections in chickens and waterfowl are rare. The virulent 9a5b mutant NDV isolate was generated by passaging the lentogenic Goose/Alaska/415/91 NDV isolate in chickens. The pathogenesis of the 9a5b isolate is unknown in both chickens and waterfowl. In this study, the virulent 9a5b mutant NDV isolate was inoculated intranasally in 32-day-old specific pathogen-free white Leghorn chickens and Japanese commercial ducks. This study compares the histopathological alterations in chickens and ducks following 9a5b NDV infection. Haematoxylin and eosin (HE) stain was used to investigate the tissue's histological alterations. NDV nucleoprotein (NDV-NP), glial fibrillary acidic protein (GFAP) and interferon (IFN)-β were detected by immunohistochemistry (IHC), apoptosis was detected by HE staining, caspase-3 IHC and the TUNEL assay. Apoptosis in buffy coat layer was detected by toluidine blue stained semi-thin section and confirmed by agarose gel electrophoresis. Unlike ducks, which remained clinically normal throughout the study, chickens were shown depression, gasping, oral discharges and greenish white soft feces. Gross and histologic lesion patterns as well as viral replication supported the differing clinical outcome. In immune organs, labelling of NDV-NP and lymphoid depletion were most marked in chicken. The pattern of apoptosis in the spleen differed between chickens and ducks. In chickens there were numerous apoptotic cells in the peri-ellipsoidal white pulp, the peri-ellipsoidal, peri-arteriolar and peri-venous lymphoid sheaths, while apoptosis in duck spleens was mainly within the germinal centers. Lymphoid depletion was the main feature in the bursal and thymic tissues of chickens, but apoptosis was marked in these organs in ducks. Ducks had slight inflammation mainly in respiratory and digestive tracts, whereas slight nonpurulent encephalitis, necrotizing pancreatitis, tubulointerstitial nephritis, and mild inflammation in respiratory and digestive tracts were detected in chickens. Expression of IFN-β appeared earlier and was more intense in the tissues from ducks compared with those from chickens. The differences in IFN-β and NDV-NP expression may reflect the relative clinical severity of the infection in the two avian species.

الْمُسْتَخْلَصْ الْعَرَبِى

الدَّجَاجُ والبَطُ مِنْ أهَمِ الطّيُورِالّتِى تُصَابُ بِفَيْرُوسِ النُّيوكَاسِل مَعَ رُدُودٍ مُتَبَايِنَةٍ لِلإِصَابَة. فِى الّبَطِ، الّعَدْوىَ بِفَيْرُوسِ النُيُوكَاسِل تَكُونُ غَالِبَاً غَيْر مَرَضِّيَة وَ مُزْمِنَة، أمَا فِى الدَّجَاجِ تَكُونُ الّعَدْوىَ ظَاهِرَةً وَعَابِرَة. قَدْ تَكُونُ هَذِهِ ألإِخْتِلافَاتُ جُزْئِيَاً تَعُودُ إلىَ مَدَىَ إسْتِجَابَة الطَائِرُ لِلإِصَابَةِ بِفَيْرُوسِ النُيُوكَاسِلْ. عِتْرَاتُ فَيْرُوس النُيُوكَاسِل الّغَيْر ضَارِيَة الّمَوْجُودَة بَيْنَ الطّيُورِ الّمَائِيَة لَدَيْهَا الّقُدْرَة عَلَى أنْ تُصْبِحَ شَدِّيدَةَ الّضَرَاوَة بَعْدَ حَقْنِهَا وَنَسْخِهَا فِى الدَّجَاج. إلىَ الأن لاَ تُوجَد دِرَاسَات مُسْتَفِيضَة تُقَارِنُ بَيْنَ مَرَضِ النُيُوكَاسِلْ فِى الدَّجَاجِ والطّيُورِ الّمَائِيَة. عِتْرَة 9a5b الّمُتَحَولَة الضَّارِية تَمَ إنْشَائُهَا مِن الّعِتْرَةِ الّغَيْرِ ضَّارِيَة Goose/Alaska/415/91 لِفَيْرُوسِ النُيُوكَاسِل بِحَقْنِهَا فِى الّحُوَيْصِلَاتِ الّهَوَائِيَةِ لِلدَّجَاجِ تِسْع مَرَّاتْ مُتَتَالِيَة ثُمَّ فِى مُخ الدَّجَاجِ خَمْس مَرَّاتْ مُتَتَالِيَة. التَطَور الّمَرَضِّى لِعِتْرَةِ 9a5b غَيْر مَعْرُوف فِي كُلٍ مِن الدَّجَاجِ وَالطّيُورِ الّمَائِيَة. فِى هَذِهِ الدِرَاسَة، تَمْتْ الّعَدْوىَ بِعِتْرَةِ 9a5b الّمُتَحولَةِ فِى كُلٍ مِنْ الدَّجَاجِ ذُو الّقُبَعَةِ الّبَيْضَاء الّخَالِي مِنْ بَعْضِ الأَمْرَاضِ الّمُحَدَدَة وَالّبَطِ الّيَابَانِى التُجَارِى أعْمَارْ 32 يَوْمَاً. وَتُقَارِنُ هَذِهِ الدِّرَاسَةُ التَّغَيُرَات التَّشْرِيحِيَةِ الّمَرَضِيَةِ فِي كُلٍ مِنْ الدَّجَاجِ وَالّبَطِ بَعْدَ الّعَدْوَى بِعِتْرَةِ 9a5b الّمُتَحولَة. صَبْغَة الّهِيمَاتُوكْسِيلِينْ وَالإِيُوزِينْ قَدْ إسْتُخْدِمَتْ لِمَعْرِفَة وَمُقَارَنَة التَّغَيُرَات الّمَرَضِّيَة فِى الأَنْسِجَةِ الّمُخْتَلِفَةِ فِى كُلٍ مِنْ الدَّجَاجِ وَالّبَط. وَقَدْ إسْتُخْدِمَتْ تِقْنِّيَة الإِمْيُونُوهِسْتُوكِمِسْتْرِى (IHC) لِلكَشْفِ عَن الْبُرُوتِّين النَّوَوِي لِفَيْرُوسِ النُيُوكَاسِل (NDV-NP) فِى الأَنْسِجَةِ الّمُخْتَلِفَة، وَالّبُرُوتِين الّلِيفِى الّحِمْضِى (GFAP) فِى خَلايَا الّمُخ وَهِرْمُون الإِنْتَرْفِيرُونْ بِيتَا (IFN-β) فِى أنْسِجَةِ الطُّحَال، وَالّغُدَة الصَعْتَرِيَة، وَجِرَاب فِبْرِيسْوَسْ وَفِى أنْسِجَةِ الرِّئَة. وَتَمَ الّكَشْف عَنْ مَوْتِ الّخَلايَا الّمُبَرْمَجْ (apoptosis) بِوَاسِطَةِ Caspase-3 IHC و TUNEL assay.وَ تَمَ الّكَشْف عَنْ مَوْتِ الّخَلايَا الّمُبَرْمَجْ أيْضَاً فِي خَلايَا الدَّمِ الّبَيْضَاء بِوَاسِطَةِ صِبْغَة الطُّولُويدِّينْ الأَزْرَقْ وَتَمَ تَأْكِيد ذَلِكَ بِوَاسِطَة الّفَصْلِ الّكَهْرَبَائِى لِلحِمْضِ النَوَوِى (gel electrophoresis). عَلَى عَكْسِ الّبَطِ، الّذِى ظَلَ طَبِيعِيَاً مِن نَاحِيَةِ الأَعْرَاضِ الظَّاهِرِيَةِ طُوَالَ فَتْرَةِ الدِّرَاسَة، كَانَ الدَّجَاجُ قَلِيلُ الّحَرَكَةِ، وَيَلْهَثُ، وَيُخْرِجُ إفْرَازَاتٍ عَن طَرِيقِ الّفَمِ وَكَانَ الّبُرَازُ طَرِيَاً وَخَلِيطٌ مِنَ الّلَوْنَيْنِ الأَبْيَضُ وَالأَخْضَر. وَكَانَت التَّغَيُرَاتُ الّعَيْنِيَةُ وَالّمَيُكْرُوسْكُوبِيَةُ وَكَذَلِكَ تَكَاثُرُ الّفَيْرُوس فِى الأَنْسِجَةِ الّمُخْتَلِفَةِ تَدْعُمُ الّفَرْقَ بَيْنَ أعْرَاضِ الّمَرَض فِى كُلٍ مِنْ الدَّجَاجِ وَالّبَط. فِي الأَجْهِزَةِ الّمَنَاعِيَةِ، تَكَاثُر فَيْرُوس النُيُوكَاسِلْ وَنُضُوب الّخَلايَا الّلِمْفَاوِيَة كَانَ أكْثَرَ وُضُوحَاً فِي الدَّجَاجْ. نَمَطْ الّخَلايَا الّمَيِتَة مَوْتَاً مُبَرْمَجَاً فِي الطُّحَالِ كَانَ مُخْتَلِفَاً بَيْنَ الدَّجَاجِ وَالّبَط. فِى الدَّجَاجِ كَانَ هُنَاكَ الّعَدِيدَ مِن الّخَلايَا الّمَيِتَة مَوْتَاً مُبَرْمَجَاً فِى كُلٍ مِن الّمَنْطِقَةِ الّمُحِيطَةِ بِاللُّبِ الّبَيْضَاوِي الأبْيَض، وَالأغْلِفَةِ الّلِمْفَاوِيَةِ لِكُلٍ مِن اللُّبِ الّبَيْضَاوِي وَالشَرَابِين وَالأوْرِدَة الصَّغِيرَة، فِي حِينْ أنَ مَوْتَ الّخَلايَا الّمُبَرْمَجْ فِي أنْسِجَةِ الطُّحَالِ فِى الّبَطِ كَانَت أسَاسَاً فِى الّمَرَاكِزِ الإِنْتَاجِيَةِ الّمُنْطَمِرَة. وَكَانَ نُضُوبَ الّخَلايَا الّلِمْفَاوِيَة هُوَ السِّمَة الرَّئِيسِيَة فِى أنْسِجَةِ جِرَاب فِبْرِيسْوَسْ وَالّغُدَةِ الصَّعْتَرِيَة فِى الدَّجَاج، وَلَكِنْ كَانَ مَوْت الّخَلايَا الّمُبَرْمَجْ فِى هَذِهِ الأجْهِزَةِ السِّمَة الّمُمَيَزَة فِي الّبَط. فِى الّبَطِ إلّتِهَابَاً طَفِيفَاً قَدْ ظَهَرَ فِى أنْسِجَةِ الجِّهَازِ التَّنَفُسِي وَالّجِهَازِ الّهَضْمِي، أمَا فِى الدَّجَاجِ، فَلَقَدْ أحْدَثَت الّعَدَوَى إلّتِهَابَاً طَفِيفَاً غَيْر صَدِيدِى فِى أنْسِجَةِ الدِّمَاغ ، وَإلّتِهَاباً نَخْرِياً فِى الّبَنْكِرْيَاس ، وإلّتِهَاباً فِى الأَنْسِجَةِ الّخِّلالِيَة الّكَلَوِيَة ،بِالإِضَافَةِ إلىَ إلّتِهَابَاً خَفِيفَاً فِي الجِّهَازِ التَّنَفُسِي وَالجِّهَازِ الّهَضْمِي. أمّا هُرْمُون الإنْتَرْفِيرُونْ بِيتَا (β-IFN) فَكَانَ إفرَازُةُ مُبَكِرَاً وَ أكْثَرَ كَثَافَةً فِي أنْسِجَةِ الّبَطِ عَنْهُ فِى أنْسِجَةِ الدَّجَاج. الإِخْتِلافَات فِى إفْرَازِ هُرْمُون الإِنْتَرْفِيرُونْ بِيتَا وَتَكَاثُر فَيْرُوس النُيُوكَاسِل فِى الأَنْسِجَةِ الّمُخْتَلِفَة قَدْ تَعْكِسُ الّفَارِقَ فِي شِّدَةِ الإِصَابَةِ فِى هَذَيْنِ النَّوْعَيْنِ مِن الطُّيُورْ.

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LIST OF ABBREVIATIONS

APMV	: Avian Paramyxovirus
BBB	: Blood Brain Barrier
dpi	: Day Post Infection
DW	: Distal Water
EID	: Egg Infective Dose
F	: Fusion
FAE	: Follicle Associated Epithelium
FAO	: Food and Agriculture Organization
GC	: Germinal Center
GFAP	: Glial Fibrillary Acidic Protein
HE	: Haematoxylin and Eosin
HI	: Haemagglutinin-Inhibition Tests
HN	: Haemagglutinin-Neuraminidase
ICPI	: Intracerebral Pathogenicity Index
IFE	: Interfollicular Epithelium
IFN-α	: Interferon-Alpha
IFN-β	: Interferon-Beta
IFS	: Interfollicular Septum
IgA	: Immunoglobulin A
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IHC	: Immunohistochemistry
IVPI	: Intravenous Pathogenicity Index
L	: Large Polymerase Protein
M	: Matrix Protein
MABs	: Monoclonal Antibodies
MDT	: Mean Death Time
MDV	: Marek's Disease Virus
Mda-5	: Melanoma Differentiation-Associated Gene 5
NBF	: Neutral Buffered Formalin
ND	: Newcastle Disease

NDV	: Newcastle Disease Virus
NDV-NP	: Newcastle Disease Virus-Nucleoprotein
NGS	: Normal Goat Serum
NP	: Nucleoprotein
NVNDV	: Neurotropic Velogenic NDV
OIE	: Office International des Epizootics
P	: Phosphoprotein
PALS	: Peri-Arteriolar Lymphoid Sheath
PB	: Phosphate Buffer
PBMC	: Peripheral Blood Mononuclear Cells
PBS	: Phosphate Buffer Saline
PELS	: Peri-Ellipsoidal Lymphoid Sheath
PPMV-1	: Pigeon Paramyxovirus Type-1
PVLS	: Peri-Venous Lymphoid Sheath
PWP	: Peri-Ellipsoidal White Pulp
RP	: Red Pulp
RIG-I	: Retinoic Acid-Inducible Gene I
RLRs	: Retinoic Acid-Inducible Gene-Like Receptors
RT	: Room Temperature
SEM	: Scanning Electron Microscopy
SPF	: Specific Pathogen-Free
TBE Buffer	: Tris/ Borate/ EDTA Buffer
TEM	: Transmission Electron Microscopy
TUNEL assay	: Terminal Deoxynucleotidyl Transferase (TdT)-: Mediated dUTP Nick End Labelling
VN	: Virus Neutralization Test
VVNDV	: Viscerotropic Velogenic NDV

LIST OF TABLES

LIST OF FIGURES

Fig. 17. Spleen; chicken, 4 dpi. Lymphoid depletion of white pulp with a huge number of apoptotic bodies in white and red pulps. Arrow indicates penicilliform capillaries. HE. Bar, 100 μm.

Fig. 18. Spleen; chicken, 4 dpi. Lymphoid depletion of white pulp with numerous apoptotic bodies (arrowhead). Arrow indicates a penicilliform capillary. HE. Bar, 50 μm.

Fig. 19. Spleen; chicken, 4 dpi. Numerous apoptotic bodies in the PALS (arrowhead). HE. Bar, 50 μm.

Fig. 20. Spleen; chicken, 4 dpi: There was intensive labelling of apoptotic cells in the PELS and PWP. Arrows indicate penicilliform capillaries. TUNEL assay. Bar, 50 μm.

Fig. 21. Spleen; duck, 1 dpi. There were intensive immunolabellings of caspase-3 in the germinal centres (GC) (arrow) and weak signals in the white pulp (arrowhead). IHC, haematoxylin counterstained. Bar 500 μm.

Fig. 22. Spleen; duck, 1 dpi. There was intensive immunolabelling of caspase-3 in the GC and weak labelling in the cells of peri-ellipsoidal lymphoid sheath and peri-ellipsoidal white pulp. Arrows refer to penicilliform capillaries; A, artery. IHC, haematoxylin counterstained. Bar 100 μm.

Fig. 23. Spleen; duck, 4 dpi. Slight lymphoid depletion around the penicilliform capillary (arrow) and numerous apoptotic cells in the GC (arrowhead). HE. Bar, 50 μm.

Fig. 24. Spleen duck, 6 dpi. There was intensive labelling of apoptotic cells in the GC (arrow). A; artery. TUNEL assay. Bar, 50 μm.

Fig. 25. Spleen; chicken, 4 dpi. There was intense labelling of NDV-NP in cells morphologically compatible with macrophages (arrow) and in lymphocytes and apoptotic bodies (arrowhead). IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

Fig. 26. Spleen; duck, 4 dpi. There were intensive immunolabellings of NDV-NP in cells morphologically compatible with macrophages in the red pulp. Arrow indicates a penicilliform capillary. A, artery; GC, germinal centre. IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

Fig. 27. Spleen; chicken, 2 dpi. There was immunolabelling for IFN-β in cells morphologically compatible with macrophages. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 28. Spleen; chicken, control. Control chickens did not show immunolabelling for IFN-β in the splenic tissues. Arrow indicates a penicilliform capillary. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 29. Spleen; duck, 1 dpi. There was intensive immunolabelling for IFN-β in cells morphologically compatible with macrophages. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 30. Spleen; duck, control. Rare immunolabelling signals for IFN-β were detected in the splenic tissues of the control ducks. Arrow indicates a penicilliform capillary. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 31. Bursa; chicken, 6 dpi. Nucleolar fragmentation in an apoptotic macrophage. TEM. Bar, 3 μm.

Fig. 32. Bursa; duck, 1 dpi. There was intense immunolabelling of caspase-3 in the cortex (C), medulla (M) and in follicle-associated epithelium (FAE). IHC, haematoxylin counterstained. Bar, 200 μm.

Fig. 33. Bursa; duck, 6 dpi. There were intense signals for apoptosis in the cortex (C), medulla (M) and in follicle-associated epithelium (FAE). TUNEL assay. IFE, interfollicular epithelium; IFS, interfollicular septum. Bar, 100 μm.

Fig. 34. Bursa; duck, 8 dpi. Numerous apoptotic bodies in the cortex(C), medulla (M) and in follicle-associated epithelium (FAE). HE. Bar, 50 μm.

Fig. 35. Bursa; chicken, 8 dpi. There was immunolabelling for NDV-NP in the cortex (C), medulla (M), and in follicle-associated epithelium (FAE). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 36. Bursa; chicken, 1 dpi. There was immunolabelling for IFN-β in macrophage- and fibroblast-like cells in the medulla (M). IHC, haematoxylin counterstained. Bar, 100 μm (inset, 15).

Fig. 37. Thymus; duck, 1 dpi. There was immunolabelling of caspase-3 in cells morphologically compatible with lymphocytes and macrophages at the corticomedullary junction (arrows). IHC, haematoxylin counterstained. C, cortex; M, medulla. Bar, 100 μm (inset, 30 μm).

Fig. 38. Thymus; duck, 10 dpi. There were intense signals for apoptosis at the corticomedullary junction. C, cortex; M, medulla; H, Hassall's corpuscles. TUNEL assay. Bar, 200 μm (inset, 30 μm).

Fig. 39. Thymus; chicken, 8 dpi. Severe lymphoid depletion of the chicken thymus. C, cortex; M, medulla. HE. Bar, 500 μm.

Fig. 40. Thymus; duck, 8 dpi. Slight lymphoid depletion of the duck thymus. C, cortex; M, medulla. HE. Bar, 500 μm.

Fig. 41. Thymus; chicken, 8 dpi. There was immunolabelling of NDV-NP in cells morphologically compatible with macrophages. C, cortex; M, medulla; S, septum. IHC, haematoxylin counterstained. Bar, 100 μm (inset, 30 μm).

Fig. 42. Thymus; ducks, 4 dpi. There was immunolabelling of NDV-NP in cells morphologically compatible with macrophages. C, cortex; M, medulla. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 43. Cerebellum; chicken, 8 dpi. Mild loss of Purkinje cells (arrows). ML, molecular layer; GL, granular layer. HE. Bar, 200 μm.

Fig. 44. Cerebellum; chicken, 8 dpi. Degenerative changes in Purkinje cells (arrows) and focal microgliosis in the molecular layer. ML, molecular layer; GL, granular layer. HE. Bar, 100 μm.

Fig. 45. Cerebellar white matter; chicken, 10 dpi. Vacuolation and demyelination in the cerebellar white matter (arrow). GL, granular layer. HE. Bar, 100 μm.

Fig. 46. Medulla oblongata; chicken, 8 dpi. Focal areas of microgliosis (main frame) and lymphocytic perivascular cuffing (inset). HE. Bar, 50 μm (inset, 50 μm).

Fig. 77. Liver; chicken, 4 dpi. Proliferation of the ectopic lymphoid tissues (asterisk) and the bile caniliculi was dilated and engorged with bile (arrows). Blood sinusoid (arrowhead). HE. Bar, 50 μm.

Fig. 78. Liver; chicken, 4 dpi. Dilatation of the bile canaliculi and the bile is engorged in the portal bile duct (BD). A, portal artery; V, portal vein. HE. Bar, 50 μm. *Inset*: Bile duct; chicken, P10. Positive signals for NDV-NP in the epithelial cells of bile duct and in macrophages around it. IHC, haematoxylin counterstained. Bar, 25 μm.

Fig. 79. Liver; chicken, 4 dpi. Signals for NDV-NP in macrophages (arrows) and in Kupffer cell (inset). IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15).

Fig. 80. Liver; duck, 4 dpi. Accumulation of heterophils in hepatic sinusoids (arrow). HE. Bar, 50 μm.

Fig. 81. Duodenum; chicken, 4 dpi. Degeneration and necrosis in the mucosal epithelium (asterisk). HE. Bar, 100 μm.

Fig. 82. Duodenum; chicken, 4 dpi. Positive signals for NDV-NP in degenerated mucosa (asterisk) and in epithelial cells (arrows) in the main frame and inset. IHC, haematoxylin counterstained. Bar, 100 μm (inset, 15 μm).

Fig. 83. Cecal tonsils; chicken, 2 dpi. Proliferation of cecal tonsils (CT) with slight haemorrhages (arrow). HE. Bar, 100 μm.

Fig. 84. Cecal tonsils; chicken, 4 dpi. Positive signals for NDV-NP in the cecal tonsils and in cecal mucosa. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 85. Duodenum; duck, 1 dpi. Proliferation of goblet cells (arrow). HE. Bar, 100 μm.

Fig. 86. Duodenum; duck, 2 dpi. Mitosis in the epithelial cells of the duodenal gland (arrow) and infiltration of heterophils (arrowhead). HE. Bar, 50 μm. *Inset*: Duodenum; duck, 4 dpi. Positive signals for NDV-NP in macrophage in the mucosa. IHC, haematoxylin counterstained. Bar, 15 μm.

INTRODUCTION

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease affecting many domestic and wild avian species, causing serious economic losses to the poultry industry worldwide (Alexander and Senne, 2008). ND is caused by ND virus (NDV), a synonymous with avian paramyxovirus type 1 (APMV-1), and it belonging to the genus *Avulavirus*, family Paramyxoviridae in the order Mononegavirales (Alexander, 1998, 2001, 2003). Historically NDVs have been classified into three major pathotypes: low virulence (lentogens), moderate virulence (mesogens), and high virulence (velogens). In chickens, lentogenic strains produce mild or inapparent respiratory infections. Mesogenic strains are associated with low mortality, acute respiratory disease, and neurologic signs in some birds. The velogenic strains are further divided into neurotropic velogenic NDVs (causing respiratory and neurologic signs with high mortality), and viscerotropic velogenic NDVs (causing acute lethal infections with necro-haemorrhagic lesions, that are most obvious in the gastrointestinal tract) (Alexander, 2003).

Among poultry, chickens are most susceptible to NDV infection, while ducks and geese are least susceptible (Kaleta and Baldauf, 1988; Wakamatsu *et al.*, 2006a). Wild birds are considered to be the natural reservoir of NDV (Zhu *et al.*, 2010). Most NDV strains isolated from waterfowl are nonpathogenic for chickens;

however, certain virulent strains from wild birds have been reported to cause outbreaks in flocks of chickens (Zhu *et al.*, 2010). Shengqing *et al.* (2002) demonstrated that even avirulent wild waterfowl viruses, possessing the typical avirulent fusion (F) gene cleavage site sequence, have the potential to become velogenic after transmission to chickens. The virulent 9a5b NDV mutant isolate was generated from the lentogenic waterfowl Goose/Alaska/415/91 isolate (wild type) by nine consecutive passages in chicken air sacs, followed by five passages in chicken brains. The wild-type isolate have been classified phylogenetically into Class I lineage 6 which includes viruses from wild waterfowl and US live bird markets (Tsunekuni *et al.*, 2010b). The wild type isolate has an intracerebral pathogenicity index (ICPI) of zero, avirulent F gene cleavage site and an inactive haemagglutinin-neuraminidase (HN) precursor. In contrast, the 9a5b NDV isolate has an ICPI of 1.88, a virulent F gene and a biologically active HN protein (Shengqing *et al.*, 2002; Tsunekuni *et al.*, 2010a). Moreover, a genetic comparison between the wild type and 9a5b isolates demonstrated 3 amino acid substitutions in the HN proteins changing the inactive HN_0 precursor (found in avirulent viruses) to biologically active HN protein (found in virulent viruses and responsible for the attachment of virus particles to the host cell and promotes fusion activity of the F protein) (Huang, Z., *et al.*, 2004; Lamb and Kolakofsky, 2001; Robert, 1993; Takimoto *et al.*, 2002; Tsunekun *et al.*, 2010). It is accepted now that, avirulent

viruses which maintained in wild waterfowl in nature have the potential to become velogenic, and a wild bird seems to have some biological properties making them a suitable reservoir as well as a constant source of NDV. Therefore, investigating the differences in the pathogenesis of NDV infection between chickens and waterfowl can help us to determine why waterfowl is a suitable reservoir for these viruses, and why ND outbreaks occurs in chickens rather than in waterfowl.

This study was initiated to compare the clinical signs, pathological lesions, and viral distribution in specific pathogen-free (SPF) chickens and commercial ducks infected with the mutant 9a5b NDV isolate. This isolate was chosen because: (1) The wild type isolate was isolated from migratory waterfowl and it was lentogenic, (2) mutation in its genome occurred by serial passages in chickens which changed it to virulent isolate, (3) and the pathogenicity index was previously determined before and after mutation. The data reported here provide, to the best of the author's knowledge, the first histopathological comparative study between chickens and ducks after experimental NDV infection.

LITERATURES REVIEW

LITERATURES REVIEW

1. History of ND

It is generally accepted that the first outbreaks of ND occurred in 1926, in Java, Indonesia (Kraneveld, 1926), and in Newcastle-upon-Tyne, England (Doyle, 1927). There are reports of disease outbreaks in Central Europe similar to what we now recognize as ND that predates 1926 (Halasz, 1912), and the disease may have been present in Korea as early as 1924 (Levine, 1964). Macpherson (1956) considered the death of all chickens in the Western Isles of Scotland in 1896 to be attributable to ND. The name "Newcastle disease" was established by Doyle, 1935, after outbreak in Newcastle-upon-Tyne, England (Doyle, 1935). Within a few years later, numerous NDV isolates that produce extremely mild or no disease in chickens were isolated around the world (Asplin, 1952; Hitchner and Johnson, 1948; McFerran and McCracken, 1988; Senne *et al.*, 1983). The history of Newcastle disease in most countries has not been well-documented. Alexander recorded the history of ND in Great Britain in detail and considered it a good example of the effect that ND may have on the poultry industry (Alexander, 2001).

2. Epidemiology, Transmission and Spread of ND

International monitoring of Newcastle disease is carried out by agencies such as the Food and Agriculture Organization (FAO) of the United Nations

and the World Organization for Animal Health (OIE). ND emerges in poultry in enzootic or regular epizootic forms throughout the world. In more developed areas, such as Western Europe, sporadic epizootics occur on a fairly regular basis despite the widespread use of vaccination. The distribution of NDV is dependent on the attempts at eradication and control made in different countries. The success of such measures is, in turn, dependent on the nature of the poultry industry (i.e., countries with mostly village chicken flocks have far greater problems than those with mostly large commercial flocks).

Alexander (2001) considered that, probably four panzootics of NDV had occurred since the first recognition of the disease. The first panzootic appears to have arisen in Southeast Asia and moved slowly through Asia to Europe and isolated in England in 1926 and Spread worldwide in the early1960s. The second panzootic appears to have begun in the Middle East in the late 1960s and reached most countries by 1973. The more rapid spread of the second panzootic could be because the major revolution in poultry industry, increase the international trade, and international trade of caged psittacine species (Francis, 1973; Walker *et al.*, 1973). Alexander (2001) considered that antigenic and genetic evidence (Alexander *et al.*, 1997; Lomniczi *et al.*, 1998) indicates that there was probably world wide spread of a third virulent virus during the late 1970s but the universal use of vaccination reduces the

symptoms of this panzootic. Vaccination had protected birds from disease but in most cases allowed replication and spread of the virus. The fourth panzootic of ND was emerged from the pigeons and doves (*Columba livia*) in the Middle East in the late 1970s (Kaleta *et al.*, 1985). This disease resembled the neurotropic form in chickens but without respiratory signs. By 1981, it had reached Europe (Biancifiori and Fioroni, 1983) and then spread worldwide rapidly.

NDV infection may take place by inhalation, ingestion or direct contact with mucous membrane (Alexander, 1988). Aerosols or large droplets derived from the respiratory tract of infected bird as well as dust and other particles, including feces, may contain the virus. These virus-laden particles may be inhaled, impinge up on the mucous membranes or swallowed, resulting in infection (Meulemans, 1988). During the course of NDV infection, large amounts of virus are excreted in the feces, ingestion of such feces results in infection; this is likely to be the main method of bird-to-bird spread for avirulent enteric NDVs (Alexander *et al.*, 1984).

