CHARACTERIZATION AND COMPARATIVE SUSCEPTIBILITY OF HIGHLY PATHOGENIC AVIAN INFLUENZA H_5N_1 VIRUS INFECTIONS IN CHICKENS AND DUCKS

BY

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ABSTRACT

Nigeria experienced outbreaks of Highly Pathogenic Avian Influenza (HPAI) H_5N_1 virus in poultry between 2006 and 2008. Mixed poultry rearing have been postulated as factors responsible for easy spread, while inadequate diagnostic specimens and procedures have militated against prompt diagnosis of the disease. This study was designed to elucidate the comparative susceptibility and diagnosis of H_5N_1 virus infection in chickens and ducks.

Clinical and pathological examinations, agar gel immunodiffusion and viral isolation were used to confirm 468 suspected chickens, ducks, turkey and geese from six (6) geopolitical zones submitted to the reference laboratory at National Veterinary Research Institute, Vom. Spatial data were mapped with Arc-view GIS. Fifty-six of the 80 confirmed H_5N_1-positive backyard poultry cases were compared for proportions (single and mixed species); the association was calculated by odds ratio using MedCalc Software. Immunohistochemistry (IHC), Real-time Reverse Transcriptase-Polymerase Chain Reaction (RRT-PCR), sequencing and alignment using the haemagglutinin cleavage site were used to detect and characterize H_5N_1 in Formalin-Fixed Paraffin-Embedded (FFPE) chicken tissues from ten of the outbreak cases. Comparative pathology; tissue virus predilection and titre were carried out using IHC and RRT-PCR in 10 Muscovy, 10 Pekin and eight Mallard 3-weeks old specific pathogen-free ducks each separately experimentally infected with clade 1 or 2.2 H_5N_1 virus genotypes. Twelve age-matched chickens served as in-contact sentinels.

Clinical signs, lesions and mortalities were severe in older birds, while younger and free-range chickens showed minimal clinical signs and lesions. Lesions were multi-systemic and characterized by severe haemorrhages and necrosis. Mortalities in birds were: 20.6% (north-central), 16.5% (north-east), 15.9% (north-west) and 6.0% (south-west). Wetland areas in north-west and north-east had more positive cases. There were higher risks (OR=3.02) of infection and mortalities in mixed than in single species farms. H_5N_1 RNA virus detection in FFPE tissues was successful in 7 of 10 while gene sequencing was possible only in four. All the viral RNA characterized belonged to the sub-clade 2.2 with >96% homology to similar virus of European origin. Along the 154 nucleotides sequenced, amino acid exchange (mutation; Ala →Thr)

occurred at position 544. Clade 1-infected Muscovy ducks shed more viruses, showed more severe nervous signs and mortality than Pekin and Mallard. Pekin ducks were moderately susceptible to clade 1 but insusceptible to clade 2.2. Mallard ducks were resistant to clinical disease from both viruses. Chickens exposed to infected ducks had 100% mortality four days post-exposure. Eyelids, combs, wattles, thymus, spinal cord, pancreas, cerebrum and bursa of Fabricius had higher RRT-PCR detection than the heart, lung, trachea, liver, spleen and intestine traditionally harvested for HPAI- H_5N_1 antigen detection.

Co-rearing of Muscovy ducks with chicken posed greater risk of transmission of Highly Pathogenic Avian Influenza to the latter. Detection of H_5N_1 virus in formalin-fixed paraffin-embedded chicken tissues was an important finding useful in retrospective diagnosis of HPAI.
Keywords; Poultry, HPAI, H_5N_1 Virus.

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CERTIFICATION

I certify that this work was carried out by AKANBI BABATUNDE OLATUNDE in the Department of Veterinary Pathology, Faculty of Veterinary Medicine University of Ibadan.

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DEDICATION

This thesis is dedicated to God the Father, God the Son and the Holy Spirit for predestinating this path and for being there all the way for me.

TABLE OF CONTENTS

List of Tables

List of Figures

Lists of Plates

Plate 4.22. Muscovy duck 3 days p.i, clade 1 HPAI. Respiratory epithelium. Necrotizing and hemorrhagic rhinitis. Mucous epithelium is hypertrophied, matted and necrotic, infiltrated by mononuclear cells with multifocal desquamation. Vascular congestion and hemorrhage HE. Bar=100µm. 105.

Plate 4.23. Airsac-Pulmonary; Pekin duck 4 days p.i, clade 1 HPAI. Airsacculitis : the mesothelium is diffusely infiltrated by mononuclear cells, mainly lymphocytes HE. Bar=100µm. 106.

Plate 4.24. Heart: Pekin duck 6 days p.i, clade 1 HPAI. Fibrinous pericarditis and myocardial necrosis, diffuse. Lung: pulmonary congestion and edema. 107.