Sources or methods of NDV spread in various epizootics may be one or more of the following: movement of live birds (feral birds, pet/exotic birds, game birds, racing pigeons and commercial poultry); contact with other animals; movement of people and equipment; movement of poultry products;

air bore spread, and contaminated poultry feed, water and vaccines. The importance of any of these factors will depend on the situation in which the epizootic occurs. In countries where poultry are kept exclusively in bird proof housing, the ability of feral birds to invade affected flocks and transfer the disease will be minimal, whereas birds kept on open range are more likely to be infected with strains carried by feral birds (Lancaster and Alexander, 1975).

3. Natural hosts of NDV

In addition to the domestic avian species, natural or experimental infection with NDV has been demonstrated in at least 241 species from 27 of the 50 orders of birds (Kaleta and Baldauf, 1988). Moreover, the number of species from which NDV has been isolated, with or without clinical signs, has greatly increased. It seems reasonable to conclude that the vast majority of, if not all, birds are susceptible to infection, but the clinical signs differ according to the virus strain and the host (Alexander and Senne, 2008).

4. Economic and Public health significance of ND

The global economic impact of NDV is enormous. Together with the highly pathogenic Asian H5N1 influenza virus NDV represented a bigger drain on the world's economy than any other animal viruses. In developed countries with established poultry industries, not only outbreaks of NDV are extremely costly,

but control measures, repeated testing and vaccination represent a continuing loss to the economy. Developing countries rely on village chickens to supply a significant portion of dietary protein in the form of eggs and meat, and it is recognized that NDV affect the quantity and quality of the poultry product. Therefore, the impact of NDV should not only measure economically but its effect on human health should be considered (Leslie, 2000; Sen *et al.*, 1998; Spradbrow, 1992).

Addition to the contribution of NDV in malnutrition, some reports described human infection. Eye infections as unilateral or bilateral reddening, excessive lachrymation, edema of the eyelids, conjunctivitis and subconjunctival hemorrhage were reported (Chang, 1981). Human infections with NDV have usually resulted from direct contact with the virus such as direct contact with infected bird, infected materials (vaccines, allantoic fluid) or contaminated utensils without taking the hygienic measures. Human-to-human infection not reported until now (Alexander and Senne, 2008).

5. Strain classification, Antigenicity and Pathogenicity of NDV

The term "strain" is generally used to mean a well-characterized isolate of the virus. All NDV isolate have been considered to represent a single antigenically homogeneous group by virus neutralization (VN) test or agar gel

diffusion technique. Monoclonal antibodies (MABs) have been employed to demonstrate antigenic variation of NDV strains and isolates. MABs may detect slight variations in antigenicity, such as single amino acid changes at the epitope to which the antibody is directed. As a result, they can detect differences not only between strains but between subpopulations of virus (Hanson, 1988).

The virulence of NDV strains varies greatly with the host. Chickens are highly susceptible. But ducks may be infected and show few or no clinical signs, even with strains lethal for chickens (Higgins, 1971). In chickens, the pathogenicity of ND is determined chiefly by the strain of the virus, although doses, route of administration, age of the chicken, and environmental conditions all have an effect. In general, the younger the chicken, the more acute the disease (Alexander and Senne, 2008). Natural routes of infection (nasal, oral, and ocular) appear to emphasize the respiratory nature of the disease (Beard and Easterday, 1967), and intramuscular, intravenous, and intracerebral routes appear to enhance the neurologic signs (Beard and Hanson, 1984).

The pathogenicity of NDV can determine by: mean death time (MDT); intracerbral pathogenivity index (ICPI); intravenous pathogenicity index (IVPI) and sequence of the F gene cleavage site.

Mean death time (MDT). In the MDT assay, strains of NDV could be grouped as "velogenic," "mesogenic," and "lentogenic" based on chicken embryo mortality at less than 60 hr, 60-90 hr and greater than 90 hr, respectively, after allantoic inoculation. Velogenic isolates have an MDT of less than 60 hours, mesogenic strains have an MDT of 60-89 hours, and lentogenic viruses have an MDT greater than 90 hours (Hanson and Brandly, 1955).

Intracerebral pathogenicity index (ICPI). Injection of 0.05 ml of the diluted virus intracerebrally into each of 10 one-day-old chicks. The birds are examined every 24 hours for 8 days. At each observation the birds are scored: 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean score per bird per observation over the 8-day period. The most virulent viruses will give a maximum score of 2.0, whereas slower strains will give values close to 0.0 (http://www.oie.int (a)).

Intravenous pathogenicity index (IVPI). IVPI test was described as the following: intravenous inoculation of 4-to-8-week-old chickens (serologically negative) with infective allantoic fluid (0.2 ml/ bird), the IVPI is determined from the number of healthy, sick, paralyzed and dead birds observed each day for 10 days post inoculation. The values in the IVPI test are from 0 to 3.0; the IVPI for velogenic strains approach 3.0, while lentogenic strains and some

mesogenic strains have IVPI values of zero. However, some viruses that can produce severe disease have IVPI values of zero; the ICPI test is generally preferred for this reason (http://www.oie.int (b); OIE, 2005)

Molecular basis for pathogenicity. During replication of NDV, the functionally important fusion protein is produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translational cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV strains. F0 molecules of virulent viruses can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in low virulence viruses are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types (Nagai *et al.*, 1976a & b; Rott and Klenk, 1988). Non-virulent NDVs have the typical avirulent cleavage motif, ^{112}R/G-R/ K-Q-G-R^{116}, with a leucine (L^{117}) at the N terminus of the F protein after cleavage (F1), which is only susceptible to trypsin-like enzymes found in limited tissues such as those in respiratory and digestive tracts, causing localized infection. On the other, hand the virulent NDVs have a virulent cleavage motif, ^{112}R/K-R-Q-R/K-R^{116}, and phenylalanine (F^{117}) at the N terminus of F1, which enables them to infect the host systemically by acquiring susceptibility to furin or other ubiquitous

intracellular host cell proteases (Collins *et al.*, 1993; de Leeuw *et al.*, 2003; Garten *et al.*, 1980; Glickman *et al.*, 1988; Kattenbelt *et al.*, 2006; Nagai, 1993; Römer-Oberdörfer *et al.*, 2003; Rott and Klenk, 1988; Shengqing *et al.*, 2002; Yusoff and Tan, 2001).

6. Forms of ND

ND is particularly complicated, in that, different isolates and strains of the virus may induce enormous variation in the severity of disease, even in a given host such as the chicken. To simplify matters, division into forms or pathotypes of disease based on clinical signs in chickens has been summarized as the following: 1) Doyle's form, which is an acute, lethal infection of all ages of chickens. Haemorrhagic lesions of the digestive tract are frequently present, and this form of disease has been termed viscerotropic velogenic ND (VVND); 2) Beach's form, which is an acute, often lethal infection of chickens of all ages. Characteristically, respiratory and neurological signs are seen, hence the term neurotropic velogenic ND (NVND); 3) Beaudette's form that appears to be a less pathogenic form of NVND in which deaths usually are seen only in young birds. Viruses causing this type of infection are of the mesogenic pathotype and have been used as secondary live vaccines; 4) Hitchner's form, represented by mild or inapparent respiratory infections caused by viruses of the lentogenic pathotype, which are commonly used as live vaccines; 5) Asymptomatic

enteric form, which is chiefly a gut infection with lentogenic viruses causing no obvious disease. Some live commercial vaccines are established from this pathotype (Beard and Hanson, 1984).

7. Morphology, Structure and Replication cycle of NDV

Negative contrast electron microscopy of NDV (Fig. 1) reveals very pleomorphic virus particles typical of members of the Paramyxovirinae subfamily. Generally, they are rounded and 100-500 nm in diameter, although filamentous forms of about 100 nm across and of variable length are often seen. The surface of the virus particle is covered with projections about 8 nm in length (Alexander and Senne, 2008).

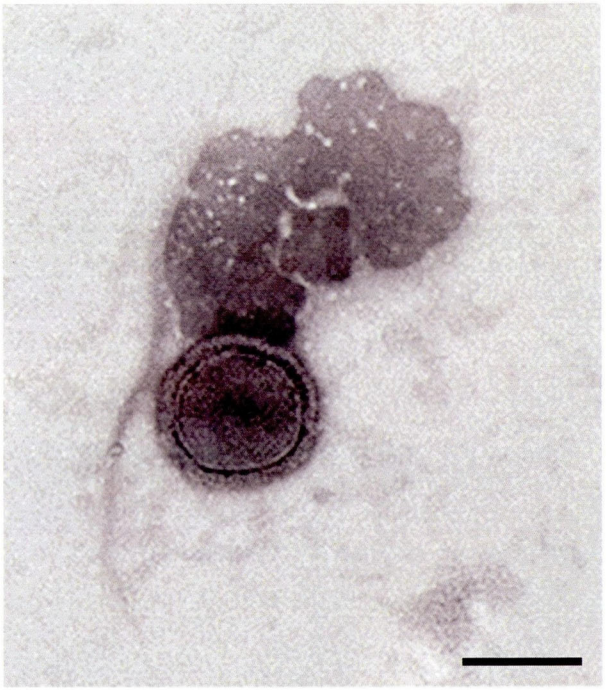


Fig. 1. Immunoelectron microscopy of purified NDV. (DiNapoli *et al.*, 2007). Bar, 100 nm.

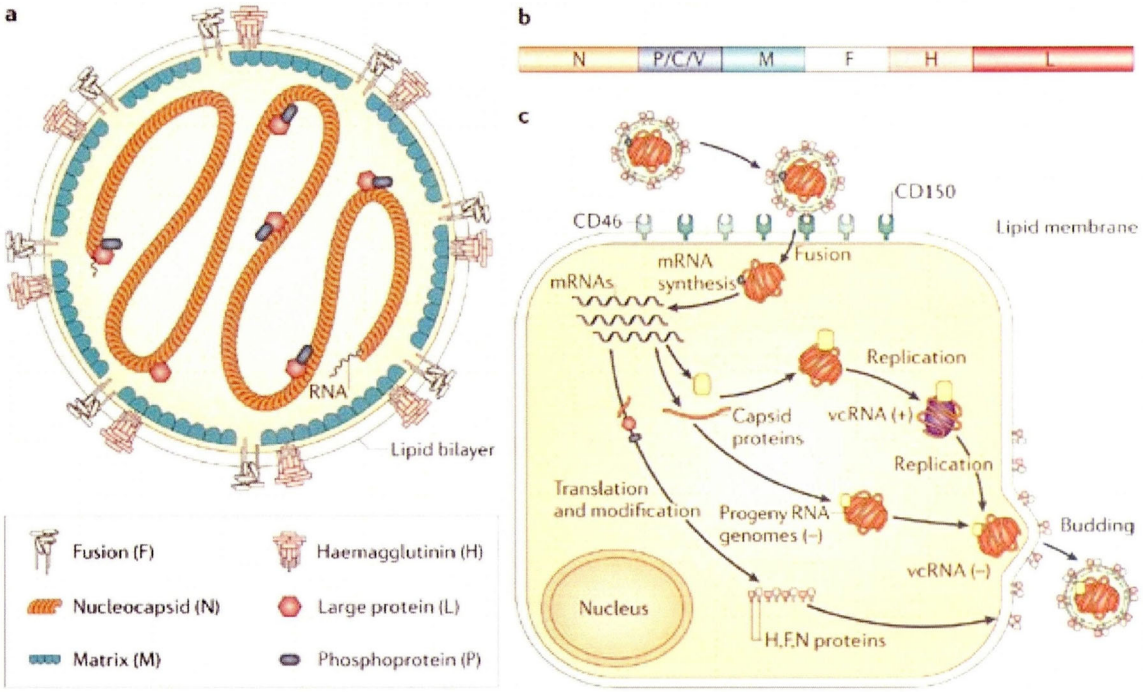


Fig. 2. (a). Diagramatic structure of paramyxoviruses. **(b).** Gene order of the paramyxoviruses genome. **(c).** Replication of measles virus as a member of paramyxovirus family. (Moss and Griffin, 2006).

NDV is an enveloped virus (Fig. 2), containing negative-sense, single stranded RNA genome which codes for six structural and two non-structural proteins (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998). Nucleotide sequencing of the NDV genome has shown it to consist of 15,186 nucleotides (Phillips *et al.*, 1998), although strains with 15,192 (Huang, Y., *et al.*, 2004) and 15,198 (Czegledi *et al.*, 2006) nucleotides have been described. The structural proteins include nucleoprotein (NP), phosphoprotein (P), and large polymerase protein (L) which form the nucleocapsid. The matrix protein (M) forms inner layer of the virion envelope. Haemagglutinin-neuraminidase (HN) and fusion (F) proteins are membrane anchored viral spike proteins that

enable cell binding and infection. The two non-structural proteins V and W; are formed by RNA editing during P gene transcription (Locke *et al.*, 2000; Steward *et al.*, 1993). Both V and W proteins have been reported to play role in virus replication and pathogenesis (Mebatsion *et al.*, 2001).

Replication strategy of NDV as a negative-sense single-strand virus is the same as other members in the paramyxovirus family, like measles virus (Fig. 2). The replication cycle was described as the following steps: *Attachment.* The virus attached to cell receptors, medialed by the HN polypeptide; *Fusion.* Fusion of the virus and cell membrane is mediated by action of the F protein; *Uncoating and Penetration.* The nucleocapsid complex enters the cell; *Transcription.* Intracellular virus replication takes place entirely within the cytoplasm. NDV virus has negative sense RNA, therefore, the RNA-polymerase (transcriptase) must produce complementary transcripts of positive sense that may act as messenger RNA; *Translation.* The virus use the cell's mechanisms in the translation into proteins and virus genomes. The F protein is synthesized as a nonfunctional precursor, F0, that requires cleavage to F1 and F2 by host proteases. The HN of some strains of NDV may also require post-translational cleavage, the viral proteins synthesized in an infected cell are transported to the cell membrane, which becomes modified by their incorporation; *Budding.* Following the alignment of the nucleocapsid close to

modified regions of the cell membrane, virus particles are budded from the cell surface (Alexander and Senne, 2008; Lamb *et al.*, 2005; Peeters *et al.*, 1999).

8. Clinical signs of NDV

Incubation period of NDV after natural exposure has been reported to vary from 2~15 days (average 5~6) depending on: virus virulence, host type and immune status, dose, route of infection, co-infection as well as the environmental conditions. The clinical sings of NDV varies widely according to many factors as those described above. The major factors are the virulence of the virus and the host.

Viscerotropic velogenic NDV (VVNDV). Sudden death and high mortality with the absence of other clinical signs were reported with high virulent NDV infection. In outbreaks in chickens due to the VVND pathotype, clinical signs often begin with listlessness, increased respiration, and weakness, ending with prostration and death. During the panzootic caused by this type of virus in 1970~1973, disease in some countries such as Great Britain and Northern Ireland was marked by severe respiratory signs, but in other countries these were absent. This type of NDV may cause edema around the eyes and head. Green diarrhea is frequently seen in birds that do not die early in infection, and prior to death, muscular tremors, torticollis, paralysis of legs and wings, and

opisthotons may be apparent. Mortality frequently reaches 100% in flocks of fully susceptible chickens (Alexander and Senne, 2008; Allan *et al.*, 1978; McFerran and McCracken, 1988).

The neurotropic velogenic NDV (NVNDV). In chickens, it is marked by sudden onset of severe respiratory disease followed a day or two later by neurologic signs. Egg production falls dramatically, but diarrhea is usually absent. Morbidity may reach 100%. Mortality is generally considerably lower, although up to 50% in adult birds and 90% in young chickens have been recorded (Alexander and Senne, 2008).

Mesogenic strains of NDV. In adult birds, there may be a marked drop in egg production that may last for several weeks. Nervous signs may occur but are not common. Mortality in fowl is usually low except in very young and susceptible birds (Alexander and Senne, 2008).

Lentogenic strains of NDV. These viruses usually do not cause disease in adults birds. In young fully susceptible birds, serious respiratory disease problems can be seen. Mortality sometimes occurs when secondary infections with other pathogens occur (Alexander and Senne, 2008).

Clinical sings in hosts other than chickens. Pigeons (usually affected by pigeon paramyxovirus type-1, PPMV-1), show diarrhea and nervous sings

(Vindevogel *et al.*, 1988); turkeys, are as susceptible as chickens to infection with NDV, but clinical signs are usually less severe (Alexander *et al.*, 1999; Box *et al.*, 1970; McFerran and McCracken, 1988); ducks and geese are resistant even to the high virulent NDV strains, However, outbreaks of severe disease in ducks infected with NDV have been described (Higgins, 1971); Outbreaks of ND have been reported in most game bird species (Lancaster, 1966; Lancaster and Alexander, 1975); young ostrich chicks may show depression and nervous signs, but adults appear unaffected (Alexander, 2000).

9. Pathology of NDV

9.1. Gross Pathology

The gross lesions depend on the strain and pathotype of the virus, in addition to the host and all other factors that may affect the severity of the disease. No pathognomonic lesions are associated with any form of the disease; gross lesions may also be absent. Nevertheless, the presence of haemorrhagic lesions in the intestine of infected chickens has been used to distinguish VVND viruses from NVND viruses (Hanson *et al.*, 1973). Haemorrhagic lesions in the mucosa of the proventriculus, ceca, and small and large intestine appear to result from necrosis of the intestinal wall or lymphoid tissues such as cecal tonsils and Peyer's patches. Generally, gross lesions are not observed in the central nervous system of birds infected

with NDV (McFerran and McCracken, 1988). Gross pathologic changes are not always present in the respiratory tract, but when observed they consist predominantly of mucosal haemorrhage and marked congestion of the trachea (Alexander and Allan, 1974). Airsacculitis may be present even after infection with relatively mild strains and thickening of the air sacs with catarrhal or caseous exudates is often observed (Beard and Hanson, 1984). Haemorrhagic conjunctivitis, focal necrosis of the spleen, and paratracheal edema, most generally observed near the thoracic inlet, were reported. Laying chickens and turkeys infected with velogenic strains usually have egg yolk in the abdominal cavity. The ovarian follicles are often flaccid and degenerative. Haemorrhage and discoloration of the other reproductive organs may occur (Alexander and Senne, 2008).

9.2. Microscopic Lesions

The microscopic lesions of ND is varied according to several factors such as the strain virulence, host type, route of infection, dose, environmental condition, immune status of the host and secondary or co-infection with other pathogens. Several descriptive reports or literature reviews have covered the histologic changes in the various organs during NDV infection. Briefly, the major histologic changes are as the following.

Nervous System. Lesions such as nonpurulent encephalomyelitis with neuronal degeneration, loss of Purkinje cells, focal gilosis, lymphocytic perivascular cuffing, and hypertrophy of endothelial cells were reported. Lesions usually are seen in the cerebellum, medulla, midbrain, brainstem, and spinal cord, but rarely in the cerebrum (Nakamura *et al.*, 2008; Piacenti *et al.*, 2006).

Vascular System. Lesions such as congestion, edema, and haemorrhage are found associated with the blood vessels of many organs. In addition, vasculitis, hydropic degeneration of the media, hyalinization of capillaries and arterioles, development of hyaline thrombosis in small vessels, fibrinoid necrosis of blood vessels as well as necrosis of endothelial cells were reported (Alexander D.J., 2003; Alexander and Senne, 2008; Nakamura *et al.*, 2008).

Lymphoid System. Previous reports have described the histopathological changes in the immune tissues of chickens after infection with different strains of NDV. The microscopical lesions such as severe lymphoid depletion, necrosis and apoptosis in the spleen, bursa of Fabricius and thymus were reported (Brown et *al.*, 1999; Harrison *et al.*, 2011; Kommers *et al.*, 2001, 2002, 2003a, b; Wakamatsu *et al.*, 2006a, b). Hyperplasia of the mononuclear phagocytic cells in various organs, especially the liver,

may take place in sub acute infections. Focal vacuolation and destruction of lymphocytes may be seen in the cortical areas and germinal centers of the spleen and thymus. Marked degeneration of lymphocytes in the medullary region is seen in the bursa of Fabricius (Stevens *et al.*, 1976).

Intestinal Tract. Haemorrhage and necrosis of mucosal lymphoid tissue (cecal tonsils and Payer's patches) are seen in the intestinal tract with infections of some virulent forms of ND. Degeneration, necrosis and ulceration of lamina propria and submucosa were reported especially in the mucosa covering the lymphoid tissues. Degeneration and necrosis of glandular epithelium of gizzard, necrosis of lymphoid tissue in the proventriculus and proliferation of macrophage in lamina propria of intestines were also reported. Other lesions related to changes in the vascular system were described also in the digestive tract (Alexander and Senne, 2008; Nakamura *et al.*, 2008; Piacenti *et al.*, 2006).

Respiratory Tract. In the mucosa of the upper respiratory tract, congestion, edema, and dense cellular infiltration of lymphocytes and macrophages may be seen, particularly following aerosol exposure. Lesions may extend throughout the length of the trachea and cilia may be lost within two days of infection. All these lesions appear to clear rapidly. Congestion and edema of the parabronchi, haemorrhage and

erythrophagocytosis in the alveolar areas of the parabronchi as well as edema, cell infiltration and increase thickness and density of the air sacs may occur in chickens (Beard and Easterday, 1967; Cheville *et al.*, 1972). Ultrastructural changes in tracheal epithelium of day-old specific pathogen-free chicks vaccinated with the La Sota strain of NDV resulted in hypertrophy of goblet cells, their rupture, and the formation of excess mucus. Activated goblet cells peaked within 4 days postvaccination. Afterward, the activation levels gradually decreased. At the level of the ciliated cells, a marked increase in the proportion of nonciliated to ciliated cells and later an almost complete deciliation of the tracheal surface were observed because a simple squamous to cuboidal epithelium replaced the original pseudostratified epithelium. Fifteen days postvaccination, all epithelial damage was restored (Mast *et al.*, 2005).

Reproductive System. Histopathologicl changes in the reproductive tract of NDV infected bird are extremely variable. The uterus and shell-forming portions of the oviduct usually functionally damaged following NDV infection. Changes in female reproductive organs included follicle atresia and infiltration of inflammatory cells as well as formation of lymphoid aggregates in the follicles and oviduct were reported (Biswal and Morrill, 1954).

Pancreas. Necrotizing pancreatitis was reported as extensive degeneration, necrosis, and depletion of acinar cells in the pancreas with infiltration of inflammatory cells (Nakamura *et al*., 2008; Wakamatsu *et al*., 2006a).

Conjunctiva. Chickens infection with VVNDV either induce; mild lesions such as focal hyperplasia of the conjunctival epithelial cells and cellular infiltration in the lamina propria; moderate lesions such as vascular necrosis with congestion and haemorrhages, hyperplasia of conjunctival epithelial cells, edema and cellular infiltration in the lamina propria of the conjunctivae and fibrin thrombi in the capillaries of the lamina propria; or marked lesions in which the whole conjunctiva was affected with more severe and extensive lesions. The mesogenic and lentogenic NDV strains induce mild conjunctivitis without vascular necrosis (Nakamura *et al*., 2004).

Other Organs. Small focal areas of degeneration and necrosis, sometimes with haemorrhage, are seen in the liver, gall bladder and heart (Alexander and Senne, 2008).

NDV and apoptosis. Previous experiments *in vivo* and *in vitro* have shown that NDV triggers apoptosis (Kommers *et al*., 2002; Ravindra *et al*.,

2008a, b; 2009a, b). Apoptosis is a multistep, multipathway process that leads to cell death. It is important both in physiological and pathological states and relies on the activation of proteolytic caspases that ultimately lead to cytoplasmic denaturation, fragmentation of the nucleus and cell destruction without induction of inflammation (Robbins and Cotran, 2009).

10. Host immunity against NDV

10.1. Active immunity

Cell-Mediated Immunity. The initial immune response to infection with NDV is cell mediated, and may be detectable as early as 2~3 days after infection with live vaccine strains (Ghumman and Bankowski, 1976; Timms and Alexander, 1977). This has been thought to explain the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen (Allan and Gough, 1976; Gough and Alexander, 1973). However, a later study concluded that the cell-mediated immune response to NDV by it-self is not protective against challenge with virulent NDV (Reynolds and Maraqa, 2000).

Humoral Immunity. Antibodies capable of protecting the host can be measured by VN test. The response of VN test appears parallel to the haemagglutinin-inhibition tests (HI) response; however, the latter test is

frequently used to assess protective response, especially after vaccination (*Allan et al.*, 1978). Antibodies directed against either of the functional surface glycopolypeptides, the HN protein and the F polypeptides can neutralize NDVs (Russell, 1988). In fact, monoclonal antibodies specific for epitopes on the F polypeptide have been shown to induce greater neutralization than those directed against HN in *in vitro* and *in vivo* tests (Millar and Emmerson, 1988). When chickens survive NDV infection long enough, antibodies usually are detectable in the serum within 6~10 days. The levels largely depend on the infecting strain, but generally, peak response is at about 3~4 weeks. Haemagglutination-inhibition antibodies may remain detectable for up to one year in birds recovered from infection with mesogenic viruses or after a series of immunizations. Reinfection or immunization some weeks after the titer begins to decline produces a secondary response (Alexander and Senne, 2008; Allan *et al.,* 1978).

Local Immunity. Antibodies appear in secretions of the upper respiratory tract and intestinal tract of chickens at the same time that the humoral antibodies can be detected. In the upper respiratory tract, IgA with some IgG are the main immunoglobulins secreted (Parry and Aitken, 1977). Similar excretions occur in the Harderian gland following ocular, but not parenteral, infection (Parry and Aitken, 1977; Powell *et al.*, 1979). Eye-

drop vaccination with Hitchner B1 resulted in replication of virus in the Harderian gland, which could be prevented by the presence of maternal IgG in lachrymal fluid. Replication of virus in the Harderian gland resulted in the production of lachrymal IgG, IgA, and IgM (Russell, 1993). In particular, the Harderian gland became the main site for IgA-antibody-forming cells in the chicken (Russell and Koch, 1993). IgM may be the most actively antibody, involved in the clearance of virus in conjunctival infections (Russell and Ezeifeka, 1995).

10.2. Passive immunity

Hens with antibodies against NDV will pass these antibodies to their progeny via the egg yolk (Heller *et al.*, 1977). Levels of antibody in day-old chicks will be directly related to titers in the parent. Maternal immunity is protective and, thus, must be taken into account when timing the primary vaccination of chicks (Alexander and Senne, 2008).

10.3. Immunosuppression

The immune response has important effects on both the viral pathogenicity and the protection levels achieved by vaccination. Under natural conditions, immunosuppression may occur due to infection with other viruses such as infectious bursal disease virus. The subsequent

immunodeficiency may result in a more severe disease caused by some NDV strains as well as failure to respond adequately to vaccination (Faragher *et al.*, 1974; Giambrone *et al.*, 1976; Pattison and Allan, 1974; Rosenberger and Gelb, 1978). Immunosuppression from chicken infectious anemia virus also has been implicated in the failure of chickens to respond well to secondary inactivated NDV vaccine (Box *et al.*, 1988).

AIM OF THE STUDY
AND
WORK PLAN

AIM OF THE STUDY

To the best of the author's knowledge, this is the first histopathological comparative study between chickens, as a fully susceptible host for NDV infection, and ducks, as one of the natural reservoir of NDV. This study was initiated to compare the clinical signs, pathological lesions, and viral distribution in specific pathogen free chickens and commercial ducks, infected with the mutant 9a5b NDV isolate.

WORK PLAN

Table 1

The work plan of this study, chickens and ducks infected experimentally with the 9a5b NDV isolate, samples and diagnostic techniques

	Chickens		Ducks	
	Control	9a5b NDV infected	Control	9a5b NDV infected
Acclimatization	One week			
Infection	-	0 day	-	0 day
1 dpi*		3 chickens		3 ducks
2 dpi		3 chickens		3 ducks
4 dpi		3 chickens		3 ducks
6 dpi		3 chickens		3 ducks
8 dpi		3 chickens		3 ducks
10 dpi	5 chickens	3 chickens	5 ducks	3 ducks
Sampling	Spleen, bursa of fabricius, thymus, brain, eyelid, nostrils, larynx, trachea, lung, air sac, heart, liver, esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon, pancreas and kidney		HE IHC (NDV-NP)	
	Spleen, bursa of fabricius and thymus		IHC (caspase-3, IFN-β) TUNEL assay	
	Brain		IHC (GFAP)	
	Bursa of fabricius		TEM	
	Trachea		SEM	
	Lung		IHC (IFN-β)	
	Buffy coat layer		Toluidine blue Semi-thin section DNA agarose gel electrophoresis	

*dpi: days post infection.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Virus

The 9a5b NDV mutant isolate was obtained from the Department of Public Health, Tottori University, Japan (Shengqing *et al.*, 2002). After propagation in SPF eggs, 0.1ml of the viral suspension which, contain $10^{8.75}$ egg infective dose $(EID)_{50}$, was inoculated intranasally.

2. Chickens and Ducks

Twenty-three 32-day-old male white Leghorn SPF chickens and twenty-three 32-day-old male Japanese commercial ducks were obtained from the Nippon Institute for Biological Science and divided into 4 groups (Table 1). The first (n = 5) and the second (n = 18) groups comprised control and infected SPF chickens, respectively. The third (n = 5) and the fourth (n = 18) groups comprised control and infected Japanese commercial ducks, respectively. All groups were reared separately in bird-bred isolators (Fig. 3) in biohazard rooms and given water and food *ad libitum* and monitored clinically.

After a 1-week acclimatization period, the infected groups were inoculated intranasally with the virus (Fig. 4). Three birds from each infected group were slaughtered on the 1^{st}, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} day post-

inoculation (dpi). Control birds were slaughtered on the last day of the experiment (10^{th} dpi). To reduce the pain, the birds were slaughtered under Halothane inhalation anesthesia. The ethics committee on animal experiments from Tottori University approved the experimental protocol, and the experiments were carried out in accordance with the guidelines for animal experiments in the same facility.

3. Sampling and Necropsy

3.1. Buffy coat isolation and preparation for semi-thin section and DNA fragmentation test.

3.1.1. *Buffy coat isolation.* Birds were slaughtered and about 20 ml blood was collected in a sterile screw caped tube contain heparin anticoagulant (20 u/ ml) and the buffy coat layer was isolated using Histopaque®-1077 (Sigma-Aldrich, Louis, USA) according to manual instructions.

3.1.2. *Buffy coat preparation for semi-thin section.* About 200 ul from the isolated buffy coat was centrifuged in Eppendorf tube to make a pellet and the pellet was fixed by 2.5 % glutraldehyde overnight/ 4 °C and processed as described later.

3.1.3. *Buffy coat preparation for DNA fragmentation test.* DNA form the buffy coat layer was isolated using MasterPure™ DNA

Purification Kit for Blood Version II (epicentre®, Madison, WI, USA) according to the manual instructions.

3.2. Necropsy examination was performed immediately after the birds were killed. The spleen, bursa of fabricius, thymus, brain, eyelid, nostrils, larynx, trachea, lung, air sac, heart, liver, esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, cecum, colon, pancreas and kidney were collected and fixed in 10% neutral buffered formalin (NBF).

3.3. Samples from the trachea and bursa of fabricius from both chickens and ducks were taken and fixed in 2.5% glutaraldehyde for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) investigation, respectively.

4. Histopathology, Immunohistochemistry (IHC) and TUNEL assay

After 72 h of fixation in NBF, samples from the above-mentioned organs were dehydrated, embedded in paraffin wax and sectioned (3μm) for haematoxylin and eosin (HE) staining. Further sections (5μm/ positively charged slides) were subjected to IHC and TUNEL assay.

4.1. ***Detection of Newcastle Disease Virus-nucleoprotein (NDV-NP) by IHC***

To detect the NDV-NP by IHC, a primary antiserum was produced as described by Kommers *et al.* (2001). Briefly, a peptide antigen was

prepared with the sequence TAYETADESETRRIC, representing residues 181–194 of the NP protein with a C addition for coupling. The peptide was conjugated to keyhole limpet haemocyanin and this conjugate was used to immunize a rabbit. Serum was collected from the immunized rabbit and the immunoglobulin (Ig) G fraction was purified by affinity chromatography (Hokudo Bioscience, Tokyo, Japan).

For IHC, tissue sections were dewaxed and then treated with H_2O_2 3% in distal water (DW) for 15 min at room temperature (RT) in order to inactivate endogenous peroxidase. Antigen retrieval was performed by microwaving the sections for 10 min at full power (400 W) in citrate buffer solution (pH 5.4), followed by blocking with 10% normal goat serum (NGS) for 5 min with microwaving at low power (250 W) without heating. Tissues were then incubated with the primary antibody (1 in 8, 000 dilution) overnight at 4°C. After stringent washing, sections were incubated with a secondary polymer reagent (Dako ChemMate INVISION Kit/HRP [DAP], Dako, Carpinteria, California, USA) for 30 min at RT. After further washing, the substrate was added (3, 3'-diaminobenzidine; Dako) and the sections were finally

counterstained with haematoxylin and coveredslipped under DPX mounting medium (Sigma Life Scienc, Steinheim, Germany).

4.2. *Detection of Interferon-beta (IFN-β) by IHC*

To detect IFN-β, we follow the above-mentioned protocol with the following modifications: microwaving antigen retrieval was done for 20 min before H_2O_2 treatment, primary antibody (1 in 400 dilution) was polyclonal rabbit anti-human IFN- β (AbD Serotec, Oxford, UK), and weak wash was applied with this antibody.

4.3. *Detection of Caspase-3 antibody by IHC*

Caspase-3 was detected by use of anti-ACTIVE® caspase-3 polyclonal antibody (Promega, Madison, WI, USA) (1 in 350 dilution) and a similar protocol described for NDV-NP except that there was no blocking with NGS, detection was done with the polyclonal biotinylated goat anti-rabbit secondary antibody (Dako) and the enzyme-substrate reaction involved a streptavidin–biotin complex (Dako).

4.4. *Detection of glial fibrillary acidic protein (GFAP) by IHC*

To detect GFAP the following protocol was used: deparaffinization, hydration with tap water for 20 min, followed by treatment with 5 % H_2O_2 for 15 min and washing with DW. Antigen retrieval was done using proteinase K for 5 min/ RT. Washing by DW then by phosphate buffer saline (PBS). Blocking was done in microwave/ 5 min at power 3 (250 W) by using 5% bovine serum albumin. Tissues were incubated with the primary antibody (Polyclonal Rabbit Anti-Glial Fibrillary Acidic Protein, Dako, USA) (1 in 500 dilution) for 30 min/ RT. All the following steps were the same as described for NDV-NP antibody.

4.5. *Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL assay)*

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL assay) was used to evaluate apoptosis. The TACS® 2TdT-DAB *in-situ* apoptosis detection kit (TREVIGEN, Helgerman, Gaithersburg, USA) was used according to the manufacturer's instructions.

5. Scanning Electron Microscopy

Tracheal samples were processed for SEM by the following method.

1. Trimming:

   Cut the sample by a new razor blade (the size and shape differ from sample to other) on a colorless plastic plat with few drops of 2.5 % glutraldehyde (4 °C) on the sample.

2. Put the samples in small well-caped labeled bottle and fix overnight at 4 °C.
3. Washing in phosphate buffer (PB) 5 min/ 3 times at RT.
4. Postfixation in 1% Osmium Tetraoxide / 1 hour.
5. Washing in filtered DW or in 0.1M PB/ until clear fluid.
6. Dehydration in alcohol.
   a. Alcohol 50%, 70%, 80%, 90%, 95%, 99%/ 20 min each.
   b. Absolute alcohol I and II / 60 min each on vibrator.
7. Absolute alcohol: t-Butyl alcohol (1:1)/ 30 min at 30 °C in incubator.
8. t-Butyl alcohol/ 30 min at 30 °C in incubator.
9. t-Butyl alcohol (just cover the tissue)/ 30 min or overnight at 4 °C.
10. Drying in machine (VFD-21, t-BuFreeze Dryer).
11. Coating with gold or silver particles.
12. Examined or stored in well closed tubes with silica gel to absorb the moisture until examination.
13. Observation was made using an SEM (Hitachi Co. Ltd., Tokyo, Japan).

6. Transmission Electron Microscopy

Fresh bursal and previously fixed buffy coat samples were processed for TEM and semi-thin sections, respectively, by the following method:

1. Sampling.

   Cut small parts from the bursal tissue by a new razor blade (0.25 cm^2) on a colorless plastic plat and add a few drops of 2.5 % glutraldehyde (4 °C) on the sample and fix overnight in 2.5 % glutraldehyde (4 °C). From this point the bursal and the previously fixed buffy coat samples were processed by the same steps.

2. Trimming.

   Cut the samples into small pieces by a new razor blade (less than 0.1 cm^2) on a colorless plastic plat.

3. Labeling. Put the samples in small well-caped labeled bottle.
4. Washing. Wash in PB 5 min/ 3 times at RT.
5. Postfixation. Post fix in 1% Osmium Tetraoxide / 1 hour.
6. Washing. Wash in filtered DW or in 0.1M PB (until clear fluid).
7. Dehydration in alcohol.

   a. Alcohol 50%, 70%, 80%, 90%, 95% and 99% / 20 min each.

   b. Absolute alcohol I and II/ 60 min each/ on vibrator.

8. QY-I (n- Butyl glycidyl ether)/ 30 min/ on vibrator.

9. QY-II (Methyl glycidyl ether) / 40 min/ on vibrator.
10. QY-I: Epon (2: 1) / 30 min/ on vibrator.
11. QY-I: Epon (1: 2)/ 30 min/ on vibrator.
12. Epon (Quelol- 812)/ 60 min/ on vibrator.
13. Epon/ 60 min or overnight/ on vibrator.
14. Embedding in Epon.
15. Put the embedded samples at 60 °C for 3 days.
16. Store the hard embedded samples in closed tube with silica gel to absorb the moisture until ultrathin and semi-thin sections preparation and examination.
17. The bursal samples were observed by a TEM (Hitachi co. Ltd., Tokoy, Japan), and the buffy coat semi-thin sections were stained with toluidine blue stain and examined by light microscopy.

Preparation of Epon

Epon 812----- Quetol 812
DDSA---------- Dodecenyl Succinic Anhydride
MNA---------- Methyl Nadic Anhydryde
DMP-30-------- dimethylaminomethyl phenol
Mix the first 3 components together in paper cub and mix on magnetic stirrer for 5 min then add the fourth component (DM-30) and continue mixing on the stirrer for about 15 min and the color will changed from dark yellow to reddish.

7. Agarose Gel Electrophoresis

The concentration of the buffy coat layer extracted-DNA was measured by Nano-Drop spectrophotometer (Thermo Scientific, USA) and about 200 ng DNA in 10 ul Tris/Borate/EDTA (TBE) buffer was subjected to electrophoresis in 1.5% agarose gel. DNA was visualized using ethidium bromide under ultraviolet light.

RESULTS

RESULTS

1. Clinical Signs

Clinical signs appeared only in infected chickens. These were mild in severity and started from the 3 dpi and included sleepiness (Fig. 5) and greenish-white soft feces, and they peaked at the fourth and the 5 dpi, with symptoms such as depression, dyspnea, and oral discharges (Fig. 6). All of these symptoms decreased from the 6 dpi, and the chickens showed only slight depression on the last day of the experiment. No clinical signs were detected in the infected duck group or in any of the 2 control groups. No deaths were recorded in this experiment.

2. Gross lesions

The infected chickens had mild congestion and haemorrhages in the nasal mucosa (Fig. 7) and lung (Fig. 8), besides swelling of the 2 ceca. The gallbladder was swollen and engorged with bile (Fig. 9). The mucosa and the content of the digestive tracts were stained with the bile pigment (Fig. 10). The air sacs were slightly thick and opaque (Fig. 11). In addition, small haemorrhages were detected in the myocardium (Fig. 12) and cecal tonsils. Furthermore, the pancreatic white necrotic foci were the most prominent gross change by the 10 dpi (Fig. 13). On the other hand, the infected ducks basically had no gross lesions but distended ceca (Fig. 14) and engorged gall bladder were

observed. In addition, the content of the digestive tracts was stained with bile pigment. No gross lesions were detected in control chickens and ducks.

3. Histopathology, Immunohistochemistry and TUNEL assay

Histopathological and immunohistochemical findings are summarized in Tables 2, 3, 4, 5, 6 and 7. Differences between chickens and ducks infected experimentally with 9a5b NDV isolate are described below.

3.1. Spleen

Apoptosis and lymphoid depletion were the main histopathological features in the splenic tissues in both chickens and ducks after 9a5b NDV isolate infection as mentioned in Table 2. The distribution and intensity of labelling of apoptotic cells in the splenic tissues is summarized in Table 3. In the infected chickens, expression of caspase-3 within the spleen was strongest in the peri-ellipsoidal white pulp (PWP), peri-ellipsoidal lymphoid sheath (PELS) and peri-arteriolar lymphoid sheath (PALS) at 1 dpi (Fig. 15 & 16). By 4 dpi a large number of apoptotic macrophages and lymphoid cells were detected by HE staining (Fig. 17, 18 & 19) and TUNEL assay (Fig. 20) in the PWP, PELS, PALS and peri-venous lymphoid sheath (PVLS), as well as in the red pulp (RP). Regeneration of the white pulp was observed by 6 dpi, and the number of apoptotic cells

started to decrease. In the infected ducks, caspase-3 expression within the spleen at 1 dpi was strongest in the germinal centers (GCs) and present in the PWP (Fig. 21 & 22). Apoptotic bodies were numerous by HE staining (Fig. 23) and TUNEL assay (Fig. 24) in the GCs from 2 to 8 dpi. In addition, large number of apoptotic cells was detected in the PWP and RP. By 8 dpi, regeneration of the white pulp was observed with a decreased number of apoptotic cells.

The degree of lymphoid depletion in the spleen is summarized in Table 4. Lymphoid depletion of splenic white pulp was moderate in the infected chickens (Fig. 18) and mild in the infected ducks (Fig. 23).

Expression of NDV-NP in the spleen is summarized in Table 5. In the infected chickens, NDV-NP was detected mainly in lymphocytes and apoptotic bodies as well as in cells morphologically compatible with macrophages. The most intense labelling for NDV-NP was in the PWP, PELS and the RP. Peak NDV-NP expression was at 4 dpi (Fig. 25). In the infected ducks, NDV-NP was detected only in cells morphologically compatible with macrophages and these cells were mainly found in the RP (Fig. 26).

The degree of IFN-β expression in the splenic tissues is summarized in Table 7. Histologically, IFN-β expression mainly detected in cells that

morphologically compatible with macrophages. In the infected chickens, IFN-β-positive cells were rare at 1 dpi, sparse at 2 dpi (Fig. 27) and peaked at 4 dpi. In the infected ducks, IFN-β expression was stronger and more intense in the spleen at 1 and 2 dpi (Fig. 29) than in the respective samples of chicken spleen.

3.2. Bursa of Fabricius

In the infected chickens, few signals for caspase-3 were detected in the bursal tissue at 1 dpi, and there was no detectable difference in HE- and TUNEL-stained samples between the infected and control chickens (Table 3). A few apoptotic macrophages were detected by TEM in the bursa of the infected chickens (Fig. 31). In the infected ducks, apoptotic cells in the cortex, medulla and follicle-associated epithelium (FAE) were detected at 1 dpi by caspase-3 IHC (Fig. 32) and later by TUNEL assay (Fig. 33) and HE staining (Fig. 34). Apoptosis in the bursal tissue of the infected ducks was present at 1 dpi and did not regress until the end of the experiment.

The degree of lymphoid depletion of the bursa of Fabricius is summarized in Table 4. Lymphoid depletion of the bursa was moderate in the infected chickens and it was mild in the infected ducks.

Expression of NDV-NP in the bursa of Fabricius is summarized in Table 5. In the infected chickens, few NDV-NP signals were detected in cells morphologically compatible with lymphocytes, macrophages and reticular cells, and in the FAE (Fig. 35). NDV-NP was not detected in the bursa of the infected ducks.

Table 7 shows that, IFN-β expression was slightly increased in infected chickens. Immunohistochemically, IFN-β mainly detected in macrophage- and fibroblast-like cells (Fig. 36). Neither the infected ducks nor the control ducks had expression of IFN-β in bursal tissue.

3.3. Thymus

The thymus of infected and control chickens had few cells labelled for caspase-3 and positive by the TUNEL assay (Table 3). In infected ducks, numerous signals for caspase-3 labelling were detected in the thymic cortex, corticomedullary junction and medulla at 1 dpi (Fig. 37). Apoptotic cells disappeared from the cortex, but were present at the corticomedullary junction (Fig. 38) and were associated with the Hassall's corpuscles, which increased in size and number.

Lymphoid depletion of the thymus is summarized in Table 4. This was severe in infected chickens and the thymic cortex was completely lost (Fig.

39). Only slight lymphoid depletion was detected in the thymus of infected ducks (Fig. 40).

Expression of NDV-NP in the thymus is summarized in Table 5. Rare positive signals were detected in cells morphologically compatible with macrophages in the thymic medulla of chickens (Fig. 41) and ducks (Fig. 42). There was no IFN-β expression in the thymic tissue of birds from any group.

3.4. Brain

Brain lesions were observed in the infected chickens only. Slight nonsuppurative lymphocytic encephalitis was detected by the 4 dpi and the most affected parts were the cerebellum and medulla oblongata. Degeneration and mild loss of Purkinje cells (Fig. 43 & 44) as well as vacuolation and demyelination in the cerebellar white matter (Fig. 45) were detected. In addition, focal areas of microgliosis and lymphocytic perivascular cuffing in the cerebrum, cerebellum, and medulla oblongata were also observed (Fig. 44 & 46). On the other hand, we did not observe any lesion in the infected duck brain tissues (Fig. 47 & 48). NDV-NP antigen could not be detected in the brain tissues of all groups by IHC.

Numerous reactive astrocytes were observed in brain sections stained for GFAP in both chickens and ducks after 9a5b NDV infection (Fig. 49 & 50). In chickens, GFAP-positive astrocytes were observed in the cerebellum, midbrain and medulla oblongata as well as in the cerebrum. These changes were more prominent in areas of inflammatory response and neuronal loss. GFAP expression was increased in the granular layer and around the Purkinje cell layer at 6 and 8 dpi in the infected chickens (Fig. 49/ A2 & A3). A few GFAP-positive cells and cell processes were detected in the cerebellar molecular layer, including the Bergmann-glia, at 10 dpi (Fig. 49/ A4). 9a5b NDV infection in chickens, intensively increased GFAP expression in cerebellar white matter (Fig. 49/ B2, B3 & B4), midbrain (Fig. 49/ C2, C3 & C4), and medulla oblongata (Fig. 49/ D2, D3 & D4). The distribution of GFAP expression in control duck is different than control chickens, while Bergmann-glia is GFAP-positive in control ducks; it appears negative in control chickens. Additionally, GFAP expression in ducks is concentrated around the Purkinje cell layer but in chickens it centered in the granular layer of cerebellum (Fig. 49/ A1 & 50/ A1).

In turn, the brain tissues of infected duck express GFAP less than infected chickens. 9a5b NDV infection in ducks slightly increased GFAP expression at 6 and 8 dpi in cerebellum (Fig. 50/ A2 & A3), cerebellar

white matter (Fig. 50/ B2 & B3), midbrain (Fig. 50/ C2 & C3), and medulla oblongata (Fig. 50/ D2 & D3) despite there is no lesions were detected by HE stain. By the 10 dpi, GFAP expression was slightly decreased in the previously mentioned brain regions (Fig. 50/ A4, B4, C4 & D4). The presence of GFAP-positive cells in the brain of the negative control groups was within normal limits.

3.5. Conjunctiva

Although lymphocytic conjunctivitis with heterophilic infiltration was mild in chickens and slight in ducks, the NDV-NP antigen could not be detected in both.

3.6. Respiratory System

The infected chickens had moderate lymphocytic rhinitis with slight haemorrhages, deciliation, and degeneration and necrosis of epithelial cells (Fig. 51) with associated NDV-NP-immunopositive signals in the epithelial cells and mononuclear cells at the same site (Fig. 52). Moderate lymphocytic laryngitis and mild tracheitis with loss of cilia and erosion were detected in the infected chickens with associated NDV-NP-immunopositive signals in the epithelial cells and mononuclear cells (Fig. 53 & 54). On the other hand, slight lymphocytic rhinitis with proliferation

of goblet cells and heterophilic infiltration was observed in the infected ducks (Fig. 55), and the NDV-NP-immunopositive signals detected in the epithelial cells and mononuclear cells (Fig. 56) were less intensive than those of chicken. Despite the NDV-NP-immunopositive signals were detected in both larynx and trachea of the infected ducks (Fig. 57 & 58), laryngitis and tracheitis were milder than those of chickens. Hyperplasia of goblet cells and infiltration of heterophils, lymphocytes, and macrophages-like cells were prominent in laryngeal and tracheal mucosa of the infected ducks.

The infected chickens had moderate lymphocytic interstitial pneumonia, which was characterized by slight haemorrhages, proliferation of periparabronchial lymphoid tissues, and infiltration of macrophages, lymphoid cells, and few heterophils (Fig. 59) with associated NDV-NP antigen in macrophage-like cells and epithelial cells (Fig. 60). Furthermore, moderate airsacculitis was developed at the late stage of the experiment. On the other hand, mild lymphocytic interstitial pneumonia, which was characterized by heterophilic infiltration and proliferation of periparabronchial and air passages submucosal lymphoid tissues, was developed in the infected ducks (Fig. 61). NDV-NP positive signals were detected in macrophage-like cells and epithelial cells of the infected duck's

lung tissues (Fig. 62). Additionally, slight airsacculitis was developed at the late stage of the infection.

The degree of IFN-β expression is summarized in Table 7. Weak IFN-β-immunopositive signals were detected in the lung tissues of the infected chickens, especially in the epithelial cells of the parabronchi (Fig. 63). On the other hand, IFN-β-immunopositive staining in the infected ducks was detected in cells morphologically consistent with fibroblasts and macrophages in lung tissues, especially in the air passages subepithelial tissues and in the epithelial cells of the parabronchi (Fig. 65), and it was more intensive than those of chickens as described in Table 7.

Tracheal Ultrastructural Changes

Infected chickens had excessive secretion of globular mucous particles on the epithelial surface at the 1 dpi (Fig. 68). By the 4 dpi, a marked increase in the proportion of non-ciliated to ciliated epithelium was observed, and disorientation of the cilia as well as adherence of the cilia to each other and to the underlying surface was seen. Deciliation of large parts of the tracheal surface and erosions in the mucosal epithelial cells were also detected (Fig. 69). In addition, scanty mucous was found on the tracheal surface as well as deciliation of large parts were detected by the 10

dpi (Fig. 70). In turn, the infected ducks had mild globular mucous particles at the 1 dpi (Fig. 72). Slight disorientation of the cilia, few globular mucous particles and fine mucous network on the cilial tips were detected by the 4 dpi (Fig. 73) and blankets of mucous were covering the underlying epithelium by the 10 dpi (Fig. 74). Ultrastructural features of tracheal mucosa in both control chickens and ducks were within normal limits (Fig. 67 & 71).

3.7. Heart

The heart lesion, which developed in the infected chickens only, was mild and appeared at the 1 dpi as scattered areas of lymphocytic myocarditis. By the 4 dpi, degeneration of myofibers, slight haemorrhages, and heterophilic infiltration were detected (Fig. 75) with associated NDV-NP-immunopositive macrophages and lymphocytes in myocardium (Fig. 76). These reactions were slightly increased by the 6 and the 8 dpi and the subsided by the 10 dpi as shown in Table 2.

3.8. Liver

The infected chickens had proliferated ectopic lymphoid tissues by the 2 dpi. By the 4, 6 and 8 dpi, the bile canaliculi and the portal bile ducts were dilated and engorged with bile (Fig. 77 & 78) with associated NDV-NP-

immunopositive macrophages and Kupffer cells in the hepatic tissues (Fig. 79) as well as NDV-NP-immunopositive macrophages and epithelial cells in the mucosa of bile duct at the 10 dpi (*inset* of fig. 78). In turn, in the infected ducks, mild proliferations of ectopic lymphoid tissues and moderate heterophilic migration in hepatic sinusoids were observed (Fig. 80).

3.9. Digestive Tract

The infected chickens had slight esophagitis and mild haemorrhagic proventriculitis with infiltration of lymphocytes, macrophages, and heterophils in the mucosa and lamina propria. In addition, infiltration of macrophages and lymphocytes in the mucosa and tunica muscularis of the gizzard, mild duodenitis with scattered areas of degeneration and necrosis in the duodenal mucosa (Fig. 81) was detected with associated NDV-NP-immunopositive macrophages and epithelial cells in the same site (Fig. 82). Furthermore, mild jejunitis, proliferation of lymphoid and goblet cells in the ileum mucosa, proliferation of cecal tonsils with slight haemorrhages (Fig. 83), and proliferation of lymphoid tissues of the colon lamina propria were also found. NDV-NP-immunopositive signals were detected in many parts of the digestive tract (Table 6). These signals were detected mainly in

macrophages and epithelial cells throughout the digestive tract as well as in the cecal tonsils (Fig. 84).

On the other hand, in the infected ducks, hyperplasia of goblet cells, proliferation of lymphoid cells, and heterophilic infiltration in the mucosa and submucosa of the digestive tract were observed. In addition, the mitotic figures were increased in the epithelial cells of mucosal gland throughout the digestive tract (Fig. 85 & 86). NDV-NP-immunopositive signals in the digestive tract of the infected ducks were less frequent and fainter than those of chickens, as described in Table 6 and as shown in macrophage-like cells between duodenal glands (*inset* of fig. 86).

3.10. Pancreas

Only in the infected chickens was the pancreatic lesion developed. Slight necrotizing pancreatitis was observed at the 1 dpi (Fig. 87) and dramatically increased at the 6 dpi and not regress until the end of the experiment (Fig. 88), with associated NDV-NP-immunopositive signals in necrotic pancreatic acinar cells (Fig. 89). In the infected ducks (Fig. 90) and both control groups, apparently normal pancreatic histological features were observed until the end of this experiment.

3.11. Kidney

Proliferations of the ectopic lymphoid tissues were observed in the infected chickens at the 2 dpi, whereas tubulointerstitial nephritis was detected by the 6 dpi and increased in severity until the end of the experiment (Fig. 91) with associated NDV-NP-immunopositive staining in the degenerated renal tubules (*inset* of Fig. 91). On the hand, renal tissues of the infected ducks had slight proliferation of ectopic lymphoid tissue (Fig. 92). The histological features in both control chickens and ducks were within the normal limits, and no NDV-NP-immunopositive signals were detected in these groups.

4. Semi-thin section and Agarose gel electrophoresis

4.1. Semi-thin section of buffy coat layer

In toluidine blue stained section, a few apoptotic cells were detected in infected chickens' buffy coat cell especially at 4 dpi (Fig. 93). In control groups as well as in the infected ducks (Fig. 94), apoptotic bodies cannot be recognized in buffy coat semi-thin sections.

4.2. Agarose gel electrophoresis of buffy coat-DNA

The stained buffy coat-DNA patterns are shown in Fig. 95 & 96. DNA extracted from the control chicken showed a smear throughout the lane

(Fig. 95/ cont.). DNA extracted from 9a5b NDV infected chicken at 1, 2 and 4 dpi showed a smear with some weak bands (Fig. 95/ 1 dpi, 2 dpi & 4 dpi) indicating mild internucleosomal DNA fragmentation which is characteristic feature of apoptosis. By the 6 dpi until the 10 dpi the DNA extracted from infected chickens showed a smear throughout the lanes as the control group (Fig. 95/ 6 dpi, 8 dpi & 10 dpi). DNA extracted from the control and infected ducks showed a smear throughout the lanes as shown in Figure 96.

5. Control groups

NDV-NP was not detected by IHC at any tissue of birds of the control group. Apoptotic cells were rare in the tissues of the control groups, except in duck bursal tissues (Table 3). Lymphoid depletion was not observed in the immune tissues of the control groups. Rare positive signals for IFN-β were detected in the bursa and spleen of control chickens and ducks, respectively. The histological features in the other tissues were within the normal limit.

Table 2
Histopathological findings in both chickens and ducks infected experimentally with the 9a5b NDV isolate [a]

Organs	Infected chickens	Infected ducks
Spleen	Marked apoptosis and moderate lymphoid depletion associated with little heterophilic infiltration (2~6 dpi)*	Marked apoptosis, mild lymphoid depletion associated with heterophilic infiltration (4~6 dpi)
Bursa	Moderate lymphoid depletion (4~8 dpi)	Mild lymphoid depletion and marked apoptosis (4~10 dpi)
Thymus	Sever lymphoid depletion with heterophilic infiltration (6~10 dpi)	Slight lymphoid depletion with heterophilic infiltration (8~10 dpi)
Brain	Slight nonpurulent encephalitis (6~10 dpi)	No significant changes
Eye lid	Mild conjunctivitis (4~6 dpi)	Slight conjunctivitis (6~8 dpi)
Nostrils	Moderate lymphocytic rhinitis with deciliation, degeneration and necrosis of epithelial cells (2~4 dpi)	Slight lymphocytic rhinitis with heterophilic infiltration (1~2 dpi)
Larynx	Moderate lymphocytic laryngitis (2~4 dpi)	Infiltration of lymphocytes/ macrophages in the mucosa (2~6 dpi)
Trachea	Mild tracheitis with deciliation of some parts (4~8 dpi)	Proliferation of goblet and lymphoid cells in the mucosa (4~6 dpi)
Lung	Moderate interstitial pneumonia (2~8 dpi)	Mild interstitial pneumonia (2~8 dpi)
Air sacs	Moderate airsacculitis (8~10 dpi)	Slight airsacculitis (8~10 dpi)
Heart	Multifocal areas of mild lymphocytic myocarditis (6~8 dpi)	No significant changes
Esophagus	Slight esophagitis (4~8 dpi)	Proliferation of lymphoid aggregates in lamina propria (2~10 dpi)
Proventriculus	Mild proventriculitis with heterophilic infiltration (2~6 dpi)	Infiltration of macrophages and lymphoid cells (4~6 dpi)
Gizzard	Mild infiltration of macrophages and lymphocytes in mucosa and tunica muscularis (6~8 dpi)	Slight heterophilic infiltration in tunica mucosa (2~6 dpi)
Duodenum	Mild duodenitis (2~6 dpi)	Heterophilic infiltration and increase mitotic figures (4~6 dpi)
Jejunum	Mild jejunitis (4~6 dpi)	Proliferation of lymphoid cells in the mucosa (P4~ P8)
Ileum	Proliferation of mucosal lymphoid and goblet cells (4~6 dpi)	Proliferation of macrophage, goblets cells, and increase mitotic figures in mucosal epithelium (2~8 dpi)
Cecum	Mild haemorrhagic tonsillitis (4~10 dpi)	Proliferation of the cecal tonsils (2~10 dpi)
Colon	Slight proliferation of lymphoid cells in lamina propria (4~10 dpi)	As chickens plus heterophilic infiltration and increased mitotic figures in epithelium (4~8 dpi)
Liver	Marked proliferation of ectopic lymphoid tissue, bile pigment in dilated bile canaliculi (4~8 dpi)	Mild proliferation of ectopic lymphoid tissue with heterophilic infiltration (4~8 dpi)
Pancreas	Severe necrotizing pancreatitis (6~10 dpi)	No significant changes
Kidney	Marked proliferation of ectopic lymphoid tissue, multifocal areas of necrotizing interstitial nephritis (8~10 dpi)	Slight proliferation of ectopic lymphoid tissue (4~10 dpi)

[a] NDV, Newcastle disease virus; * (X~Y dpi) means the peak period of histological findings; dpi, days post infection.

Table 3

Distribution and intensity of apoptotic cells in the immune organs of both chickens and ducks infected experimentally with the 9a5b NDV isolate

		Chickens											Ducks										
		Spleen						Bursa			Thymus		Spleen						Bursa			Thymus	
		PELS	PWP	PALS	PVLS	GC	RP	C	M	FAE	C	M	PELS	PWP	PALS	PVLS	GC	RP	C	M	FAE	C	M
1 dpi	Caspase-3[a]	+++	++++	+++	++	+/-	+	+	+	+	-	+	+	+++	+/-	+/-	++++	+	+++	+++	+	+++	++
	TUNEL[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	++	++	+	+	+
	HE[c]	+	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	+/-	+/-	++	+	+	+	+/-	+/-	+/-
2 dpi	Caspase-3	++	+++	++	+	+/-	+	-	-	-	-	+	+	++	+	+/-	++	+	+/-	+/-	+/-	++	++
	TUNEL	++	+++	+++	++	+	+++	+	+	+	+	+	+	+	+	+	++++	++	++	++	+	++	++
	HE	+	++	+	+	+/-	++	+/-	+/-	+/-	+/-	+/-	+/-	+	+	+	+++	+	++	++	+/-	+	+
4 dpi	Caspase-3	+	+	+	+	+/-	+/-	-	-	+/-	-	+	-	+	+/-	+	+/-	+/-	-	-	-	++	+
	TUNEL	+++	++++	+++	++++	+	++++	+	+	+	+	+	+	++	++	++	++++	++	+++	+++	+	++	++
	HE	++	+++	++	+++	+/-	+++	+/-	+/-	+/-	+/-	+/-	-	+	+	+	+++	+	++	++	+/-	++	+
6 dpi	Caspase-3	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	+	-	+/-	-	-	+/-	+/-	-	-	-	-	-
	TUNEL	++	++++	++	++	+	+++	+	+	+	+	+	+	+++	+	+	++++	+++	+++	+++	++	+++	+++
	HE	+	+++	+	+	+/-	++	+/-	+/-	+/-	+/-	+/-	-	++	+/-	+/-	++	++	++	+++	+/-	++	++
8 dpi	Caspase-3	-	+/-	-	-	-	-	-	-	-	-	+	-	-	-	-	+/-	-	-	-	-	-	-
	TUNEL	++	+++	+++	++	+	++	+	+	+	+	+	+	++	+	+	++++	++	+++	+++	+	++	++
	HE	+	++	++	+	+/-	+	+/-	+/-	+/-	+/-	+/-	-	+	+/-	+/-	+++	+	+++	+++	+/-	++	+
10 dpi	Caspase-3	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	TUNEL	+	+++	++	++	+	+	+	+	+	+	+	+	+	+	+	++	+	+++	+++	+	(+++)*	
	HE	+/-	++	+	+	+/-	+	+/-	+/-	+/-	+/-	+/-	-	+	+/-	+/-	+	+/-	+++	+++	+/-	(++)*	
Cont.	Caspase-3	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
	TUNEL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	+	+	+
	HE	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	+/-	+/-	+	+/-	+	+	+/-	+/-	+/-

PELS, peri-ellipsoidal lymphoid sheath; PWP, peri-ellipsoidal white pulp; PALS, peri-arteriolar lymphoid sheath; PVLS, peri-venous lymphoid sheath; GCs, germinal centers; RP, red pulp; C, cortex; M, medulla; FAE, follicle associated epithelium; dpi, days post infection; Cont., control group; a, caspase-3 immunohistochemistry (IHC); b, TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling; c, HE, haematoxylin and eosin.

[a, b] - = no labelling ; +/- = rare positive cells; + = few positive cells; ++ = positive cells seen, < 50% of all high-power field (HPF); +++ = positive signal seen in 50 to 75% of HPF; ++++ = abundant positive signals in > 75% of HPF.

[c] Condensed or fragmented cells: - = no cells; +/- = rare cells; + = few cells; ++ = frequent cells; +++ = regular clusters of condensed or fragmented cells.

* Thymic corticomedullary junction.

Table 4

The degree of lymphoid depletion* in the immune organs of both chickens and ducks infected experimentally with the 9a5b NDV isolate

	Chickens			Ducks		
	Spleen	**Bursa**	**Thymus**	**Spleen**	**Bursa**	**Thymus**
1 dpi	-	-	-	-	-	-
2 dpi	+/-	-	+/-	-	-	-
4 dpi	++	+/-	+	+/-	+/-	-
6 dpi	+	++	++	+	+	-
8 dpi	+	++	+++	+	+	+/-
10 dpi	+	+	+++	+/-	+	+/-
Cont.	-	-	-	-	-	-

dpi, days post infection.

The degree of lymphoid depletion: - = no; +/- = slight; + = mild; ++ = moderate; +++ = severe.

Table 5

Distribution and intensity of NDV-NP immunolabelling* in the immune organs of both chickens and ducks infected experimentally with the 9a5b NDV isolate

	Chickens											Ducks										
	Spleen						Bursa			Thymus		Spleen						Bursa			Thymus	
	PELS	PWP	PALS	PVLS	GC	RP	C	M	FAE	C	M	PELS	PWP	PALS	PVLS	GC	RP	C	M	FAE	C	M
1 dpi	+/-	+/-	-	-	-	-	+/-	+/-	+/-	-	+/-	-	+/-	+/-	+/-	+/-	+	-	-	-	-	-
2 dpi	+	+	-	-	-	+/-	+/-	+/-	+/-	-	+/-	-	+/-	+	+	+/-	++	-	-	-	-	+/-
4 dpi	++	+++	++	+	+	++	+/-	+/-	+/-	-	+/-	+/-	+	+	+	+/-	+++	-	-	-	-	+/-
6 dpi	++	+++	+	+	+/-	++	+	+	+	-	+/-	-	+/-	+	+	-	++	-	-	-	-	+/-
8 dpi	-	+	+	-	-	+	+	+	+	-	+/-	-	-	+/-	+/-	-	+	-	-	-	-	-
10 dpi	-	+/-	+/-	-	-	+/-	+/-	+/-	+/-	-	+/-	-	-	+/-	+/-	-	+/-	-	-	-	-	-
Cont.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PELS, peri-ellipsoidal lymphoid sheath; PWP, peri-ellipsoidal white pulp; PALS, peri-arteriolar lymphoid sheath; PVLS, peri-venous lymphoid sheath; GCs, germinal centers; RP, red pulp; C, cortex; M, medulla; FAE, follicle associated epithelium; dpi, days post infection; Cont., control group.

*- = no labelling; +/- = rare positive cells; + = few positive cells; ++ = frequent positive cells; +++ = regular clusters of positive cells.

Table 6
Immunolabellings* for Newcastle disease viral nucleoprotein antigen by IHC in both chickens and ducks infected experimentally with the 9a5b NDV isolate

Organs	Infected chickens						Infected ducks					
	1 dpi	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi	1 dpi	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi
Brain	-	-	-	-	-	-	-	-	-	-	-	-
Eye lid	-	-	-	-	-	-	-	-	-	-	-	-
Nostrils	+++	+++	+++	++	++	+	+	+	+	+	+	+
Larynx	-	-	+	+	+	+	+	+	+	-	-	-
Trachea	-	-	+	+	+	+	-	+	+	-	-	-
Lung	+	++	++	++	++	+	+	+	+	+	+	+
Air sacs	-	-	+	+	+	+	-	-	-	-	+	+
Heart	-	-	+	+	+	-	-	-	-	-	-	-
Esophagus	-	-	-	-	-	-	-	-	-	-	-	-
Proventriculus	-	-	+	+	+	+	-	-	-	+	+	-
Gizzard	-	-	+	+	++	+	-	-	-	-	-	-
Duodenum	+	+	++	+	++	++	+	+	+	+	+	+
Jejunum	+	+	+	+	+	+	-	-	+	+	+	+
Ileum	+	+	++	+	++	++	-	-	+	-	-	-
Cecum	+	+	++	+	++	++	-	-	+	-	-	-
Colon	-	-	+	+	+	+	-	-	-	-	+	+
Liver	-	-	+	+	+	+	-	-	-	-	-	-
Pancreas	-	-	-	-	++	++	-	-	-	-	-	-
Kidney	-	-	-	-	-	+	-	-	-	-	-	-

dpi, days post infection.

* - = no labelling; + = rare positive cells; ++ = frequent positive cells; +++ = regular clusters of positive cells.

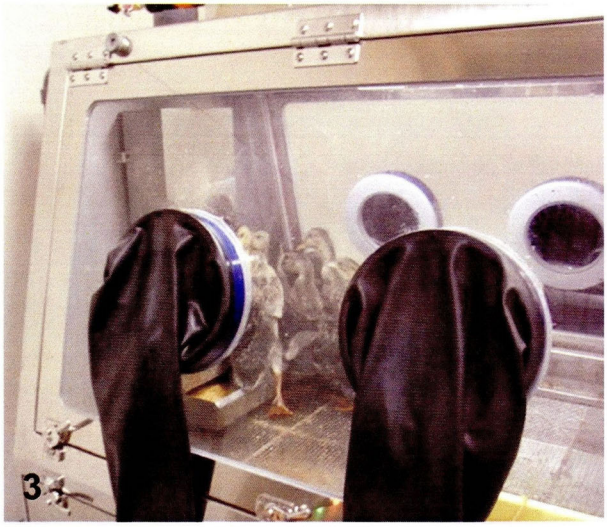
Table 7

Immunolabelling* for IFN-β antibody in the immune organs and lung of both chickens and ducks infected experimentally with the 9a5b NDV isolate

	Chickens				Ducks			
	Spleen	**Bursa**	**Thymus**	**Lung**	**Spleen**	**Bursa**	**Thymus**	**Lung**
1 dpi	+	+	-	+/-	++	-	-	+
2 dpi	++	+/-	-	+	+++	-	-	+
4 dpi	+++	+/-	-	+	+/-	-	-	++
6 dpi	+	+	-	ND	-	-	-	ND
8 dpi	+	+/-	-	ND	-	-	-	ND
10 dpi	+	+/-	-	ND	-	-	-	ND
Cont.	-	+/-	-	-	+/-	-	-	-

dpi, days post infection.

*- = no labelling; +/- = rare positive cells; + = few positive cells; ++ = frequent positive cells; +++ = regular clusters of positive cells; ND = not determined.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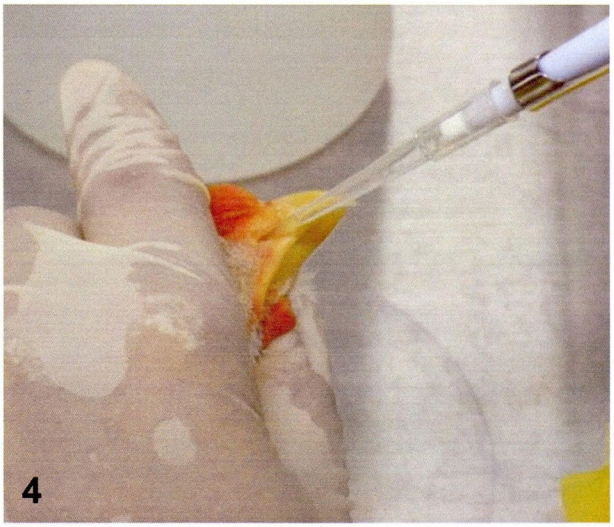
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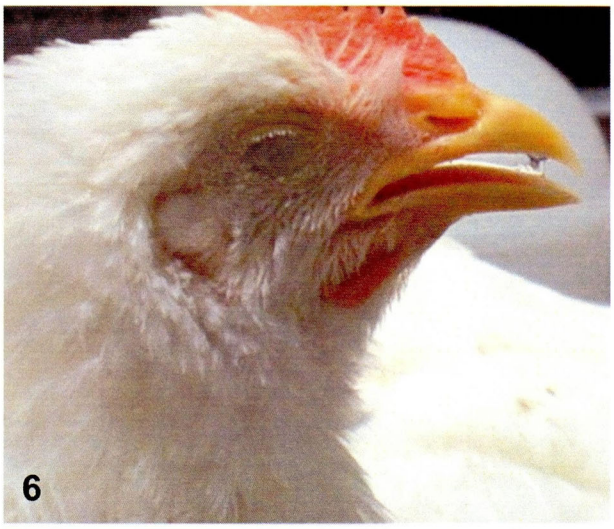
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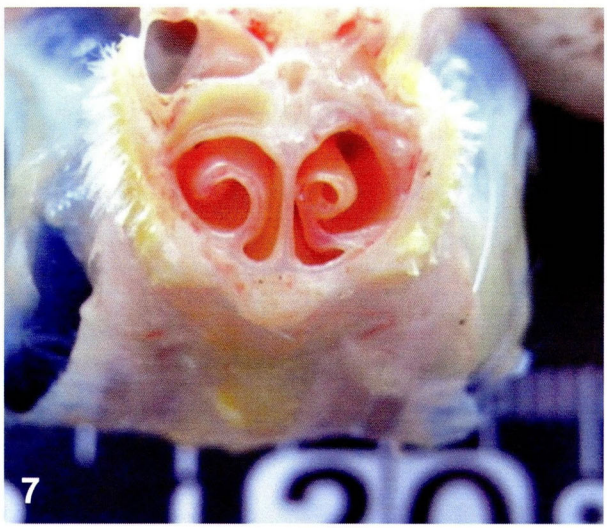
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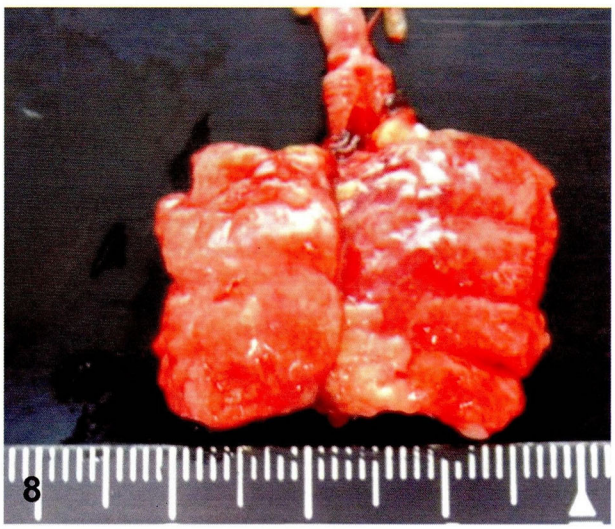
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Fig. 3. Grouping of birds in a bird-bred isolator.

Fig. 4. Intranasal inoculation of the virus suspension.

Fig. 5. Chickens; 3 dpi. Depression and sleepiness.

Fig. 6. Chickens; 5 dpi. Depression, dyspnoea, and oral discharges.

Fig. 7. Nostrils; chickens, 4 dpi. Mild congestion and haemorrhages in the nasal mucosa.

Fig. 8. Lung; chickens, 4 dpi. Mild congestion and haemorrhages.

Fig. 9. Gallbladder; chickens, 4 dpi. The gallbladder swollen and engorged with bile.

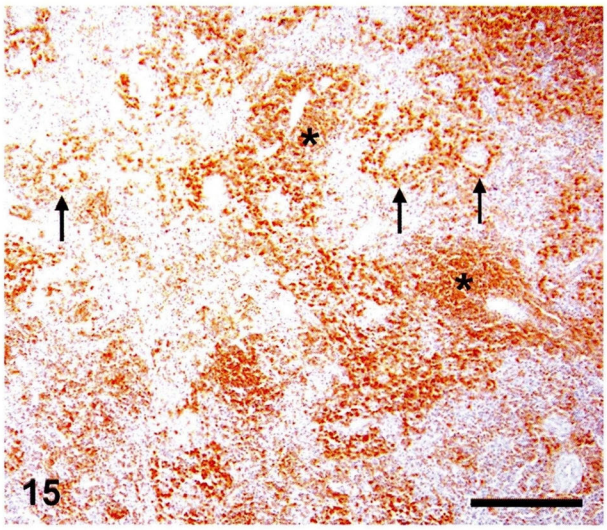
Fig. 10. Gizzard; chickens, 4 dpi. The mucosa and the content of the digestive tracts were stained with the bile pigment.

Fig. 11. Air sacs; chickens, 8 dpi. The air sacs were slightly thick and opaque.

Fig. 12. Heart; chickens, 8 dpi. Small haemorrhages in the myocardium.

Fig. 13. Pancreas; chickens, 10 dpi. White necrotic foci in the pancreas.

Fig. 14. Ceca; Ducks, 8 dpi. Distended ceca.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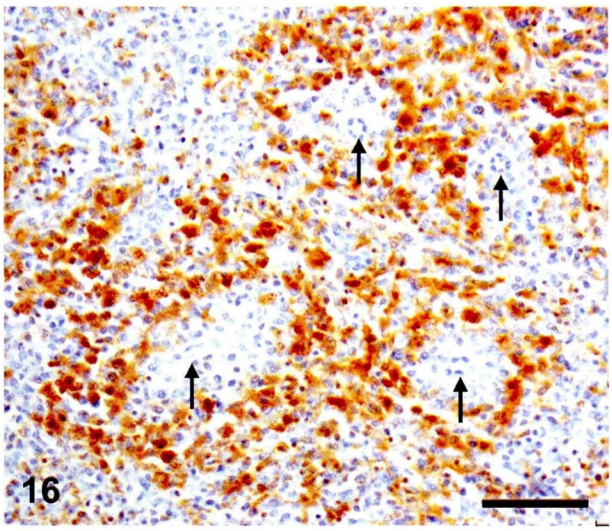
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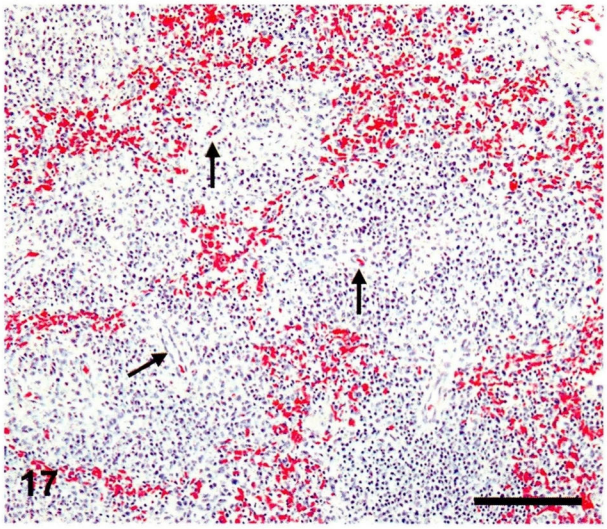
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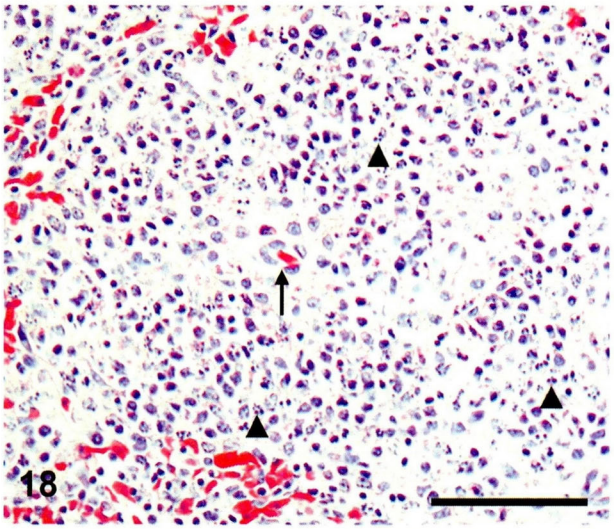
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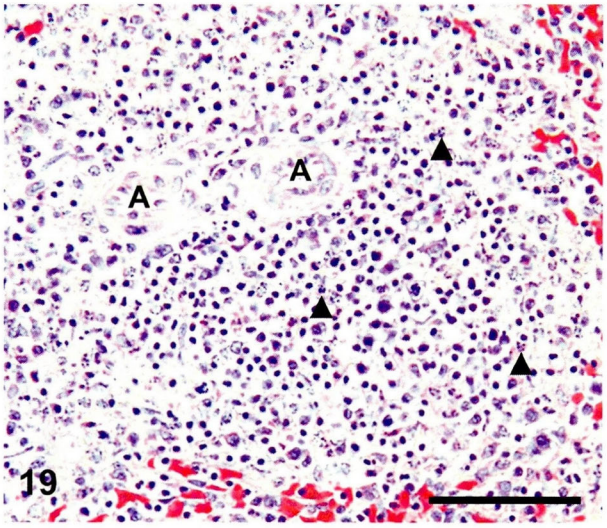
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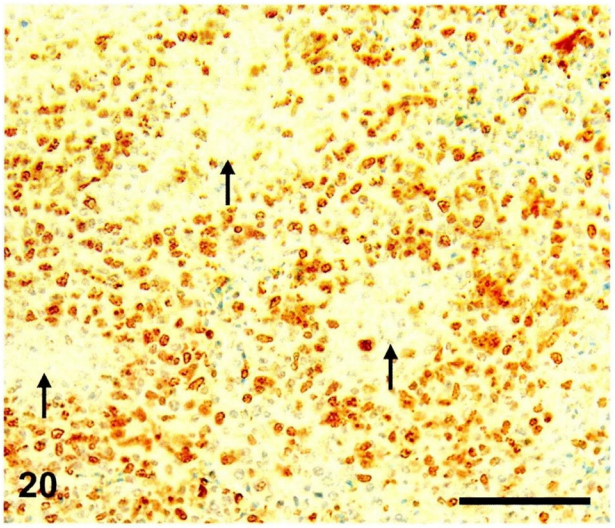
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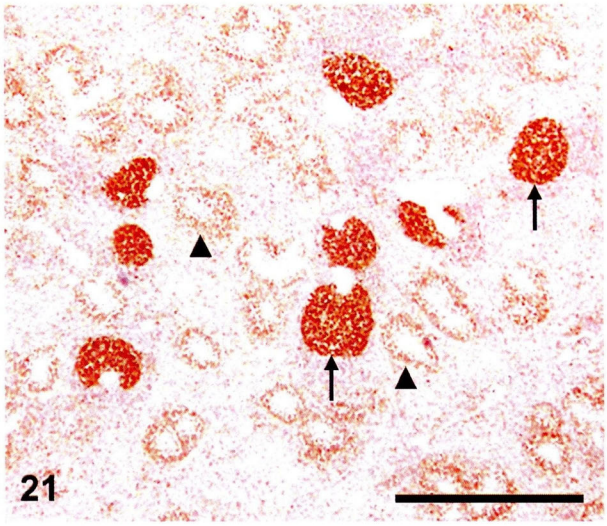
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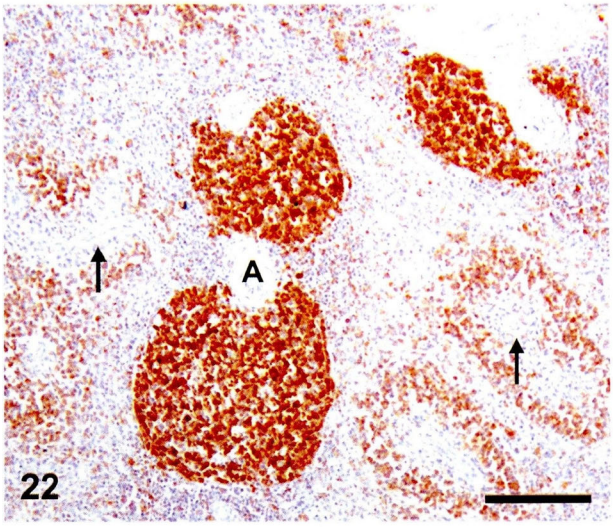
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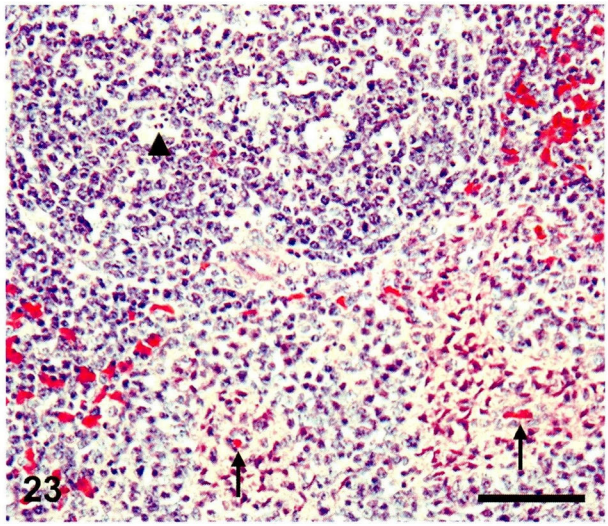
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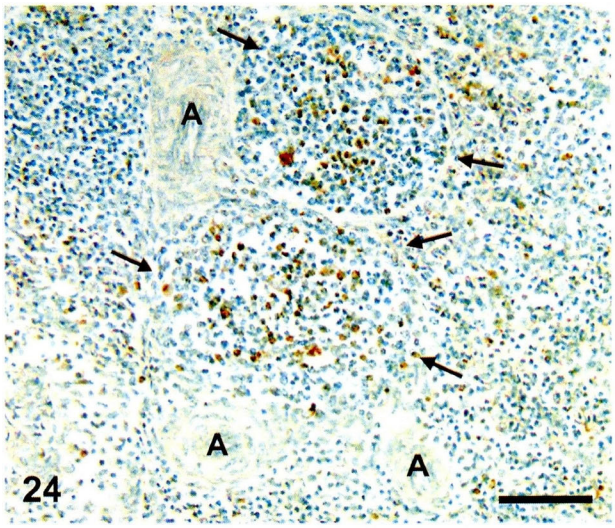
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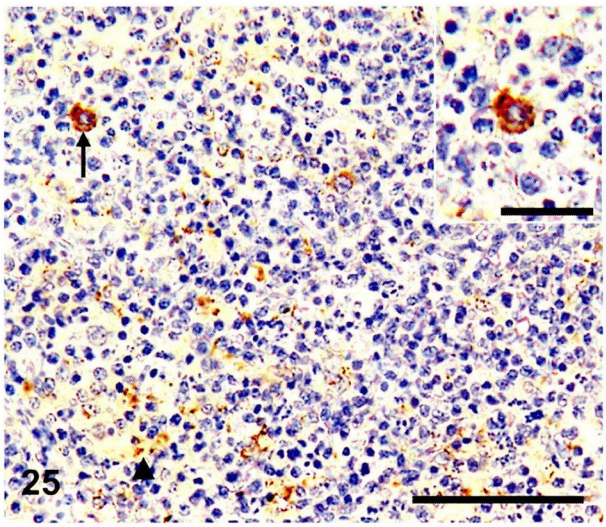
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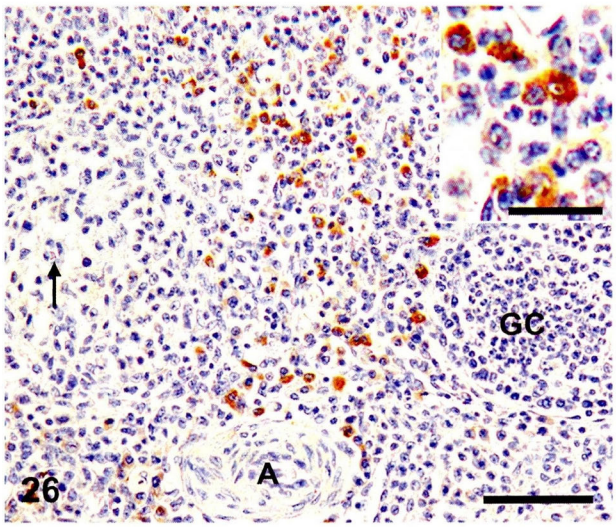
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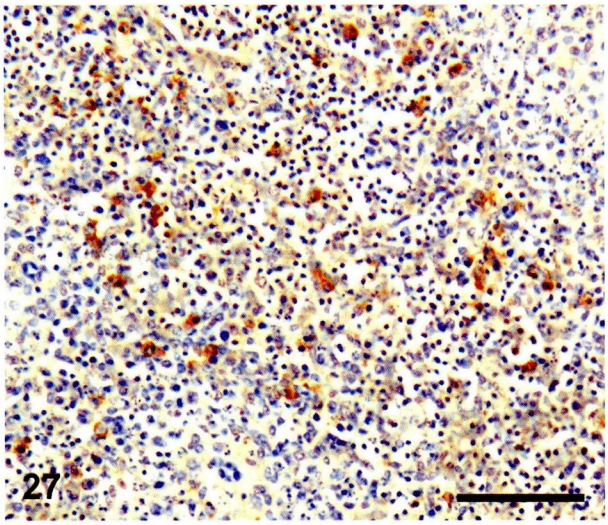
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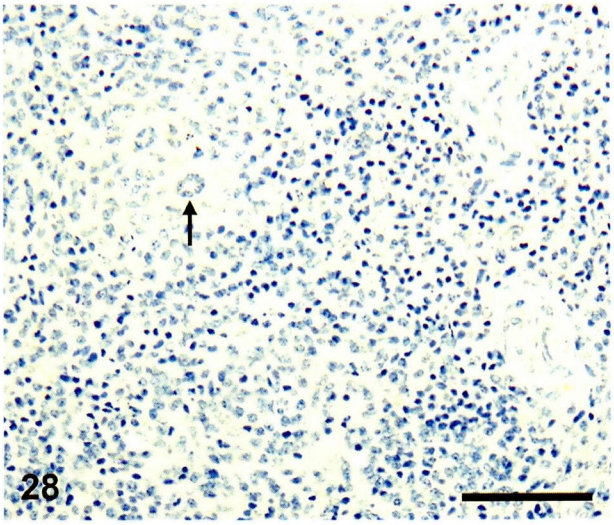
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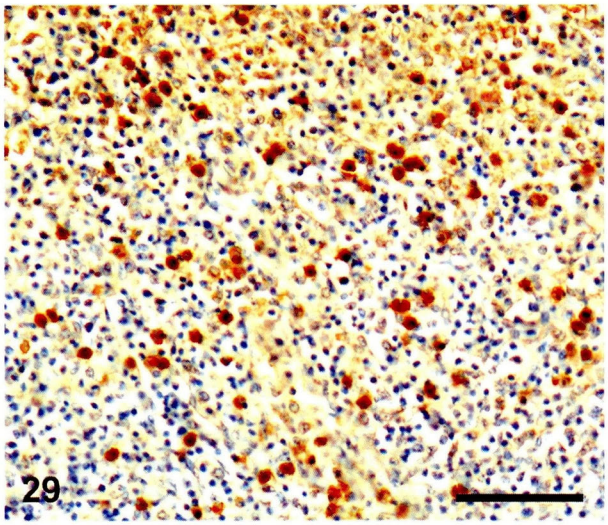
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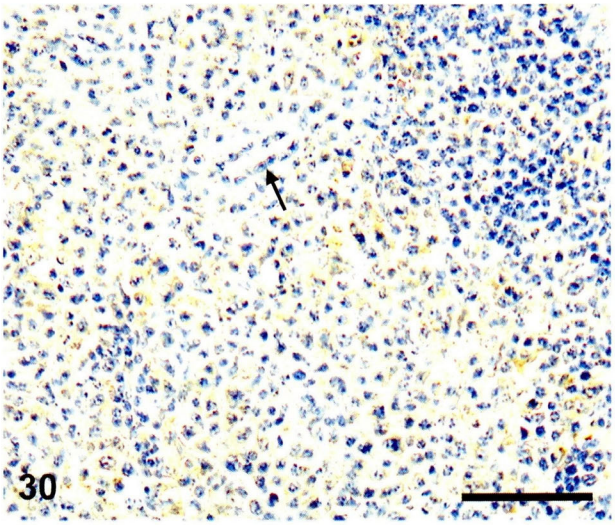
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Fig. 15. Spleen; chicken, 1 dpi. There was intensive immunolabelling of caspase-3 in the cells of peri-ellipsoidal lymphoid sheath (PELS), peri-ellipsoidal white pulp (PWP) (arrow) and peri-arteriolar lymphoid sheath (PALS) (asterisk). IHC, haematoxylin counterstained. Bar, 200 μm.

Fig. 16. Spleen; chicken, 1 dpi. There was intensive immunolabelling of caspase-3 in the cells of PELS and PWP. Arrows indicate penicilliform capillaries. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 17. Spleen; chicken, 4 dpi. Lymphoid depletion of white pulp with a huge number of apoptotic bodies in white and red pulps. Arrow indicates penicilliform capillaries. HE. Bar, 100 μm.

Fig. 18. Spleen; chicken, 4 dpi. Lymphoid depletion of white pulp with numerous apoptotic bodies (arrowhead). Arrow indicates a penicilliform capillary. HE. Bar, 50 μm.

Fig. 19. Spleen; chicken, 4 dpi. Numerous apoptotic bodies in the PALS (arrowhead). HE. Bar, 50 μm.

Fig. 20. Spleen; chicken, 4 dpi: There was intensive labelling of apoptotic cells in the PELS and PWP. Arrows indicate penicilliform capillaries. TUNEL assay. Bar, 50 μm.

Fig. 21. Spleen; duck, 1 dpi. There were intensive immunolabellings of caspase-3 in the germinal centres (GC) (arrow) and weak signals in the white pulp (arrowhead). IHC, haematoxylin counterstained. Bar, 500 μm.

Fig. 22. Spleen; duck, 1 dpi. There was intensive immunolabelling of caspase-3 in the GC and weak labelling in the cells of peri-ellipsoidal lymphoid sheath and peri-ellipsoidal white pulp. Arrows refer to penicilliform capillaries; A, artery. IHC, haematoxylin counterstained. Bar, 100 μm.

Fig. 23. Spleen; duck, 4 dpi. Slight lymphoid depletion around the penicilliform capillary (arrow) and numerous apoptotic cells in the GC (arrowhead). HE. Bar, 50 μm.

Fig. 24. Spleen duck, 6 dpi. There was intensive labelling of apoptotic cells in the GC (arrow). A; artery. TUNEL assay. Bar, 50 μm.

Fig. 25. Spleen; chicken, 4 dpi. There was intense labelling of NDV-NP in cells morphologically compatible with macrophages (arrow) and in lymphocytes and apoptotic bodies (arrowhead). IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

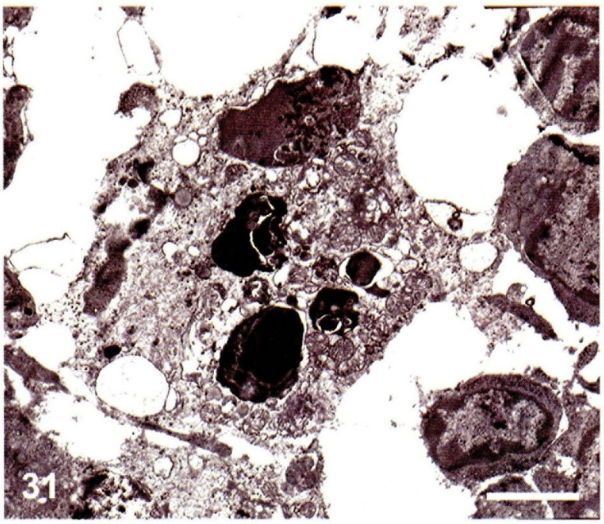
Fig. 26. Spleen; duck, 4 dpi. There were intensive immunolabellings of NDV-NP in cells morphologically compatible with macrophages in the red pulp. Arrow indicates a penicilliform capillary. A, artery; GC, germinal centre. IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

Fig. 27. Spleen; chicken, 2 dpi. There was immunolabelling for IFN-β in cells morphologically compatible with macrophages. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 28. Spleen; chicken, control. Control chickens did not show immunolabelling for IFN-β in the splenic tissues. Arrow indicates a penicilliform capillary. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 29. Spleen; duck, 1 dpi. There was intensive immunolabelling for IFN-β in cells morphologically compatible with macrophages. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 30. Spleen; duck, control. Rare immunolabelling signals for IFN-β were detected in the splenic tissues of the control ducks. Arrow indicates a penicilliform capillary. IHC, haematoxylin counterstained. Bar, 50 μm.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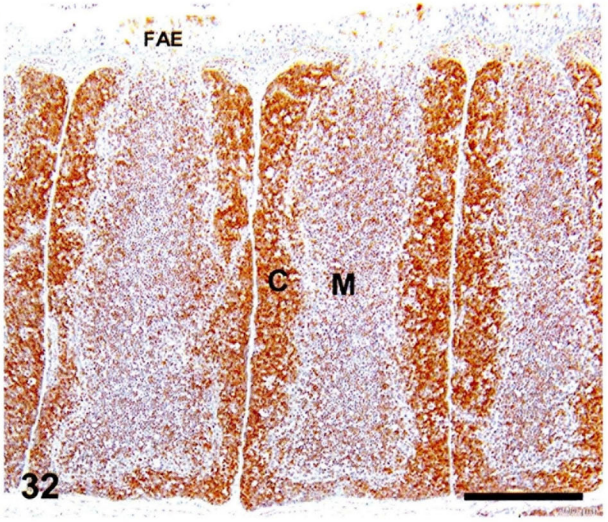
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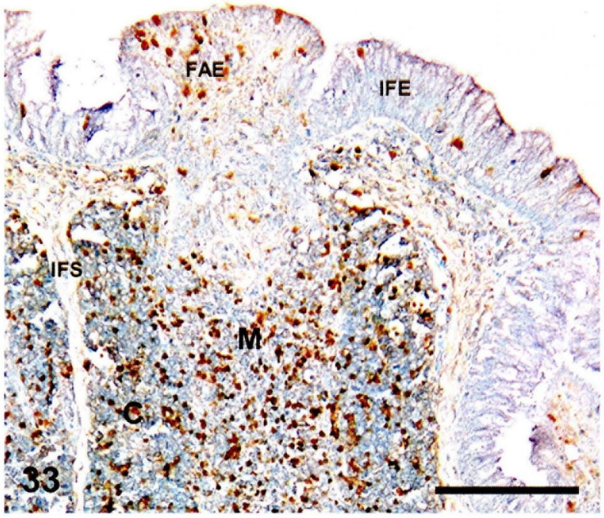
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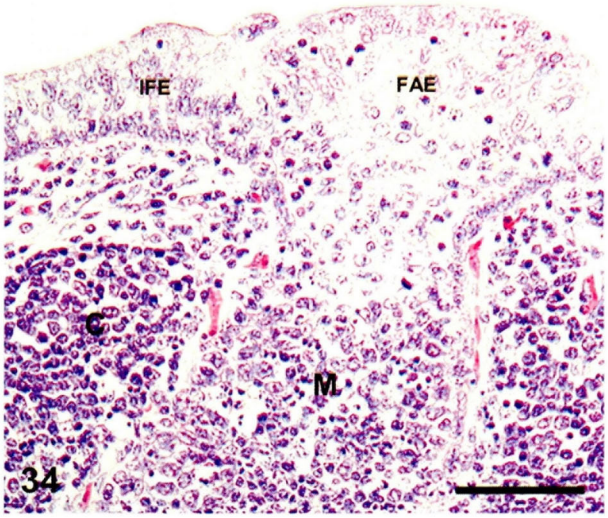
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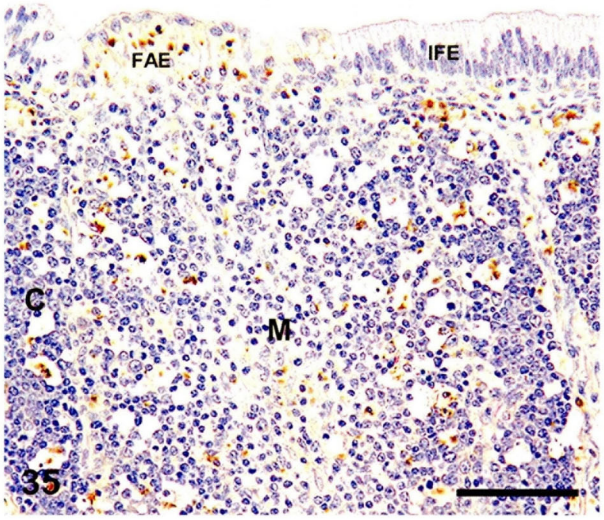
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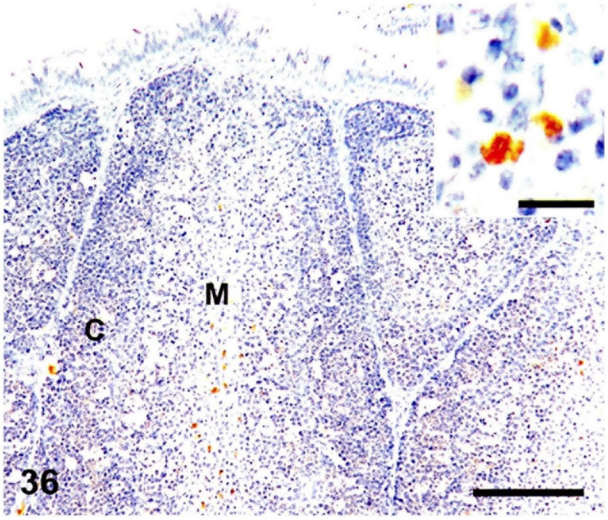
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Fig. 31. Bursa; chicken, 6 dpi. Nucleolar fragmentation in an apoptotic macrophage. TEM. Bar, 3 μm.

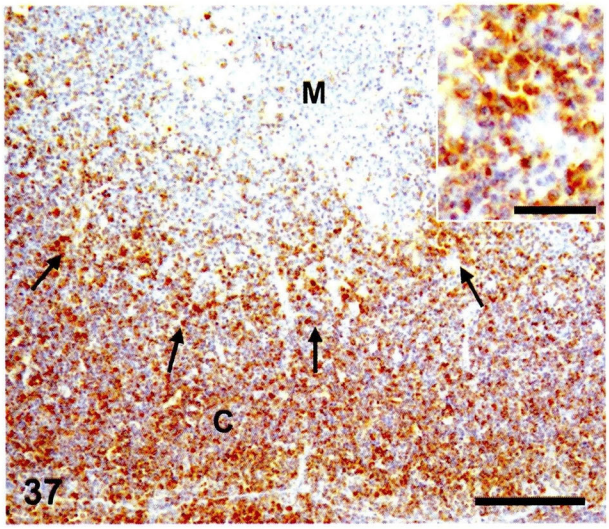
Fig. 32. Bursa; duck, 1 dpi. There was intense immunolabelling of caspase-3 in the cortex (C), medulla (M) and in follicle-associated epithelium (FAE). IHC, haematoxylin counterstained. Bar, 200 μm.

Fig. 33. Bursa; duck, 6 dpi. There were intense signals for apoptosis in the cortex (C), medulla (M) and in follicle-associated epithelium (FAE). TUNEL assay. IFE, interfollicular epithelium; IFS, interfollicular septum. Bar, 100 μm.

Fig. 34. Bursa; duck, 8 dpi. Numerous apoptotic bodies in the cortex(C), medulla (M) and in follicle-associated epithelium (FAE). HE. Bar, 50 μm.

Fig. 35. Bursa; chicken, 8 dpi. There was immunolabelling for NDV-NP in the cortex (C), medulla (M), and in follicle-associated epithelium (FAE). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 36. Bursa; chicken, 1 dpi. There was immunolabelling for IFN-β in macrophage- and fibroblast-like cells in the medulla (M). IHC, haematoxylin counterstained. Bar, 100 μm (inset, 15).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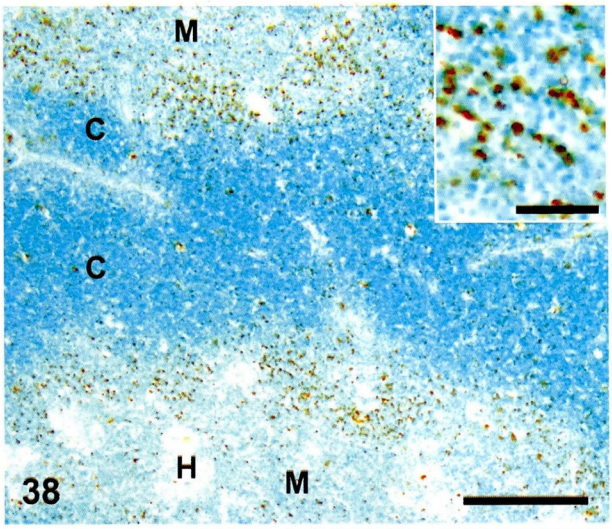
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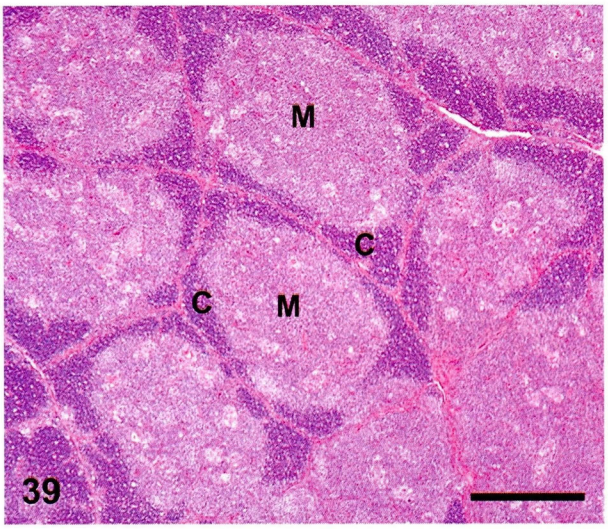
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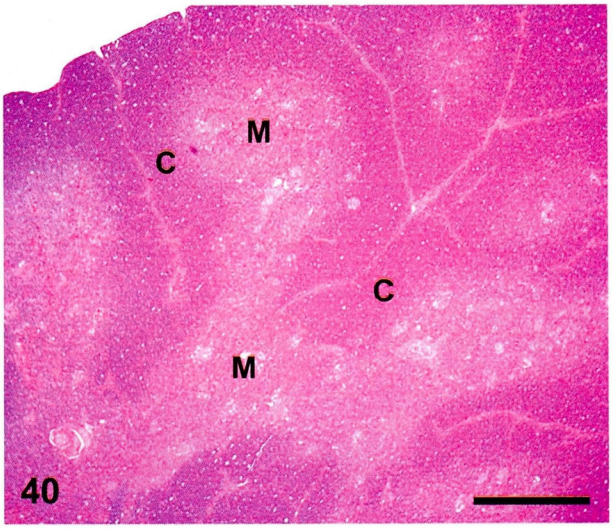
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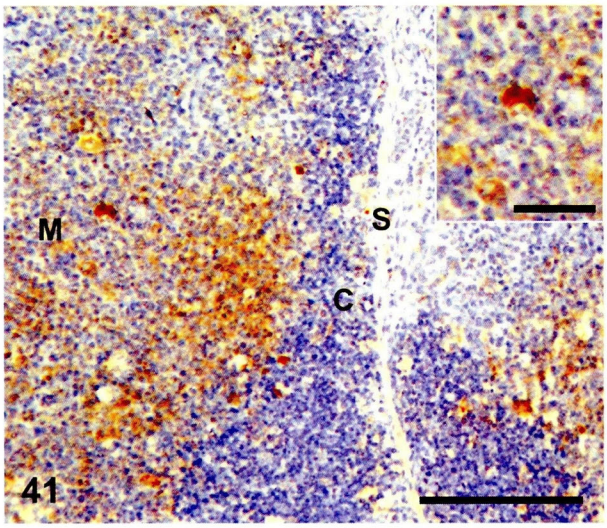
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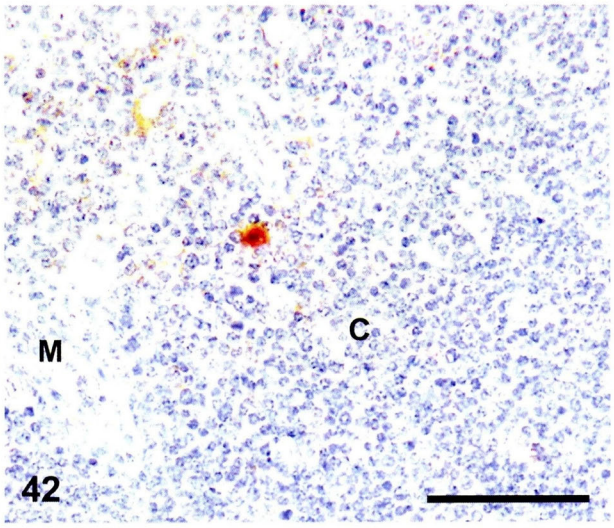
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Fig. 37. Thymus; duck, 1 dpi. There was immunolabelling of caspase-3 in cells morphologically compatible with lymphocytes and macrophages at the corticomedullary junction (arrows). IHC, haematoxylin counterstained. C, cortex; M, medulla. Bar, 100 μm (inset, 30 μm).

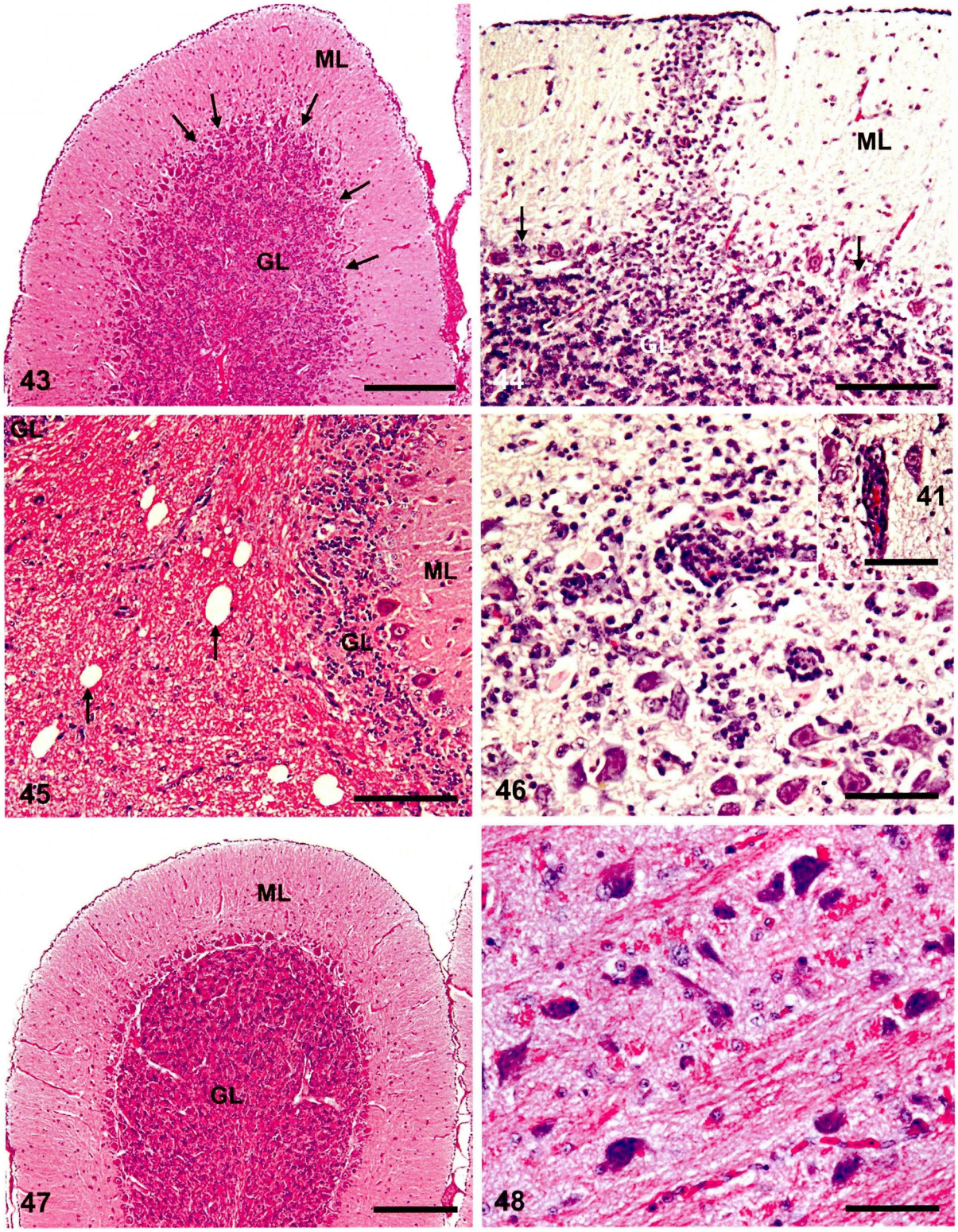
Fig. 38. Thymus; duck, 10 dpi. There were intense signals for apoptosis at the corticomedullary junction. C, cortex; M, medulla; H, Hassall's corpuscles. TUNEL assay. Bar, 200 μm (inset, 30 μm).

Fig. 39. Thymus; chicken, 8 dpi. Severe lymphoid depletion of the chicken thymus. C, cortex; M, medulla. HE. Bar, 500 μm.

Fig. 40. Thymus; duck, 8 dpi. Slight lymphoid depletion of the duck thymus. C, cortex; M, medulla. HE. Bar, 500 μm.

Fig. 41. Thymus; chicken, 8 dpi. There was immunolabelling of NDV-NP in cells morphologically compatible with macrophages. C, cortex; M, medulla; S, septum. IHC, haematoxylin counterstained. Bar, 100 μm (inset, 30 μm).

Fig. 42. Thymus; ducks, 4 dpi. There was immunolabelling of NDV-NP in cells morphologically compatible with macrophages. C, cortex; M, medulla. IHC, haematoxylin counterstained. Bar, 50 μm.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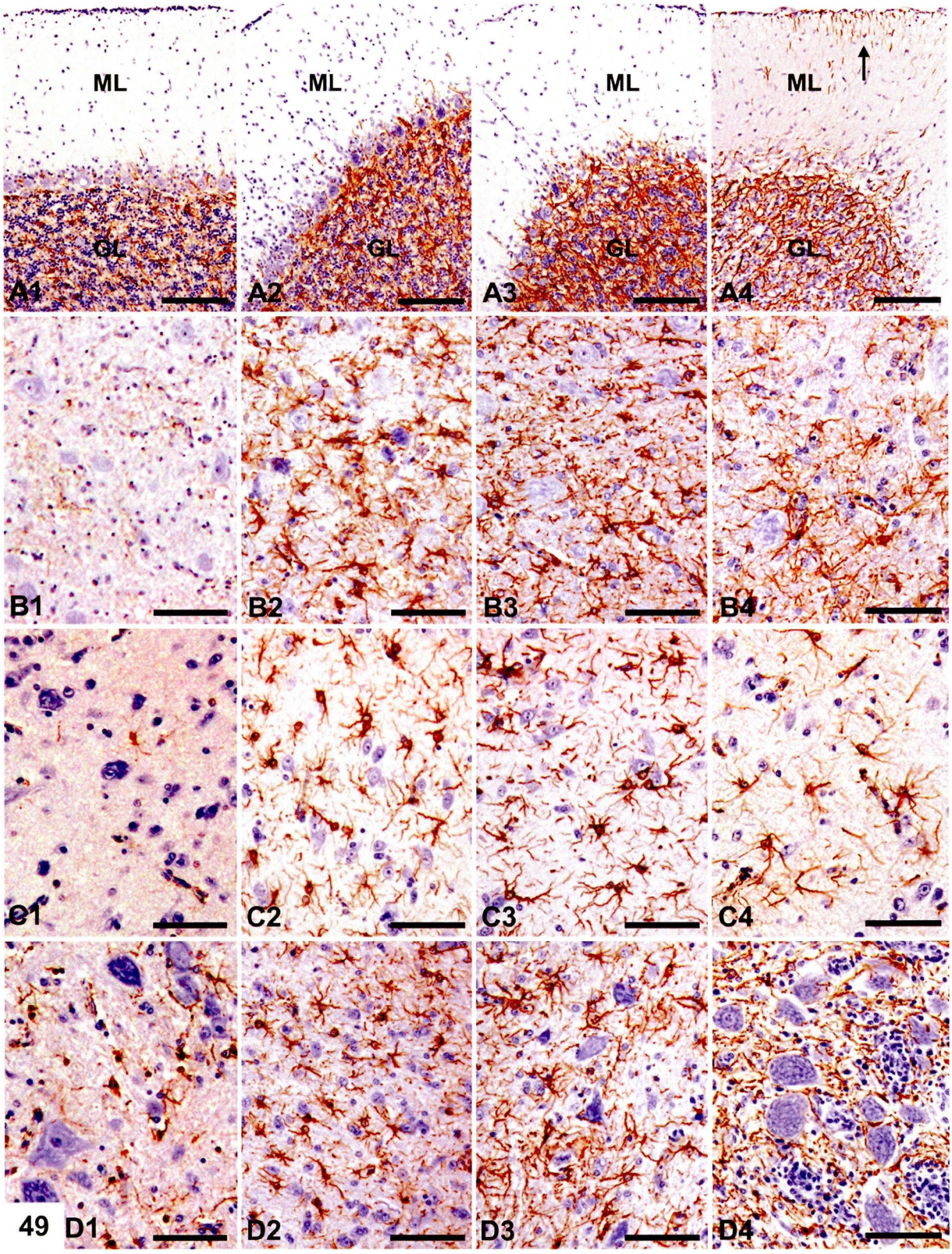
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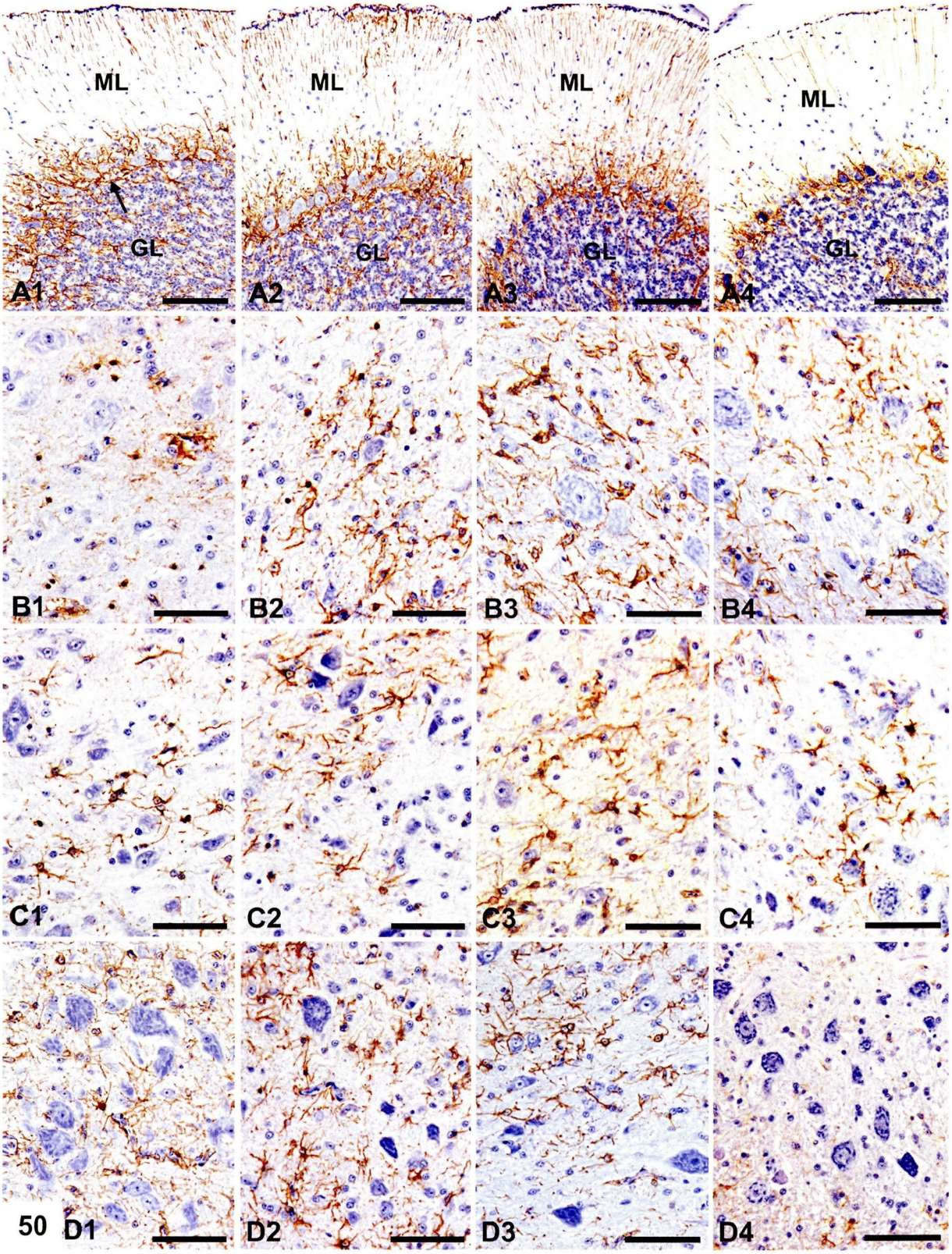
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Fig. 43. Cerebellum; chicken, 8 dpi. Mild loss of Purkinje cells (arrows). ML, molecular layer; GL, granular layer. HE. Bar, 200 μm.

Fig. 44. Cerebellum; chicken, 8 dpi. Degenerative changes in Purkinje cells (arrows) and focal microgliosis in the molecular layer. ML, molecular layer; GL, granular layer. HE. Bar, 100 μm.

Fig. 45. Cerebellar white matter; chicken, 10 dpi. Vacuolation and demyelination in the cerebellar white matter (arrow). GL, granular layer. HE. Bar, 100 μm.

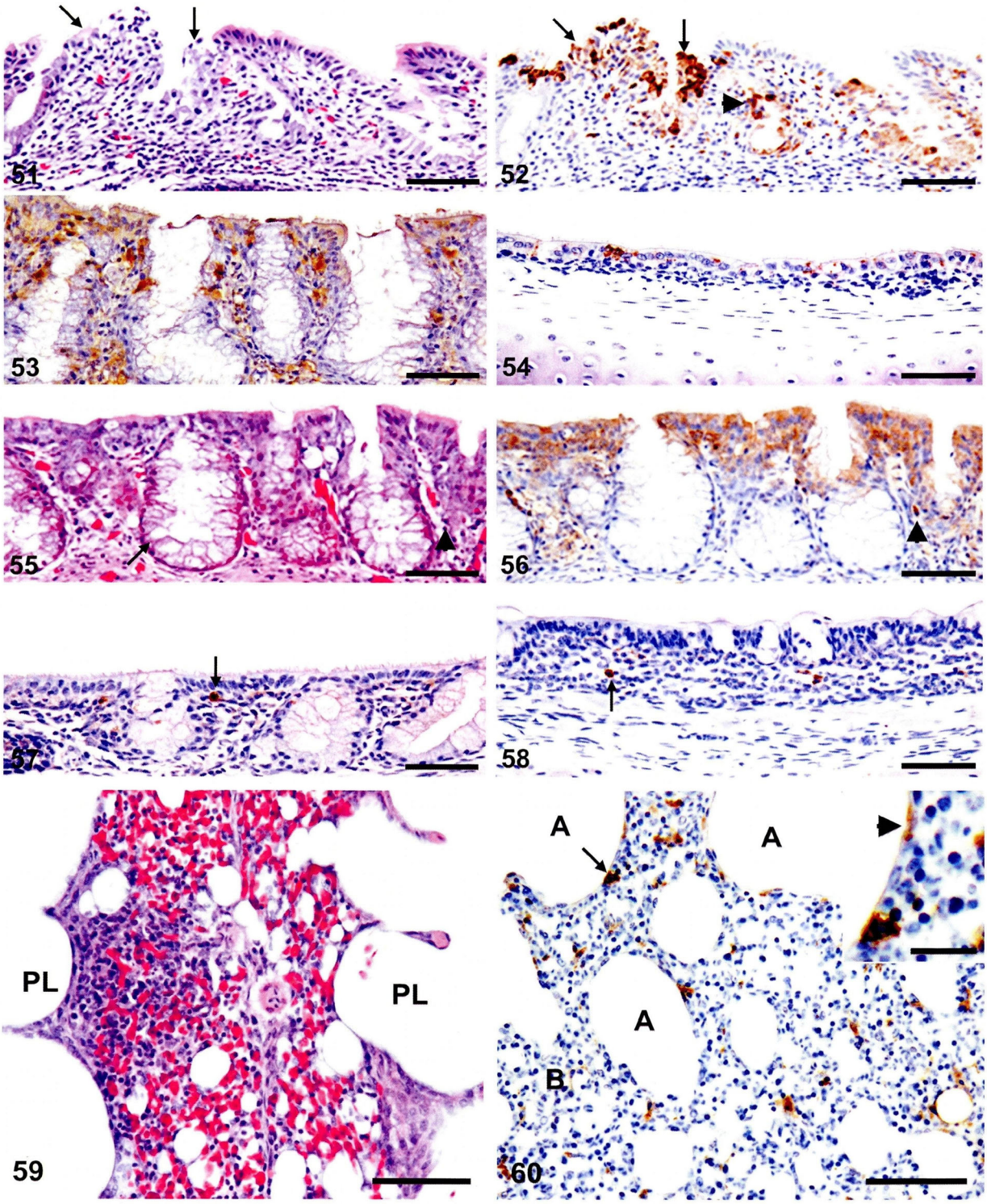
Fig. 46. Medulla oblongata; chicken, 8 dpi. Focal areas of microgliosis (main frame) and lymphocytic perivascular cuffing (inset). HE. Bar, 50 μm (inset, 50 μm).

Fig. 47. Cerebellum; duck, 10 dpi. No detectable changes. ML, molecular layer; GL, granular layer. HE. Bar, 200 μm.

Fig. 48. Medulla oblongata; duck, 10 dpi. No detectable changes in the medulla oblongata at the last day of the experiment. HE. Bar, 50 μm.

Fig. 49. Brain; chicken, **A**, **B**, **C** & **D** represent: cerebellum, cerebellar white mater, midbrain & medulla oblongata respectively. **1**, **2**, **3** & **4** represent: control chicken, 6, 8 & 10 dpi of infected chicken, respectivly. Intense increase of the expression of Glial fibrillary acidic protein (GFAP) in the granular layer (GL) of cerebellum by the 6 dpi (A2) and peaked at 8 dpi (A3) and appeared in the Bergmann-glia cell and some cell processes in the molecular layer (ML) at the 10 dpi (A4) (arrow). GFAP-immunolabellings were intensive in cerebellar white matter, midbrain and medulla oblongata by the 6, 8 dpi and 10 dpi. IHC, haematoxylin counterstained. Bars: **A**, 100 μm; **B**, **C**, & **D** 50 μm.

Fig. 50. Brain; duck, **A**, **B**, **C** & **D** represent: cerebellum, cerebellar white mater, midbrain & medulla oblongata respectively. **1**, **2**, **3** & **4** represent: control duck, 6, 8 & 10 dpi of infected duck, respectively. In duck cerebellum, **A**, the distribution of GFAP expression was different than chicken which concentrated around the Purkinje cell layer and expressed in the Bergmann-glia in the molecular layer (A1). Slight increase of GFAP expression was detected in the infected ducks at 6 & 8 dpi in cerebellum, cerebellar white matter, midbrain and medulla oblongata. GFAP immunolabelling was slightly decreased at the 10 dpi. IHC, haematoxylin counterstained. Bars: **A**, 100 μm; **B**, **C**, & **D** 50 μm.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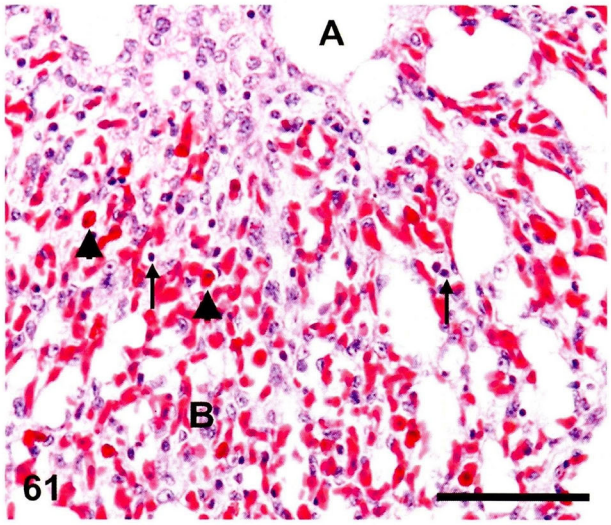
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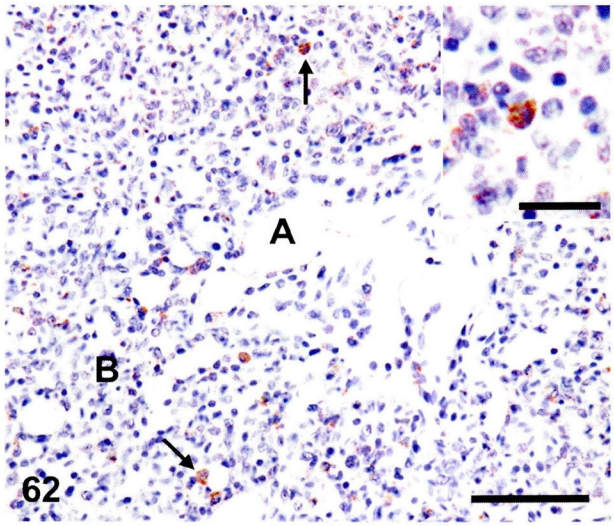
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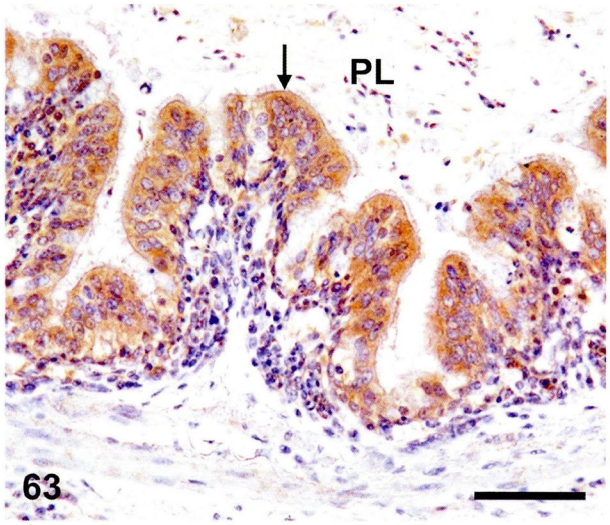
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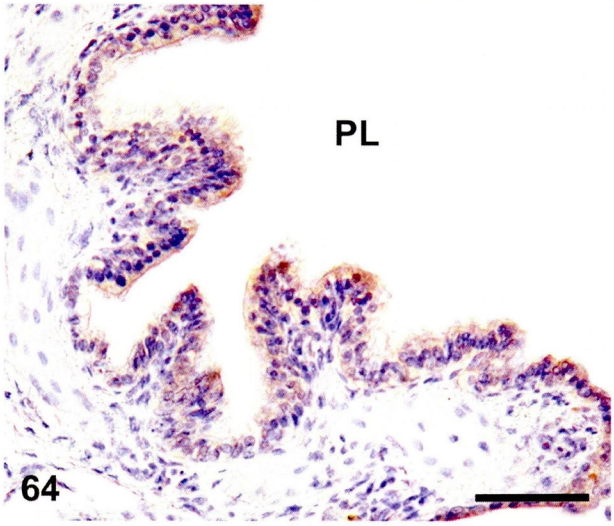
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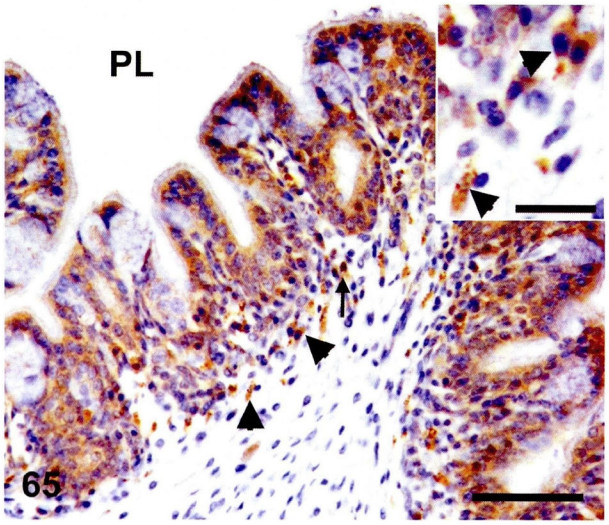
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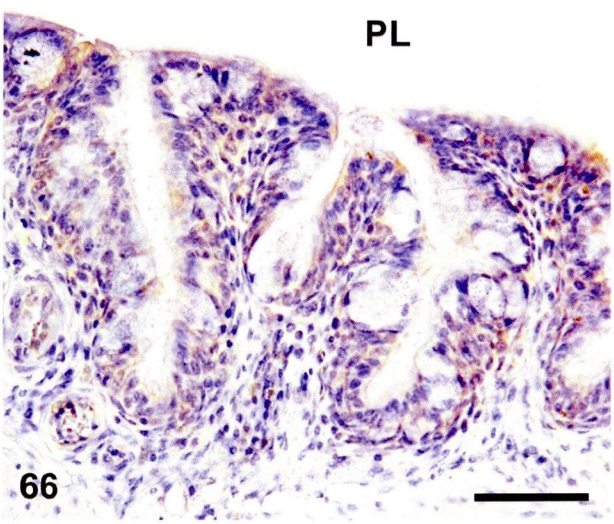
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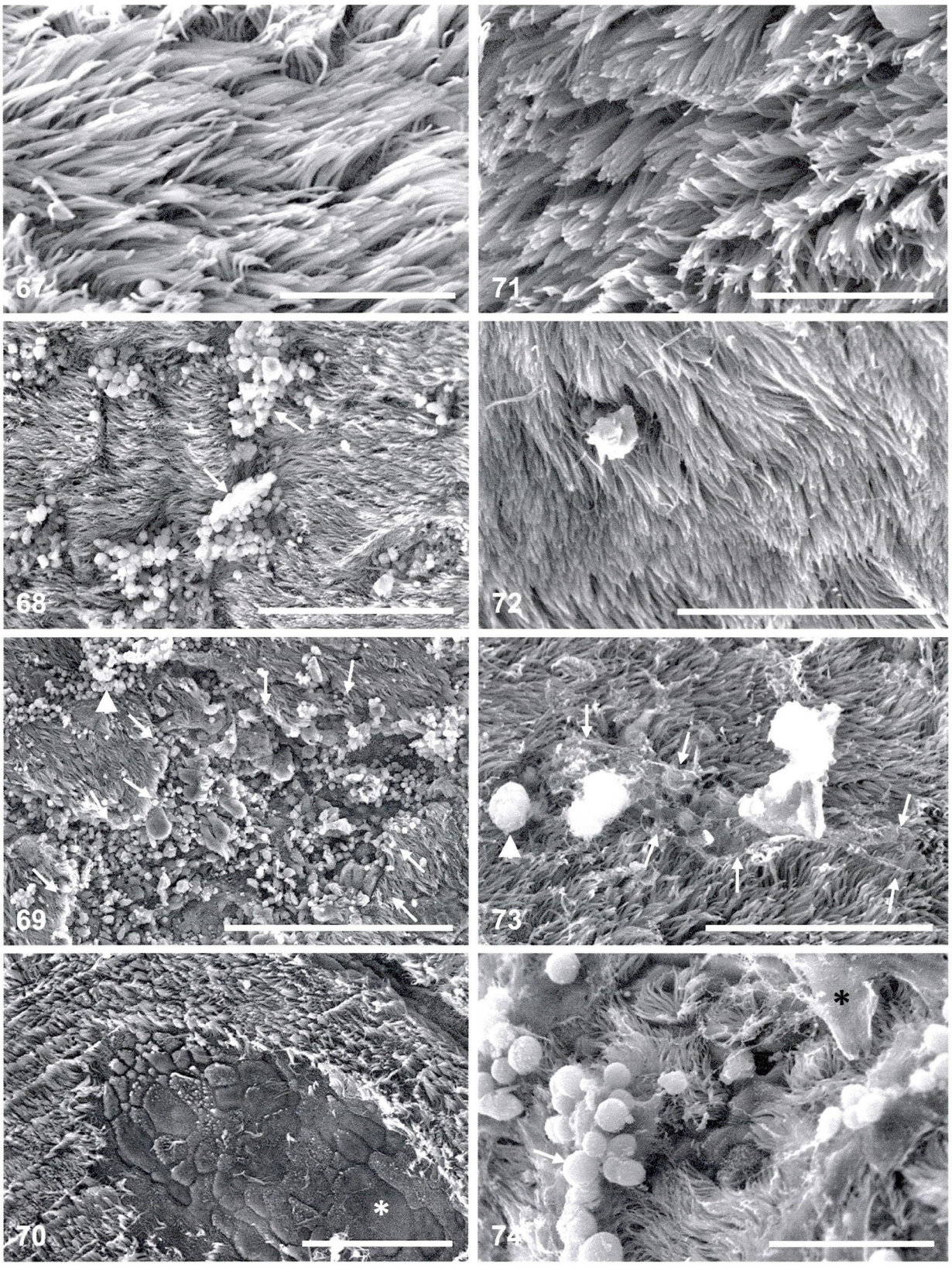
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Fig. 51. Nostrils; chicken, 1 dpi. Mild lymphocytic rhinitis with degeneration and necrosis of the mucosal epithelial cells (arrows). HE. Bar, 50 μm.

Fig. 52. Nostrils; chicken, 1 dpi. Positive signals for NDV-NP in the mucosal epithelial cells (arrows) and mononuclear cells (arrowhead). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 53. Larynx; chicken, 4 dpi. Positive signals for NDV-NP in the mucosal epithelial cells. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 54. Trachea; chicken, 4 dpi. Positive signals for NDV-NP in the mucosal epithelial cells. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 55. Nostrils; duck, 1 dpi. Hyperplasia of goblet cells (arrow) and infiltration of mononuclear cells (arrowhead). HE. Bar, 50 μm.

Fig. 56. Nostrils; duck, 1 dpi. Positive signals for NDV-NP in the mucosal epithelial cells and in mononuclear cells (arrowhead). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 57. Larynx; duck, 4 dpi. Positive signals for NDV-NP in mononuclear cells in the laryngeal mucosa (arrow). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 58. Trachea; duck, 2 dpi. Positive signals for NDV-NP in mononuclear cells in the Tracheal mucosa (arrow). IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 59. Lung; chicken, 2 dpi. Lymphocytic interstitial pneumonia with proliferation of the periparabronchial lymphoid tissues, and infiltration of macrophages and lymphoid cells. PL, parabroncheal lumen. HE. Bar, 50 μm.

Fig. 60. Lung; chicken, 2 dpi. Positive signals for NDV-NP in macrophage (arrow) and in epithelial cell (arrowhead). IHC, haematoxylin counterstained. A, Atria; B, air capillaries. Bar, 50 μm (inset, 15 μm).

Fig. 61. Lung; duck, 2 dpi. Mild interstitial pneumonia with infiltration of lymphocytes (arrows) and heterophils (arrowhead). HE. A, Atria; B, air capillaries. Bar, 50 μm.

Fig. 62. Lung; duck, 2 dpi. Positive signals for NDV-NP in macrophage (arrows). IHC, haematoxylin counterstained. A, Atria; B, air capillaries. Bar, 50 μm (inset, 15 μm).

Fig. 63. Lung; chicken, 4 dpi. Positive signals for interferon-beta (IFN-β) in epithelial cells of parabronchi (arrow). PL, Parabronchial lumen. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 64. Lung; chicken, negative control. PL, Parabronchial lumen. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 65. Lung; duck, 4 dpi. Positive signals for IFN-β in macrophage-like cell (arrow), fibroblast-like cells (arrowhead) and epithelial cells of parabronchial lumen (PL). IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

Fig. 66. Lung; duck, negative control. PL, Parabronchial lumen. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 67. Trachea; chicken, contraol. Normal cilia of the surface epithelium. SEM. Bar, 10 μm.

Fig. 68. Trachea; chicken, 1 dpi. Excessive mucous secretion on the surface mucosa (white arrows). SEM. Bar, 50 μm.

Fig. 69. Trachea; chicken, 4 dpi. Excessive globular mucous particles (white arrowhead), erosions of the tracheal epithelial cells (white arrows). SEM. Bar, 100 μm.

Fig. 70. Trachea; chicken, 10 dpi. Deciliation (asterisk), disorientation of the cilia, adherence of the cilia to each other and scanty mucous was found on the tracheal surface epithelium. SEM. Bar, 50 μm.

Fig. 71. Trachea; duck, contraol. Normal cilia of the surface epithelium. SEM. Bar, 10 μm.

Fig. 72. Trachea; duck, 1 dpi. Few globular mucous particles on the surface epithelium. SEM. Bar, 20 μm.

Fig. 73. Trachea; duck, 4 dpi. Globular mucous particles (white arrowhead), fine mucous network (white arrows) and slight disorientation of the cilia. SEM. Bar, 20 μm.

Fig. 74. Trachea; duck, 10 dpi. Globular mucous particles (white arrow), blankets of mucous were covering the underlying epithelium (asterisk) and slight disorientation of the cilia. SEM. Bar, 20 μm.

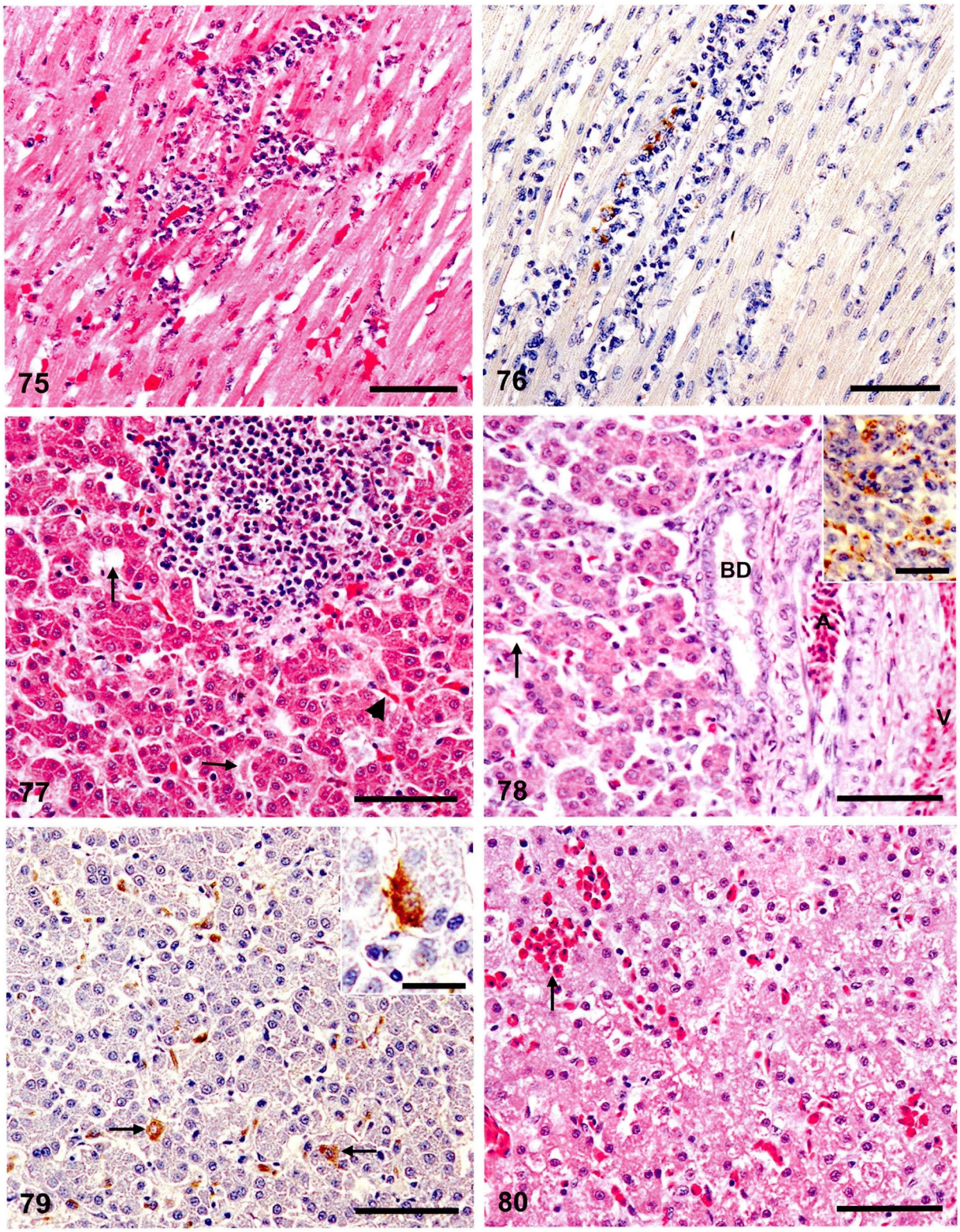
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Fig. 75. Heart; chicken, 4 dpi. Multifocal myocardial degeneration with infiltration of inflammatory cells. HE. Bar, 50 μm.

Fig. 76. Heart; chicken, 4 dpi. Positive signals for NDV-NP in lymphocytes and macrophages in degenerated area in myocardium. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 77. Liver; chicken, 4 dpi. Proliferation of the ectopic lymphoid tissues (asterisk) and the bile caniliculi was dilated and engorged with bile (arrows). Blood sinusoid (arrowhead). HE. Bar, 50 μm.

Fig. 78. Liver; chicken, 4 dpi. Dilatation of the bile canaliculi and the bile is engorged in the portal bile duct (BD). A, portal artery; V, portal vein. HE. Bar, 50 μm. *Inset*: Bile duct; chicken, P10. Positive signals for NDV-NP in the epithelial cells of bile duct and in macrophages around it. IHC, haematoxylin counterstained. Bar, 25 μm.

Fig. 79. Liver; chicken, 4 dpi. Signals for NDV-NP in macrophages (arrows) and in Kupffer cell (inset). IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15).

Fig. 80. Liver; duck, 4 dpi. Accumulation of heterophils in hepatic sinusoids (arrow). HE. Bar, 50 μm.

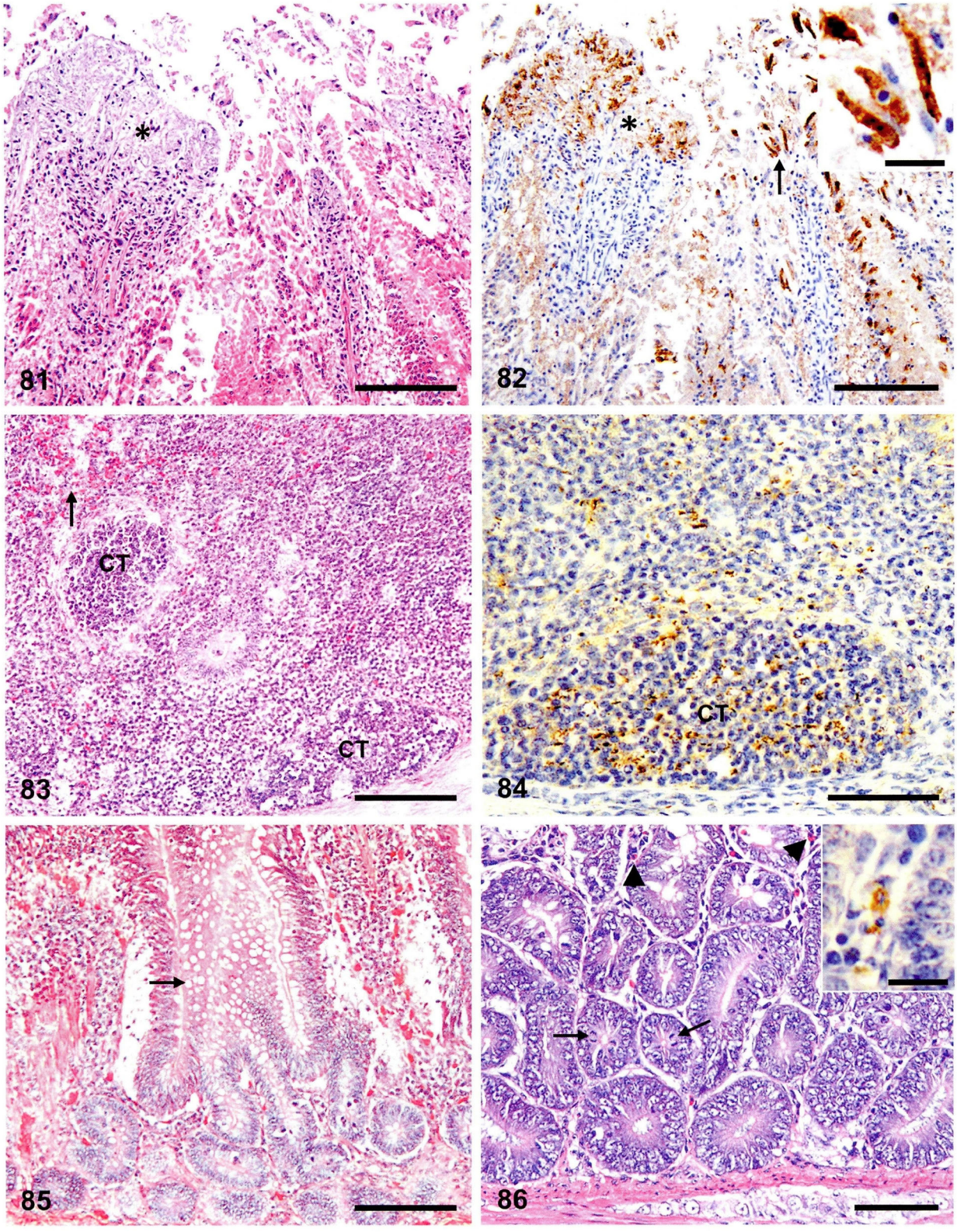
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Fig. 81. Duodenum; chicken, 4 dpi. Degeneration and necrosis in the mucosal epithelium (asterisk). HE. Bar, 100 μm.

Fig. 82. Duodenum; chicken, 4 dpi. Positive signals for NDV-NP in degenerated mucosa (asterisk) and in epithelial cells (arrows) in the main frame and inset. IHC, haematoxylin counterstained. Bar, 100 μm (inset, 15 μm).

Fig. 83. Cecal tonsils; chicken, 2 dpi. Proliferation of cecal tonsils (CT) with slight haemorrhages (arrow). HE. Bar, 100 μm.

Fig. 84. Cecal tonsils; chicken, 4 dpi. Positive signals for NDV-NP in the cecal tonsils and in cecal mucosa. IHC, haematoxylin counterstained. Bar, 50 μm.

Fig. 85. Duodenum; duck, 1 dpi. Proliferation of goblet cells (arrow). HE. Bar, 100 μm.

Fig. 86. Duodenum; duck, 2 dpi. Mitosis in the epithelial cells of the duodenal gland (arrow) and infiltration of heterophils (arrowhead). HE. Bar, 50 μm. *Inset*: Duodenum; duck, 4 dpi. Positive signals for NDV-NP in macrophage in the mucosa. IHC, haematoxylin counterstained. Bar, 15 μm.

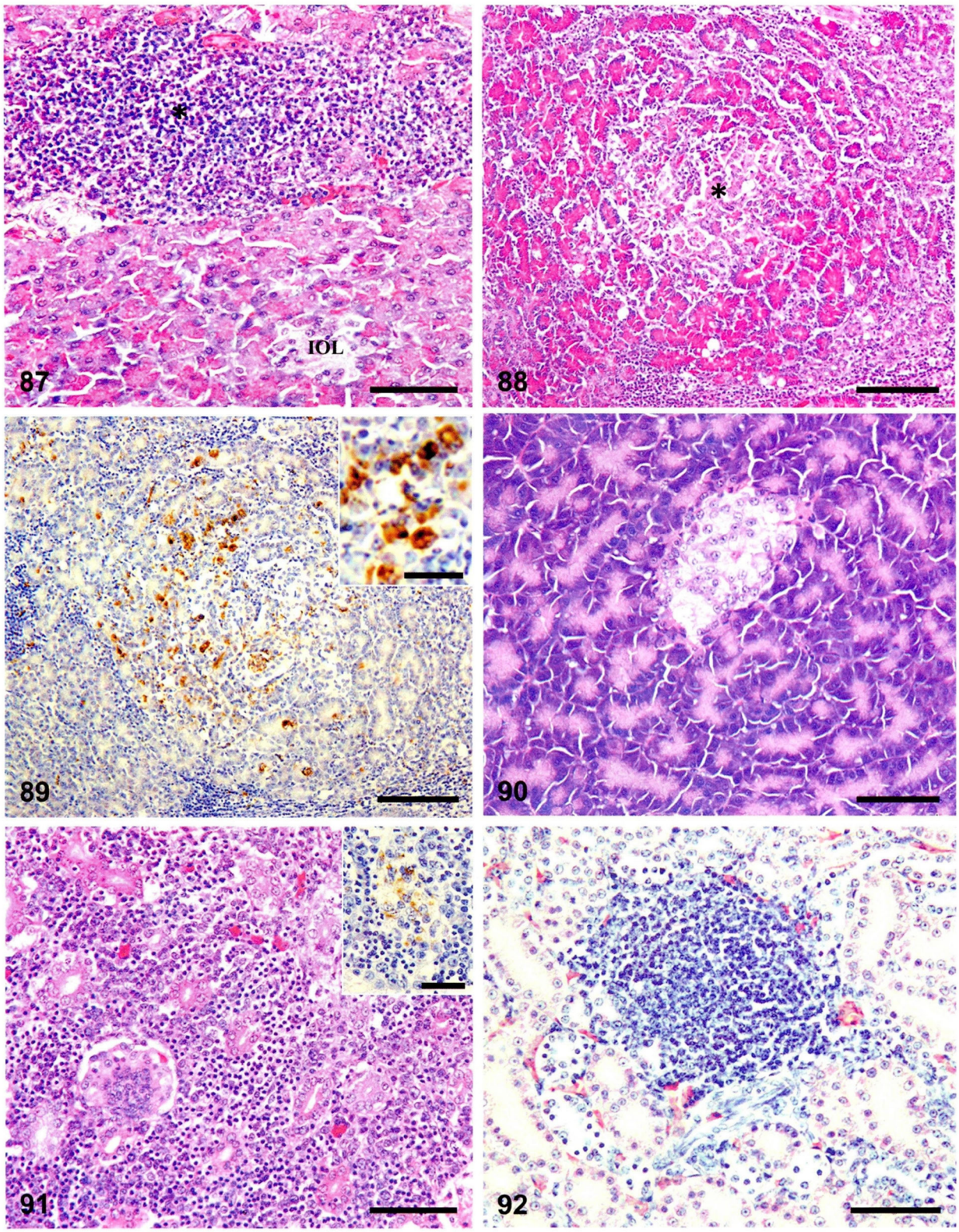
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Fig. 87. Pancreas; chicken, 1 dpi. Necrotizing pancreatitis with infiltration of inflammatory cells (asterisk). IOL, islets of Langerhans. HE. Bar, 50 μm.

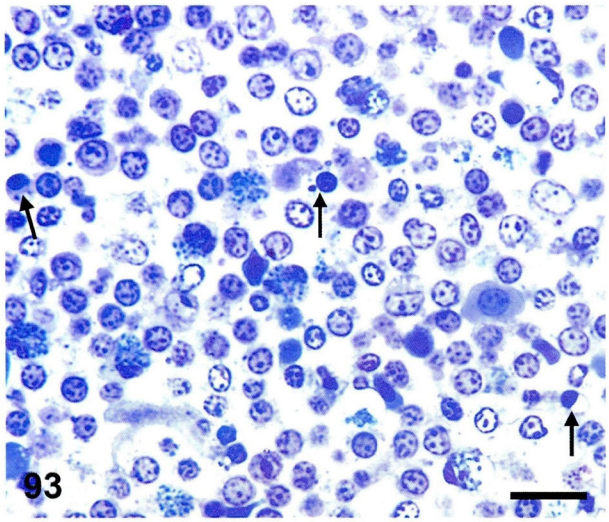
Fig. 88. Pancreas; chicken, 10 dpi. Necrotizing pancreatitis with marked infiltration of inflammatory cells (asterisk). HE. Bar, 50 μm.

Fig. 89. Pancreas; chicken, 10 dpi. Signals for NDV-NP in the degenerated pancreatic acinar cells in the same site of fig. 88. IHC, haematoxylin counterstained. Bar, 50 μm (inset, 15 μm).

Fig. 90. Pancreas; duck, 10 dpi. No detectable pathologic changes comparing to the control group. HE. Bar, 50 μm.

Fig. 91. Kidney; chicken, 10 dpi. Tubulointerstitial nephritis. HE. *Inset*: Positive signals for NDV-NP in degenerated renal tubules. IHC, haematoxylin counterstained. Bar, 50 μm (inset, 25 μm).

Fig. 92. Kidney; duck, 8 dpi. Proliferation of the ectopic lymphoid tissues in the kidney. HE. Bar, 50 μm.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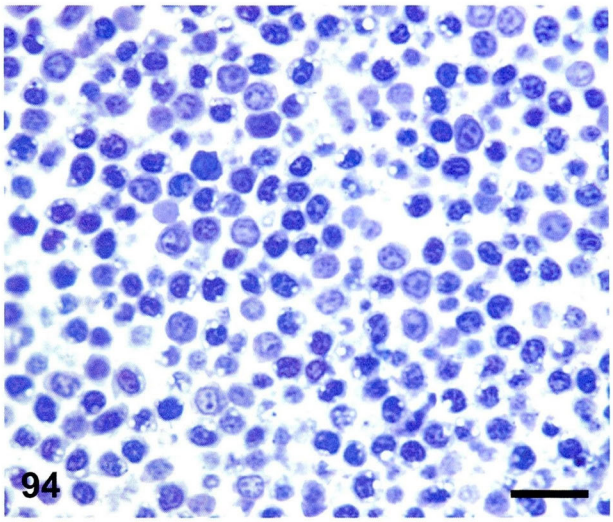
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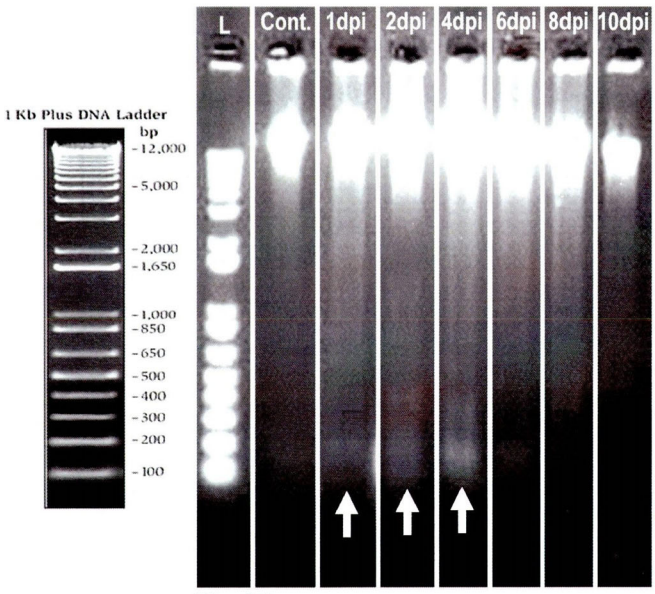
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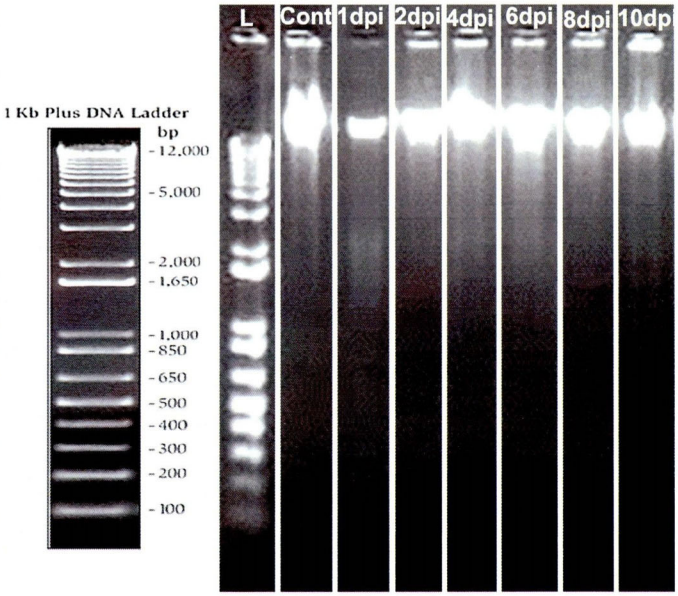
Fig. 95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6dpi
8dpi
10dp

Fig. 96

Fig. 93. Buffy coat; chicken, 4 dpi. Few apoptotic cells (arrows). Toluidine blue stain. Bar, 15 μm.

Fig. 94. Buffy coat; duck, 6 dpi. Apoptotic cells cannot be detected in the buffy coat section in duck. Toluidine blue stain. Bar, 15 μm.

Fig. 95. Agarose gel electrophoresis, chicken Buffy coat DNA. L, ladder; cont., control group; dpi, day post infection. Note DNA fragmentation especially at 4 dpi.

Fig. 96. Agarose gel electrophoresis, duck Buffy coat DNA. L, ladder; cont., control group; dpi, day post infection. DNA fragments cannot be detected in the control group or in the infected duck.

DISCUSSION

DISCUSSION

In this study, 9a5b NDV isolate (which has a virulent F gene cleavage site and a high ICPI of 1.88) (Shengqing *et al.*, 2002) induced clinical signs in the infected chickens only, and they were mild. In agreement, the chicken's lesions were more severe than the duck's lesions, and the distribution and intensity of the NDV-NP-positive signals were greater in chickens than in ducks. NDV virulence groups (lentogen, mesogen and velogen) are very broad categories, and the degree of clinical disease does not always segregate with ICPI. There are cases, for example, in which a strain is considered "virulent" by ICPI but does not cause much in the way of clinical disease (Susta *et al.*, 2011; Wakamatsu *et al.*, 2006b & c). In addition, the pathogenic effects of NDV are not dependent on the fusion cleavage site alone (Wakamatsu *et al.*, 2006b). As described previously, NDV isolates that had a virulent fusion protein cleavage site and high ICPI differed in their ability to cause clinical signs, in their lesions, and in their viral distribution in SPF chickens (Nakamura *et al.*, 2008; Susta *et al.*, 2011). Moreover, chickens and ducks vary in their response to virulent NDV infection (Aldous *et al.*, 2010). Among poultry, chickens are the most susceptible to NDV infection, while ducks are considered to be one of the natural reservoirs for this virus (Kaleta and Baldauf, 1988; Wakamatsu *et al.*, 2006a).

Immunohistochemical expression of NDV-NP was more intense in the lymphoid and non-lymphoid organs of chickens than those of ducks. The spleen was consistently positive for NDV-NP in both chickens and ducks in the present study and in chickens in previous studies (Brown *et al.*, 1999; Kommers *et al.*, 2001). NDV can replicate in avian macrophages, leading to functional alteration with subsequent viral dissemination (Qureshi, 1998; Brown *et al.*, 1999). In the present study, lymphocytes, macrophages and apoptotic bodies were positive for NDV-NP in the splenic tissues of infected chickens, but macrophages were the only cells labelled in the spleen of infected ducks. These findings may indicate wider dissemination of NDV in chicken tissues.

In general, NDV-NP and apoptotic cells were more abundant in the spleen of chickens than in ducks, but the noteworthy finding in the present study was the distinct apoptotic patterns in the spleen when chickens and ducks were compared at 1 dpi. There were numerous apoptotic cells in the PWP, PELS and PALS of infected chickens, which are in agreement with the results of a previous study (Harrison *et al.*, 2011). In contrast, apoptosis in the infected ducks was largely restricted to the GCs. The GCs of ducks were larger in number and in size than those of chickens. Further studies will be required to investigate the role of the GC in the pathogenesis of NDV infection and the cause of the distinct apoptotic pattern between chickens and ducks.

Macrophages expressing NDV-NP were numerous in the spleen, rare in the thymus and absent from the bursa of infected ducks. In contrast, apoptosis was abundant in macrophages, lymphocytes and other lymphoid cells in these organs. Moreover, despite the abundant apoptosis in the GCs of infected ducks, NDV-NP was rarely expressed in the GCs. A possible role for indirect viral effects in triggering apoptosis might be considered. This finding has also been observed in infections with other paramyxoviruses (e.g. those caused by measles and distemper viruses) in which apoptosis is initiated by immune modulation rather than by direct viral damage (Moss *et al.*, 2004; Schneider-Schaulies *et al.*, 2003; Schobesberger *et al.*, 2005).

Apoptosis was abundant in the bursa and thymus of infected ducks after 9a5b NDV infection, but lymphoid depletion was the main feature in the thymus and bursa of infected chickens. Lymphoid depletion is described as a sequela to chronic necrosis or apoptosis (Elmore, 2006). In the present study, apoptosis was rare in the thymic and bursal tissues of infected chickens and lymphoid depletion is unlikely to have been due to cell necrosis because NDV-NP was found rarely in these organs. In man, B and T lymphocytes can migrate and home to sites of inflammation after activation by antigen presentation by dendritic cells (Koboziev *et al.*, 2010; Luster *et al.*, 2005; von Andrian and Mackay, 2000). Therefore, it is possible to explain the depletion of T cells from the thymus and B cells from the

bursa by this phenomenon, but this would require confirmation. The question remains as to why lymphoid depletion does not occur in the bursa and thymus of ducks despite the large number of apoptotic cells. In the present study, numerous mitoses were observed in the duck tissues, which may suggest rapid tissue regeneration in these birds.

This study shows that, 9a5b NDV induce apoptosis in buffy coat cells of chickens. A few apoptotic cells were detected by toluidine blue stained semi-thin section, especially at 4 dpi; and this results were confirmed by agarose gel electropherosis. Weak DNA laddering pattern was observed at the same day. In previous studies, GB NDV strain causes apoptosis and necrosis in peripheral blood lymphocytes (Lam and Vasconcelos, 1994) and Marek's disease virus (MDV) causes apoptosis in T cell in peripheral blood mononuclear cells (PBMC) in chicken resulting in immunosuppression (Morimura *et al.*, 1995). In our study, infected ducks did not show any apoptotic pattern neither by buffy coat semi-thin sections nor by DNA electrophoresis; and this may indicate that, ducks after NDV infection did not suffer from immunosuppression while chickens suffer.

In the present study, the respiratory and digestive lesions started at an early stage (except airsacculitis) and subsided at a late stage in both chickens and ducks. On the other hand, necrotizing pancreatitis and tubulointerstitial nephritis were detected only in chickens; they dramatically increased at late stage and did not

subside until the 10 dpi. We are unaware of what the sequelae of these lesions might have been if the birds were studied for a longer period. Moreover, mild lymphocytic myocarditis and slight nonpurulent encephalitis were detected only in chickens, and these lesions were described previously (Kommers *et al.*, 2001; Kommers *et al.*, 2003b). Immunohistochemical NDV-NP-positive signals were detected in infected chickens at a late stage in the degenerated pancreatic acinar cells and degenerated renal tubules, at the middle of the experimental period in the myocardium, but not in the brain, indicating no or too low virus replication in the brain tissue to be detected by IHC, in addition to the direct effect of NDV on the pancreatic acinar cells, renal epithelial cells and myocardial fibers. This finding is consistent with a previous observation in SPF chickens infected with NDV isolates of different virulence (Brown *et al.*, 1999).

Ducks in the present study had no lesions in the brain, heart, pancreas, or kidney; the NP-positive signals were not detected in these organs either. These findings indicate wider dissemination of the 9a5b isolate in tissues of chicken than in those of duck. Chickens, turkeys, and ducks are varies in their response to virulent NDV infection. Although asymptomatic infection occurred in ducks and turkeys even at the highest dose (10^6 EID_{50}) without any deaths noted, chickens were shown to be extremely susceptible, and all died even with a low dose ($10^{1.5}$ EID_{50}) (Aldous *et al.*, 2010). In line with that, in SPF chickens, the mesogenic Anhinga NDV isolate

induced eyelid edema, multifocal myofiber disruption with infiltrates of lymphocytes, and macrophages in the heart. The cerebellum had multifocal gliosis with Purkinje cell loss and epithelial necrosis in the comb as well as NDV-NP positive signals in some organs, including brain (Kommers *et al.*, 2003b). Chickens are the most susceptible to NDV infection among poultry species, whereas ducks and geese are the least susceptible (Kaleta and Baldauf, 1988), for this reason, wild birds are considered to be the natural reservoir of NDV (Zhu *et al.*, 2010). Unfortunately, pathological studies of Newcastle disease in ducks are rare, so it is difficult to compare between our results and previous data.

In this study, GFAP expression was more intense in brain tissues of chickens than those of ducks. GFAP is expressed in astrocytes, where it is thought to help maintain mechanical strength, shape of cells, as well as its role in cell motility and migration. Recently, it is established that GFAP plays a key role in modulating astrocytic and neuronal glutamate transporter trafficking and function and in the control of glutamine production. The exact function of GFAP remains an enigma, despite the huge number of studies using it as a marker for astrocytes. Recently, there is a growing realization that astrocytes have a much broader function than only supporting the neurons in the brain, as they have specialized functions in inducing and regulating the blood brain barrier (BBB), protect neurons against neurotransmitter excesses, promote synaptic plasticity, coordinate neuronal activity

via direct communication with neurons and are the neural stem cells in the adult brain (Elobeid *et al.*, 2000; Gomi *et al.*, 1995; Lepekhin *et al.*, 2001; Liedtke *et al.*, 1996; McCall *et al.*, 1996; Middeldorp and Hol, 2011; Pekny *et al.*, 1995).

The enlargement of astrocytes and the increased expression of GFAP is an indication of reactive gliosis, a process which has shown to be highly related to brain damage and aging (Nichols *et al.*, 1993). Brain damage may induced by different causes such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease and Alexander disease) and different neurological conditions including developmental, infectious and inflammatory, and vascular disorders (Johnston- Wilson *et al.*, 2000; Miguel-Hidalgo *et al.*, 2000; Muller *et al.*, 2001).

In our study, 9a5b NDV induce intensive GFAP expression in chicken's brain tissues, and the expression was mostly related to the areas of microgliosis and neuron degeneration/ necrosis in cerebellum, midbrain and medulla oblongata. GFAP-positive reaction was observed in the areas of gliosis adjacent to the degenerated Purkinje cells as well as in Bergmann-glia at 10 dpi, and these results in agreement with previous study on NDV (Kommers *et al.*, 2002). Normally in chickens, the Bergmann-glia, a highly specialized astrocytic cell type found in the cerebellar molecular layer, is GFAP negative (Kalman *et al.*, 1993). Reactive gliosis following a stab wound lesion was described in the cerebellar molecular layer in chickens. In that case, the Bergmann-glia showed a marked

immunopositivity to GFAP around the lesion site (Ajtai and Kalman, 1998). In this study, 9a5b NDV infected ducks had shown a slight increase of the GFAP expression despite the histologically normal features of duck's brain tissues by HE stain and negative staining of NDV-NP antigen. Therefore, the expression of GFAP in duck's brain tissues still needs farther studies to explain the real causes underlying this expression.

In our study, histopathological lesions were more severe in infected chickens. On the other hand, heterophilic infiltration, proliferation of lymphoid tissues, and hyperplasia of goblet cells were more oven observed in the infected ducks. Avian heterophils are highly phagocytic and capable of a broad spectrum of antimicrobial activity, and they form the first line of cellular defense against invading microbial pathogens in the lungs and air sacs, where resident macrophages are typically lacking (Brune *et al.*, 1972; Harmon, 1998). In this study, SEM investigation of tracheal tissues revealed that 9a5b NDV infection induced rapid and abundant mucous secretion at 1 dpi in chickens, but scanty mucous was observed at the 10 dpi, possibly indicating severe exhaustion of goblet cells, which is in line with previous studies (Lai and Ibrahim, 1983; Mast *et al.*, 2005). In infected ducks, the mucous secretion was mildly increased from the 1 to the 10 dpi. Moreover, deciliation and mucosal destruction were greater in chickens than in ducks.

Therefore, in field condition, secondary infection may occur easily in chickens rather than in ducks.

As described previously, chicken eye-lid inoculation with 3 virulent NDV isolates did not cause any lesions at the 1 dpi (Susta *et al.*, 2011). In contrast, in the present study, at the 1 dpi, the lesions in the infected chickens were not restricted to the respiratory and the digestive tracts. A few localized areas of lymphocytic myocarditis and pancreatitis were detected even though NDV-NP could not be detected by IHC in the heart and pancreas at that day (Table 6). Therefore, the early detectable lesions in our experiment may depend on the route of inoculation as well as virulence differences. Further studies to detect the virus by *in situ* hybridization or viral isolation and to explain the development of early lesions in the heart and pancreas are needed.

In viral infections the induction of IFN-α and IFN-β helps to mediate and regulate protective immune responses (Biron, 1998). In the present study, immunohistochemical expression of IFN-β was detected in the lung and immune organs, and it was early and intense in duck's tissues. IFN-β expression in lung tissues was mainly expressed in the epithelial cells of the parabronchi as well as in the macrophages- and fibroblaste-like cells. In the immune organs IFN-β expression was mainly in macrophage-like cells and the greatest expression was in the spleen, in which there was also the greatest expression of NDV-NP. IFN-β

mRNA expression is also reported in the spleen after H5N1 influenza virus infection of chickens (Karpala *et al.*, 2008). IFN-β can be produced by cells of the innate immune system (e.g. monocyte/macrophages, natural killer cells and neutrophils), and can also be produced by non-immune cells within hours or days of viral infection (Ahmed and Biron, 1998). NDV failed to induce chicken IFN genes in a macrophage cell line, but the primary macrophages were the most effective producers of virus-induced IFNs under the same conditions (Sick *et al.*, 1998). IFN-β has a role in the redistribution of nucleated cells from the red to the white pulp areas of the spleen. Such trafficking changes could facilitate innate immune cell localization for delivery of antimicrobial and/or immunoregulatory functions (Ishikawa and Biron, 1993; Salazar-Mather *et al.*, 1996).

In the present experiment, IFN-β expression after NDV infection was earlier, stronger and more intense in tissues from ducks compared with those from chickens. IFN-β appears to be protective during the early stage of influenza infection and this effect cannot be compensated for by IFN-α, and therefore superior innate immunity might protect the duck during this critical period (Barber *et al.*, 2010). Retinoic acid-inducible gene-like receptors (RLRs) including retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (Mda)-5 are cytoplasmic RNA sensors (Yoneyama *et al.*, 2004) that can detect influenza virus (Loo *et al.*, 2008) and NDV (Sick *et al.*, 1998), leading to production of IFN-

β and expression of downstream IFN-stimulated antiviral genes (Loo *et al.*, 2008). RLRs play central roles in viral recognition and subsequent induction of antiviral immune responses in conventional dendritic cells, macrophages and fibroblasts (Kumar *et al.*, 2006; Sun *et al.*, 2006). RIG-I is present in the duck and absent in chickens (Barber *et al.*, 2010), while the V proteins of paramyxoviruses directly interact with Mda-5 to block RLR signaling (Andrejeva *et al.*, 2004). This indicates that after NDV infection, chickens cannot produce IFN-β via the RLR pathway, which gives the duck an advantage in this point (Andrejeva *et al.*, 2004; Barber *et al.*, 2010; Loo *et al.*, 2008; Sick *et al.*, 1998; Yoneyama *et al.*, 2004). Based on this information, after NDV infection, ducks may have an innate immune response that is faster and stronger than that of chickens. This may reflect the subclinical NDV infection that occurs in ducks and the clinically apparent infection in chickens.

In summary, the results of the present study have shown that the 9a5b mutant isolate induced a mild infection in SPF chickens and asymptomatic infection in Japanese commercial ducks. Accordingly, the pathological lesions and virus dissemination was grater in chickens than ducks. Regarding the immune organs, the spleen was the most affected organ. The pattern of apoptosis in the spleen was distinct between chickens and ducks. Apoptotic cells were abundant in the PWP, PELS, PALS and PVLS of infected chickens, but apoptosis was concentrated in

the GCs of the infected ducks. NDV may induce apoptosis by direct and indirect means in lymphoid tissues. Apoptosis and necrosis may be not the only causes of lymphoid depletion in the immune organs and cell migration from these organs may be another possible explanation for the observed lymphoid depletion. Immunosuppression may develop in chicken by the effect of NDV on peripheral blood mononuclear cells. Nonpurulent encephalitis, lymphocytic myocarditis, necrotizing pancreatitis and tubuleinterstitial nephritis were developed in infected chicken only. The expression of GFAP was increased in chicken and ducks after 9a5b NDV infection; in chicken it was intense and related to pathological alterations in the chicken's brain. Despite the lack of pathological findings in brain tissues of ducks, GFAP expression was slightly increased after infection. IFN-β immunopositivity was more intensive in immune and lung tissues of ducks which may indicate stronger duck innate immunity against NDV infection. Further studies to compare chickens and waterfowl are needed, which may help us to elucidate the natural mechanisms underlying waterfowl resistance to NDV infection.

REFERENCES

REFERENCES

Ahmed, R. & Biron, C.A. (1998) Immunity to viruses. In: *Fundamental Immunology*, 4th Edit., Paul, W.E. (ed.), Raven Press, New York, pp. 1295-1334.

Ajtai, B.M. & Kalman, M. (1998) Glial fibrillary acidic protein expression but no glial demarcation follows the lesion in the molecular layer of cerebellum. *Brain Res*, **802**, 285-288.

Aldous, E.W., Seekings, J.M., McNally, A., Nili, H., Fuller, C.M., Irvine, R.M., Alexander, D.J. & Brown, I.H. (2010) Infection dynamics of highly pathogenic avian influenza and virulent avian paramyxovirus type 1 viruses in chickens, turkeys and ducks. *Avian Pathology*, **39**, 265-273.

Alexander, D.J. (2001) *Gordon Memorial Lecture*, Newcastle disease. *British Poultry Science*, **42**(1), 5-22.

Alexander, D.J. (1988) Newcastle disease: Methods of spread. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 256-272.

Alexander, D.J. (1998) Newcastle disease and other paramyxoviruses. In: *A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens*, 4th Edit., Swayne, D.E., Glisson, J.R., Jackwood, M.W. & Reed, W.M. (ed.), American of Avian Pathologists, Kennett Square, pp. 156-163.

Alexander, D.J. (2000) Newcastle disease in ostriches (Struthio camelus) - a review. *Avian Pathol*, **29**, 95-100.

Alexander, D.J. (2003) Newcastle disease, other avian paramyxoviruses, and pneumovirus infection. In: *Diseases of Poultry*, 11th Edit., Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDouglad, J.R. *et al.* (ed.), Iowa State University Press, Ames, pp. 63-92.

Alexander, D.J. & Allan, W.H. (1974) Newcastle disease virus pathotypes. *Avian Pathol*, **3**, 269-278.

Alexander, D.J., Manvell, R.J., Banks, J., Collins, M.S., Parsons, G., Cox, B., Frost, K.M., Speidel, E.C., Ashman, S. & Aldous, E.W. (1999) Experimental assessment of the pathogenicity of the Newcastle disease viruses from outbreaks in Great Britain in 1997 for chickens and turkeys, and the protection afforded by vaccination. *Avian Pathology*, **28**, 501-511.

Alexander, D.J., Manvell, R.J., Lowings, J.P., Frost, K.M., Collins, M.S., Russell, P.H. & Smith, J.E. (1997) Antigenic diversity and similarities detected in avian paramyxovirus type 1 (Newcastle disease virus) isolates using monoclonal antibodies. *Avian Pathol*, **26**, 399-418.

Alexander, D.J., Parsons, G. & Marshall, R. (1984) Infection of fowls with Newcastle disease virus by food contaminated with pigeon faeces. *Veterinary Record*, **115**, 601-602.

Alexander, D.J. & Senne, D.A. (2008) Newcastle disease, other avian Paramyxoviruses and Pneumovirus infection. In: *Disease of Poultry*, 12th Edit., Saif, Y.M. (ed), Iowa state university press, 75-100.

Allan, W.H. & Gough, R.E. (1976) A comparison between the haemagglutination inhibition and complement fixation tests for Newcastle disease. *Res Vet Sci*, **20**, 101-103.

Allan, W.H., Lancaster, J.E. & Toth, B. (1978) Newcastle disease vaccines-Their production and use. FAO Animal Production and Health. Series No.10. FAO: Rome, Italy.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S. & Randall, R.E. (2004) The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-β promoter. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 17264-17269.

Asplin, F.D. (1952) Immunization against Newcastle disease with a virus of low virulence (strain F) and observations on subclinical infection in partially resistant fowls. *Veterinary Record*, **64**, 245-249. *Cited in*: Alexander and Senne, 2008.

Barber, M.R.W., Aldridge, J.R., Webster, R.G. & Magor, K.E. (2010) Association of RIG-I with innate immunity of ducks to influenza. *Proceedings of the National Academy of Sciences*, **107**, 5913-5918.

Beard, C.W. & Easterday, B.C. (1967) The influence of the route of administration of Newcastle disease virus on host response. I. Serological and virus isolation studies. *J Infect Dis*, **117**, 55-61.

Beard, C.W. & Hanson, R.P. (1984) Newcastle disease. In: *Diseases of Poultry*, 8th Edit., Hofstad, M.S., Barnes, H.J., Calnek, B.W., Reid, W.M., Yoder, H.W. (ed.), Iowa State University Press, Ames, 452-470.

Biancifiori, F. & Fioroni, A. (1983) An occurrence of Newcastle disease in pigeons: virological and serological studies on the isolates. *Comp Immunol Microbiol Infect Dis*, **6**, 247-252.

Biron, C.A. (1998) Role of early cytokines, including alpha and beta interferons (IFN-α\β), in innate and adaptive immune responses to viral infections. *Seminars in Immunology*, **10**, 383-390.

Biswal, G. & Morrill, C.C. (1954) The Pathology of the Reproductive Tract of Laying Pullets Affected with Newcastle Disease. *Poultry Science*, **33**, 880-897.

Box, P., Helliwell, B. & Halliwell, P. (1970) Newcastle disease in turkeys. Determination of the 50 per cent. Lethal dose of the Herts (1933) Weybridge strain of Newcastle disease virus and the potency of B.P.L. inactivated Newcastle disease vaccine in turkeys. *Veterinary Record*, **86**, 524-527.

Box, P.G., Holmes, H.C., Bushell, A.C. & Finney, P.M. (1988) Impaired response to killed Newcastle disease vaccine in chicken possessing circulating antibody to chicken anaemia agent. *Avian Pathol*, **17**, 713-723.

Brown, C., King, D.J. & Seal, B.S. (1999) Pathogenesis of Newcastle Disease in Chickens Experimentally Infected with Viruses of Different Virulence. *Veterinary Pathology Online*, **36**, 125-132.

Brune, K., Leffell, M.S. & Spitznagel, J.K. (1972) Microbicidal activity of peroxidaseless chicken heterophile leukocytes. *Infect Immun*, **5**, 283-287.

Chang, P.W. (1981) Newcastle disease. In: *CRC Handbook Series in Zoonoses*, Section B,Viral Zoonoses, Volume II., Beran, G.W. (ed.), CRC Press, Baton Raton, 261-274.

Cheville, N.F., Stone, H., Riley, J. & Ritchie, A.E. (1972) Pathogenesis of virulent Newcastle disease in chickens. *J Am Vet Med Assoc*, **161**, 169-179.

Collins, M.S., Bashiruddin, J.B. & Alexander, D.J. (1993) Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Archives of Virology*, **128**, 363-370.

Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O. & Lomniczi, B. (2006) Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Res*, **120**, 36-48.

de Leeuw, O. & Peeters, B. (1999) Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. *Journal of General Virology*, **80**, 131-136.

de Leeuw, O.S., Hartog, L., Koch, G. & Peeters, B.P.H. (2003) Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. *Journal of General Virology*, **84**, 475-484.

DiNapoli, J.M., Yang, L., Suguitan, A., Elankumaran, S., Dorward, D.W., Murphy, B.R., Samal, S.K., Collins, P.L. & Bukreyev, A. (2007) Immunization of Primates with a Newcastle Disease Virus-Vectored Vaccine via the Respiratory Tract Induces a High Titer of Serum Neutralizing Antibodies against Highly Pathogenic Avian Influenza Virus. *Journal of Virology*, **81**, 11560-11568.

Doyle, T.M. (1927) A hitherto unrecorded disease of fowls due to a filter-passing virus. *J Comp Pathol Therap*, **40**,144-169. *Cited in*: Alexander and Senne, 2008.

Doyle, T.M. (1935) Newcastle disease of fowls. *J Comp Pathol Therap*, **48**,1-20. *Cited in*: Alexander and Senne, 2008.

Elmore, S.A. (2006) Histopathology of the Lymph Nodes. *Toxicologic Pathology*, **34**, 425-454.

Elobeid, A., Bongcam-Rudloff, E., Westermark, B. & Nistér, M. (2000) Effects of inducible glial fibrillary acidic protein on glioma cell motility and proliferation. *Journal of Neuroscience Research*, **60**, 245-256.

Faragher, J., Allan, W. & Wyeth, P. (1974) Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. *Veterinary Record*, **95**, 385-388.

Francis, D.W. (1973) Newcastle and psittacines, 1970-1971. *Poult Dig*, **32**, 16-19. *Cited in*: Alexander and Senne, 2008.

Garten, W., Berk, W., Nagai, Y., Rott, R. & Klenk, H.-D. (1980) Mutational Changes of the Protease Susceptibility of Glycoprotein F of Newcastle Disease Virus: Effects on Pathogenicity. *Journal of General Virology*, **50**, 135-147.

Ghumman, J.S. & Bankowski, R.A. (1976) In Vitro DNA Synthesis in Lymphocytes from Turkeys Vaccinated with LaSota, TC, and Inactivated Newcastle Disease Vaccines. *Avian Diseases*, **20**, 18-31.

Giambrone, J.J., Eidson, C.S., Page, R.K., Fletcher, O.J., Barger, B.O. & Kleven, S.H. (1976) Effect of infectious bursal agent on the response of chickens to Newcastle disease and Marek's disease vaccination. *Avian Dis*, **20**, 534-544.

Glickman, R.L., Syddall, R.J., Iorio, R.M., Sheehan, J.P. & Bratt, M.A. (1988) Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *Journal of Virology*, **62**, 354-356.

Gomi, H., Yokoyama, T., Fujimoto, K., Ikeda, T., Katoh, A., Itoh, T. & Itohara, S. (1995) Mice devoid of the glial fibrillary acidic protein develop normally and are susceptible to scrapie prions. *Neuron*, **14**, 29-41.

Gough, R. & Alexander, D. (1973) The speed of resistance to challenge induced in chickens vaccinated by different routes with a B1 strain of live NDV. *Veterinary Record*, **92**, 563-564.

Halasz, F. (1912) Contributions to the knowledge of fowl pest. Vet Doctoral Dissertation. Commun Hungar Roy Vet Schl: Patria, Budapest, 1-36. *Cited in*: Alexander and Senne, 2008.

Hanson, R.P. (1988) Heterogeneity within strains of Newcastle disease virus: Key to survival. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 113-130.

Hanson, R.P. & Brandly, C.A. (1955) Identification of vaccine strains of Newcastle disease virus. *Science*, **122**,156-157.

Hanson, R.P., Spalatin, J. & Jacobson, G.S. (1973) The viscerotropic pathotype of Newcastle disease virus. *Avian Dis*, **17**, 354-361.

Harmon, B. (1998) Avian heterophils in inflammation and disease resistance. *Poultry Science*, **77**, 972-977.

Harrison, L., Brown, C., Afonso, C., Zhang, J. & Susta, L. (2011) Early Occurrence of Apoptosis in Lymphoid Tissues from Chickens Infected with Strains of Newcastle Disease Virus of Varying Virulence. *Journal of Comparative Pathology*, **145**, 327-335.

Heller, E.D., Nathan, D.B. & Perek, M. (1977) The transfer of Newcastle serum antibody from the laying hen to the egg and the chick. *Res Vet Sci*, **22**, 376-379.

Higgins, D.A. (1971) Nine disease outbreaks associated with myxoviruses among ducks in Hong Kong. *Trop Anim Health Prod*, **3**, 232-240.

Hitchner, S.B. & Johnson, E.P. (1948) A virus of low virulence for immunizing fowls against Newcastle disease (avian pneumoencephalitis). *Vet Med*, **43**, 525-530. *Cited in*: Alexander and Senne, 2008.

http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf , (a).

http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf, (b).

Huang, Y., Wan, H.Q., Liu, H.Q., Wu, Y.T. & Liu, X.F. (2004) Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a

novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report. *Arch Virol*, **149**, 1445-1457.

Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D. & Samal, S.K. (2004) The Hemagglutinin-Neuraminidase Protein of Newcastle Disease Virus Determines Tropism and Virulence. *Journal of Virology*, **78**, 4176-4184.

Ishikawa, R. & Biron, C.A. (1993) IFN induction and associated changes in splenic leukocyte distribution. *The Journal of Immunology*, **150**, 3713-3727.

Johnston-Wilson, N.L., Sims, C.D., Hofmann, J.P., Anderson, L., Shore, A.D., Torrey, E.F. & Yolken, R.H. (2000) Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium. *Mol Psychiatry*, **5**, 142-149.

Kaleta, E.F., Alexander, D.J. & Russell, P.H. (1985) The first isolation of the avian PMV-1 virus responsible for the current panzootic in pigeons ? *Avian Pathol*, **14**, 553-557.

Kaleta, E.F. & Baldauf, C. (1988) Newcastle disease in free living and pet birds. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publisher, Boston, pp. 197-246.

Kalman, M., Szekely, A.D. & Csillag, A. (1993) Distribution of glial fibrillary acidic protein-immunopositive structures in the brain of the domestic chicken (Gallus domesticus). *J Comp Neurol*, **330**, 221-237.

Karpala, A.J., Lowenthal, J.W. & Bean, A.G. (2008) Activation of the TLR3 pathway regulates IFNβ production in chickens. *Developmental & Comparative Immunology*, **32**, 435-444.

Kattenbelt, J.A., Stevens, M.P. & Gould, A.R. (2006) Sequence variation in the Newcastle disease virus genome. *Virus Research*, **116**, 168-184.

Koboziev, I., Karlsson, F. & Grisham, M.B. (2010) Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. *Annals of the New York Academy of Sciences*, **1207**, E86-E93.

Kommers, G.D., King, D.J., Seal, B.S. & Brown, C.C. (2001) Virulence of Pigeon-Origin Newcastle Disease Virus Isolates for Domestic Chickens. *Avian Diseases*, **45**, 906-921.

Kommers, G.D., King, D.J., Seal, B.S. & Brown, C.C. (2003a) Pathogenesis of chicken-passaged Newcastle disease viruses isolated from chickens and wild and exotic birds. *Avian Dis*, **47**, 319-329.

Kommers, G.D., King, D.J., Seal, B.S. & Brown, C.C. (2003b) Virulence of six heterogeneous-origin Newcastle disease virus isolates before and after sequential passages in domestic chickens. *Avian Pathology*, **32**, 81-93.

Kommers, G.D., King, D.J., Seal, B.S., Carmichael, K.P. & Brown, C.C. (2002) Pathogenesis of Six Pigeon-Origin Isolates of Newcastle Disease Virus for Domestic Chickens. *Veterinary Pathology Online*, **39**, 353-362.

Kraneveld, F.C. (1926) A poultry disease in the Dutch East Indies. *Ned Indisch Bl* Diergeneeskd, **38**,448-450. *Cited in*: Alexander and Senne, 2008.

Krishnamurthy, S. & Samal, S.K. (1998) Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *Journal of General Virology*, **79**, 2419-2424.

Kumar, H., Kawai, T., Kato, H., Sato, S., Takahashi, K., Coban, C., Yamamoto, M., Uematsu, S., Ishii, K.J., Takeuchi, O. & Akira, S. (2006) Essential role of IPS-1 in innate immune responses against RNA viruses. *The Journal of Experimental Medicine*, **203**, 1795-1803.

Lai, M.C. & Ibrahim, A.L. (1983) Scanning electron microscopy of tracheal epithelium of chickens infected with velogenic viscerotropic Newcastle disease virus. *Avian Dis*, **27**, 393-404.

Lam, K.M. & Vasconcelos, A.C. (1994) Newcastle disease virus-induced apoptosis in chicken peripheral blood lymphocytes. *Veterinary Immunology and Immunopathology*, **44**, 45-56.

Lamb, R.A., Collins, P.L., olakofsky, D.K., Melero, J.A., Nagai, Y., Oldstone, M.B.A., Pringle, C.R. & Rima, B.K. (2005) Family Pararmyxoviridae. In: *Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses*. Fauquet, C.M., Mayo, M.A., Maniloff, J.,

Desselberger, U., & Ball, L.A. (ed.) Elsevier Academic Press, San Diego, 65-68.

Lamb, R.A., Kolakofsky, D. (2001) Paramyxoviridae: the virus and their replication. In: *Fields Virology*, 4th Edit., Knipe, D.M. & Howley, P.M. (ed.) Lippincott Williams and Wilkins, Philadelphia, 1305-1340.

Lancaster, J.E. (1966) Newcastle disease a review 1926-1964. *Monograph No. 3*. Canadian Department of Agriculture, Ottawa.

Lancaster J.E. & Alexander D.J. (1975) Newcastle disease: virus and spread. *Monograph No. 11*. Canadian Department of Agriculture, Ottawa.

Lepekhin, E.A., Eliasson, C., Berthold, C.H., Berezin, V., Bock, E. & Pekny, M. (2001) Intermediate filaments regulate astrocyte motility. *J Neurochem*, **79**, 617-625.

Leslie, J. (2000) Newcastle disease: outbreak losses and control policy costs. *Veterinary Record*, **146**, 603-606.

Levine, P.P. (1964) World dissemination of Newcastle disease. In: *Newcastle Disease, An Evolving Pathogen*, Hanson, R. P. (ed.), University of Wisconsin Press, Madison, WI, 65-69.

Liedtke, W., Edelmann, W., Bieri, P.L., Chiu, F.C., Cowan, N.J., Kucherlapati, R. & Raine, C.S. (1996) GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. *Neuron*, **17**, 607-615.

Locke, D.P., Sellers, H.S., Crawford, J.M., Schultz-Cherry, S., King, D.J., Meinersmann, R.J. & Seal, B.S. (2000) Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. *Virus Research*, **69**, 55-68.

Lomniczi, B., Wehmann, E., Herczeg, J., Ballagi-Pordany, A., Kaleta, E.F., Werner, O., Meulemans, G., Jorgensen, P.H., Mante, A.P., Gielkens, A.L., Capua, I. & Damoser, J. (1998) Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Arch Virol*, **143**, 49-64.

Loo, Y.-M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M.A., García-Sastre, A., Katze, M.G. & Gale, M. (2008) Distinct RIG-I and MDA5 Signaling by RNA Viruses in Innate Immunity. *Journal of Virology*, **82**, 335-345.

Luster, A.D., Alon, R. & von Andrian, U.H. (2005) Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol*, **6**, 1182-1190.

Macpherson, L.W. (1956) Some Observations On The Epizootiology Of NewCastle Disease. *Can J Comp Med Vet Sci*, **20**, 155-168.

Mast, J., Nanbru, C., van den Berg, T. & Meulemans, G. (2005) Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. *Vet Pathol*, **42**, 559-565.

McCall, M., Gregg, R., Behringer, R., Brenner, M., Delaney, C., Galbreath, E., Zhang, C., Pearce, R., Chiu, S. & Messing, A. (1996) Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. *Proceedings of the National Academy of Sciences*, **93**, 6361-6366.

McFerran, J.P. & McCracken, R.M. (1988) Newcastle disease. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 161-183.

Mebatsion, T., Verstegen, S., De Vaan, L.T.C., Römer-Oberdörfer, A. & Schrier, C.C. (2001) A Recombinant Newcastle Disease Virus with Low-Level V Protein Expression Is Immunogenic and Lacks Pathogenicity for Chicken Embryos. *Journal of Virology*, **75**, 420-428.

Meulemans, G. (1988) Control by vaccination. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 318-332.

Middeldorp, J. & Hol, E.M. (2011) GFAP in health and disease. *Prog Neurobiol*, **93**, 421-443.

Miguel-Hidalgo, J.J., Baucom, C., Dilley, G., Overholser, J.C., Meltzer, H.Y., Stockmeier, C.A. & Rajkowska, G. (2000) Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. *Biol Psychiatry*, **48**, 861-873.

Millar, N.S. & Emmerson, P.T. (1988) Molecular cloning and nucleotide sequencing of Newcastle disease virus. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 79-97.

Morimura, T., Hattori, M., Ohashi, K., Sugimoto, C. & Onuma, M. (1995) Immunomodulation of peripheral T cells in chickens infected with Marek's disease virus: involvement in immunosuppression. *The Journal of general virology*, **76 (Pt 12)**, 2979-2985.

Moss, W.J. & Griffin, D.E. (2006) Global measles elimination. *Nat Rev Microbiol*, **4**, 900-908.

Moss, W.J., Ota, M.O. & Griffin, D.E. (2004) Measles: immune suppression and immune responses. *The International Journal of Biochemistry & Cell Biology*, **36**, 1380-1385.

Muller, M.B., Lucassen, P.J., Yassouridis, A., Hoogendijk, W.J., Holsboer, F. & Swaab, D.F. (2001) Neither major depression nor glucocorticoid treatment affects the cellular integrity of the human hippocampus. *Eur J Neurosci*, **14**, 1603-1612.

Nagai, Y. (1993) Protease-dependent virus tropism and pathogenicity. *Trends in microbiology*, **1**, 81-87.

Nagai, Y., Klenk, H.D. & Rott, R. (1976a) Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology*, **72**, 494-508.

Nagai, Y., Ogura, H. & Klenk, H.-D. (1976b) Studies on the assembly of the envelope of Newcastle disease virus. *Virology*, **69**, 523-538.

Nakamura, K., Ohta, Y., Abe, Y., Imai, K. & Yamada, M. (2004) Pathogenesis of conjunctivitis caused by Newcastle disease viruses in specific-pathogen-free chickens. *Avian Pathol*, **33**, 371-376.

Nakamura, K., Ohtsu, N., Nakamura, T., Yamamoto, Y., Yamada, M., Mase, M. & Imai, K. (2008) Pathologic and Immunohistochemical Studies of Newcastle Disease (ND) in Broiler Chickens Vaccinated with ND: Severe Nonpurulent Encephalitis and Necrotizing Pancreatitis. *Veterinary Pathology Online*, **45**, 928-933.

Nichols, N.R., Day, J.R., Laping, N.J., Johnson, S.A. & Finch, C.E. (1993) GFAP mRNA increases with age in rat and human brain. *Neurobiol Aging*, **14**, 421-429.

OIE, Office International des Epizootics. (2005) Evolution of the animal health situation with regard to avian influenza. Aug 29, 2005.

Parry, S.H. & Aitken, I.D. (1977) Local immunity in the respiratory tract of the chicken. II. The secretory immune response to newcastle disease virus and the role of IgA. *Veterinary Microbiology*, **2**, 143-165.

Pattison, M. & Allan, W. (1974) Infection of chicks with infectious bursal disease and its effect on the carrier state with Newcastle disease virus. *Veterinary Record*, **95**, 65-66.

Peeters, B.P.H., de Leeuw, O.S., Koch, G. & Gielkens, A.L.J. (1999) Rescue of Newcastle Disease Virus from Cloned cDNA: Evidence that Cleavability of the Fusion Protein Is a Major Determinant for Virulence. *Journal of Virology*, **73**, 5001-5009.

Pekny, M., Leveen, P., Pekna, M., Eliasson, C., Berthold, C.H., Westermark, B. & Betsholtz, C. (1995) Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. *Embo J*, **14**, 1590-1598.

Phillips, R.J., Samson, A.C. & Emmerson, P.T. (1998) Nucleotide sequence of the 5'-terminus of Newcastle disease virus and assembly of the complete genomic sequence: agreement with the "rule of six". *Arch Virol*, **143**, 1993-2002.

Piacenti, A.M., King, D.J., Seal, B.S., Zhang, J. & Brown, C.C. (2006) Pathogenesis of Newcastle disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. *Vet Pathol*, **43**, 168-178.

Powell, J.R., Aitken, I.D. & Survashe, B.D. (1979) The response of the Harderian gland of the fowl to antigen given by the ocular route. II. Antibody production. *Avian Pathol*, **8**, 363-373.

Qureshi, M. (1998) Role of macrophages in avian health and disease. *Poultry Science*, **77**, 978-982.

Ravindra, P.V., Tiwari, A.K., Ratta, B., Chaturvedi, U., Palia, S.K., Subudhi, P.K., Kumar, R., Sharma, B., Rai, A. & Chauhan, R.S. (2008a) Induction of apoptosis in Vero cells by Newcastle disease virus requires viral replication, de-novo protein synthesis and caspase activation. *Virus Research*, **133**, 285-290.

Ravindra, P., Tiwari, A., Sharma, B., Rajawat, Y., Ratta, B., Palia, S., Sundaresan, N., Chaturvedi, U., Aruna Kumar, G., Chindera, K., Saxena, M., Subudhi, P., Rai, A. & Chauhan, R. (2008b) HN protein of Newcastle disease virus causes apoptosis in chicken embryo fibroblast cells. *Archives of Virology*, **153**, 749-754.

Ravindra, P.V., Tiwari, A.K., Ratta, B., Bais, M.V., Chaturvedi, U., Palia, S.K., Sharma, B. & Chauhan, R.S. (2009a) Time course of Newcastle disease virus-induced apoptotic pathways. *Virus Research*, **144**, 350-354.

Ravindra, P.V., Tiwari, A.K., Ratta, B., Chaturvedi, U., Palia, S.K. & Chauhan, R.S. (2009b) Newcastle disease virus-induced cytopathic effect in infected cells is caused by apoptosis. *Virus Research*, **141**, 13-20.

Reynolds, D.L. & Maraqa, A.D. (2000) Protective Immunity against Newcastle Disease: The Role of Antibodies Specific to Newcastle Disease Virus Polypeptides. *Avian Diseases*, **44**, 138-144.

Robert A, L. (1993) Paramyxovirus Fusion: A Hypothesis for Changes. *Virology*, **197**, 1-11.

Robbins, S.L., & Cotran, R.S. (2009) Cellular adaptations, cell injury and cell death. In: *Pathologic Basis of Disease*, 8th Edit., Kumar, V., Abbas, A.K., Fausto, N. (ed.), Saunders-Elsevier, Philadelphia, pp. 26-32.

Römer-Oberdörfer, A., Werner, O., Veits, J., Mebatsion, T. & Mettenleiter, T.C. (2003) Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. *Journal of General Virology*, **84**, 3121-3129.

Rosenberger, J.K. & Gelb, J., Jr. (1978) Response to several avian respiratory viruses as affected by infectious bursal disease virus. *Avian Dis*, **22**, 95-105.

Rott, R. & Klenk, H.D. (1988) Molecular basis of infectivity and pathogenicity of Newcastle disease virus. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.) Boston, Kluwer Academic Publishers, 98-112.

Russell, P.H. (1988) Monoclonal antibodies in research, diagnosis and epizootiology of Newcastle disease. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA, 131-146.

Russell, P.H. (1993) Newcastle disease virus: virus replication in the harderian gland stimulates lacrimal IgA; the yolk sac provides early lacrimal IgG. *Vet Immunol Immunopathol*, **37**, 151-163.

Russell, P.H. & Ezeifeka, G.O. (1995) The Hitchner B1 strain of Newcastle disease virus induces high levels of IgA, IgG and IgM in newly hatched chicks. *Vaccine*, **13**, 61-66.

Russell, P.H. & Koch, G. (1993) Local antibody forming cell responses to the Hitchner B1 and Ulster strains of Newcastle disease virus. *Veterinary Immunology and Immunopathology*, **37**, 165-180.

Salazar-Mather, T.P., Ishikawa, R. & Biron, C.A. (1996) NK cell trafficking and cytokine expression in splenic compartments after IFN induction and viral infection. *The Journal of Immunology*, **157**, 3054-3064.

Schneider-Schaulies, S., Klagge, I.M. & ter Meulen, V. (2003) Dendritic cells and measles virus infection. *Curr Top Microbiol Immunol*, **276**, 77-101.

Schobesberger, M., Summerfield, A., Doherr, M.G., Zurbriggen, A. & Griot, C. (2005) Canine distemper virus-induced depletion of uninfected lymphocytes is associated with apoptosis. *Veterinary Immunology and Immunopathology*, **104**, 33-44.

Sen, S., Shane, S.M., Scholl, D.T., Hugh-Jones, M.E. & Gillespie, J.M. (1998) Evaluation of alternative strategies to prevent Newcastle disease in Cambodia. *Prev Vet Med*, **35**, 283-295.

Senne, D.A., Pearson, J.E., Miller, L.D. & Gustafson, G.A. (1983) Virus Isolations from Pet Birds Submitted for Importation into the United States. *Avian Diseases*, **27**, 731-744.

Shengqing, Y., Kishida, N., Ito, H., Kida, H., Otsuki, K., Kawaoka, Y. & Ito, T. (2002) Generation of Velogenic Newcastle Disease Viruses from a Nonpathogenic Waterfowl Isolate by Passaging in Chickens. *Virology*, **301**, 206-211.

Sick, C., Schultz, U., Münster, U., Meier, J., Kaspers, B. & Staeheli, P. (1998) Promoter Structures and Differential Responses to Viral and Nonviral Inducers of Chicken Type I Interferon Genes. *Journal of Biological Chemistry*, **273**, 9749-9754.

Spradbrow, P.B. (1992) Newcastle disease in village chickens. Control with thermostable oral vaccines. Proceedings of an International Workshop, Kuala Lumpur, Malaysia 1991. ACIAR, Canberra.

Stevens, J.G., Nakamura, R.M., Cook, M.L. & Wilczynski, S.P. (1976) Newcastle disease as a model for paramyxovirus-induced neurological syndromes: Pathogenesis of the respiratory disease and preliminary characterization of the ensuing encepbalitis. *Infect Immun*, **13**, 590-599.

Steward, M., Vipond, I.B., Millar, N.S. & Emmerson, P.T. (1993) RNA editing in Newcastle disease virus. *Journal of General Virology*, **74**, 2539-2547.

Sun, Q., Sun, L., Liu, H.-H., Chen, X., Seth, R.B., Forman, J. & Chen, Z.J. (2006) The Specific and Essential Role of MAVS in Antiviral Innate Immune Responses. *Immunity*, **24**, 633-642.

Susta, L., Miller, P.J., Afonso, C.L. & Brown, C.C. (2011) Clinicopathological Characterization in Poultry of Three Strains of Newcastle Disease Virus Isolated From Recent Outbreaks. *Veterinary Pathology Online*, **48**, 349-360.

Takimoto, T., Taylor, G.L., Connaris, H.C., Crennell, S.J. & Portner, A. (2002) Role of the Hemagglutinin-Neuraminidase Protein in the Mechanism of Paramyxovirus-Cell Membrane Fusion. *Journal of Virology*, **76**, 13028-13033.

Timms, L. & Alexander, D.J. (1977) Cell-mediated immune response of chickens to Newcastle disease vaccines. *Avian Pathol*, **6**, 51-59.

Tsunekuni, R., Ito, H., Kida, H., Otsuki, K. & Ito, T. (2010a) Increase in the Neuraminidase Activity of a Nonpathogenic Newcastle Disease Virus Isolate during Passaging in Chickens. *The Journal of Veterinary Medical Science*, **72**, 453-457.

Tsunekuni, R., Ito, H., Otsuki, K., Kida, H. & Ito, T. (2010b) Genetic comparisons between lentogenic Newcastle disease virus isolated from waterfowl and velogenic variants. *Virus Genes*, **40**, 252-255.

Vindevogel, H. & Ducnate, J.P. (1988) Panzootic Newcastle disease virus in pigeons. In: *Newcastle Disease*, 1st Edit., Alexander, D.J. (ed.), Kluwer Academic Publishers, Boston, MA,184-196.

von Andrian, U.H. & Mackay, C.R. (2000) T-Cell Function and Migration: Two Sides of the Same Coin. *New England Journal of Medicine*, **343**, 1020-1034.

Wakamatsu, N., King, D.J., Kapczynski, D.R., Seal, B.S. & Brown, C.C. (2006a) Experimental Pathogenesis for Chickens, Turkeys, and Pigeons of Exotic Newcastle Disease Virus from an Outbreak in California during 2002-2003. *Veterinary Pathology Online*, **43**, 925-933.

Wakamatsu, N., King, D.J., Seal, B.S., Samal, S.K. & Brown, C.C. (2006b) The pathogenesis of Newcastle disease: A comparison of selected Newcastle disease virus wild-type strains and their infectious clones. *Virology*, **353**, 333-343.

Wakamatsu, N., King, D.J., Seal, B.S., Peeters, B.P. & Brown, C.C. (2006c) The effect on pathogenesis of Newcastle disease virus LaSota strain from a mutation of the fusion cleavage site to a virulent sequence. *Avian Dis*, **50**, 483-488.

Walker, J.W., Heron, B.R. & Mixson, M.A. (1973) Exotic Newcastle Disease Eradication Program in the United States. *Avian Diseases*, **17**, 486-503.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S. & Fujita, T. (2004) The RNA helicase RIG-I has an

essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*, **5**, 730-737.

Yusoff, K. & Tan, W.S. (2001) Newcastle disease virus: Macromolecules and opportunities. *Avian Pathology*, **30**, 439-455.

Zhu, W., Dong, J., Xie, Z., Liu, Q. & Khan, M. (2010) Phylogenetic and pathogenic analysis of Newcastle disease virus isolated from house sparrow (<i>Passer</i> <i>domesticus</i>) living around poultry farm in southern China. *Virus Genes*, **40**, 231-235.