Plate 4.25. Heart: Pekin duck 5 days p.i, clade 1 HPAI. Myocarditis, diffuse and coronary vascular congestion. 108

Plate 4.26. Heart; Pekin duck 6 days p.i, clade 1 HPAI. Myocarditis, severe diffuse: Cardiomyocyte necrosis and mononuclear cell infiltration, mainly lymphocyte and plasma cells . HE. Bar=100µm. 109.

Plate 4.27. Pancreas: Muscovy duck 3 days p.i, clade 1 HPAI. Necrotic pancreatitis, multifocal with vascular congestion. 110.

Plate 4.28. Bursa of Fabricius; Pekin duck 4 days p.i, clade 1 HPAI. Bursitis, follicular necrosis and lymphoid depletion and lymphocytic repopulation. HE. Bar=100µm. 111.

Plate 4.29. Bursa of Fabricius; Muscovy duck 3 days p.i, clade 1 HPAI. Follicular mononuclear cells are focally and strongly immunoreactive to H5 nuclear protein. Bar=100µm. 112.

Plate 4.30. Trigerminal nerve-Nasal septum; Pekin duck 4 days p.i, clade 1 HPAI. Neuronal and axonal necrosis with focally diffuse infiltration of lymphocytes. The neurons are diffusely and strongly immunoreactive to H5 nuclear protein. Bar=100µm. 113.

CHAPTER ONE

1.0 INTRODUCTION

Nigeria experienced outbreaks of highly pathogenic avian influenza virus (HPAIV) H5N1 infection causing high mortalities in both commercial and local poultry populations between January 2006 and October 2007 as an extension of the global outbreaks (Akanbi *et al.,* 2007). By mid-January 2007 Nigeria had lost an estimated 945,862 birds of various species (Fasina *et al.,* 2007) and as at the end of 2007, about 1,264,191 birds had either died or were depopulated. The compensation that was paid out to farmers amounted to N631 million, equivalent to US$5.43 million (Maina*,* 2008). By the year 2003, there was already the awareness on the need to prepare for a possible incursion of HPAIV into Nigeria (Maina, 2006), following global outbreaks of the virus. As far back as early February 2004, the then President of the Federal Republic of Nigeria, President Olusegun Obasanjo, at the 2nd Investiture of College of Veterinary Surgeons Nigeria (CVSN) challenged the Veterinary profession on the need to ensure that HPAI did not enter Nigeria (Maina, 2006). Also in 2004, a committee of experts was set up to prepare a prevention strategy document, which in November 2005 came up with an Emergency Preparedness and Response Plan (EPP) (Maina, 2006). The features of Agricultural sector Emergency Preparedness Plan, included a risk analysis of the 24 wetlands in Nigeria and the 2 major migratory routes of wild birds, evaluation and upgrading of Veterinary services, ban on importation of poultry and poultry products and increased surveillance for the virus (Maina, 2006). Surveillance and Disease Reporting included passive and active surveillance, and regular disease reporting. The active surveillance component involved the epidemiological surveillance of network of 170 points within the country and targeted surveillance of wetlands and farms (Maina, 2006). This included, wild fowl and migrating birds surveillance conducted by a team from the National Veterinary Research Institute (NVRI), between September and November 2005 at the Nguru-Hadejia wetlands covering an area of about 4,125 km^2 (Joannis *et al*., 2008). Similarly, active surveillance was carried out in the same period in the high risk agro-ecological farming areas and amongst live bird (poultry) markets. All these surveillance failed to detect H5 or H7 avian influenza virus (Joannis *et al*., 2008). The EPP also had an immediate/daily disease reporting component, which was implemented and complied with. All of these were put in place before 2006, when there was no official report of HPAIV in Nigeria. First suspicion of HPAIV in Nigeria was at the Sambawa Farm in Kaduna- ABU/NVRI 16/01/06 (Joannis *et al*., 2006; Maina, 2006). Suspected

avian influenza sample was subsequently sent on the 3rd of February, 2006 to the Food and Agricultural Organisation of the United Nations (FAO) Reference Laboratory on Avian Influenza and Newcastle disease in Padova, Italy and the confirmation of HPAIV (H5N1 Profile PQGERRRKKRGLFG) infection was made on the 6th of February, 2006. While the Federal Government of Nigeria made an official declaration of the outbreak on 8th February, 2006 a day after receiving the result (Maina, 2006). In response to the outbreak of HPAI (H5N1) in the poultry population in 2006, the Nigerian government set up an inter-ministerial committee comprising- Health (Federal Ministry of Health), Veterinary/Agricultural (Federal Ministry of Agriculture and Rural Development) and Information Personnel (Federal Ministry of Information) - to tackle the HPAI outbreaks (Joannis *et al*., 2008). Several routine surveillance efforts were jointly carried out at various times by the national team in collaboration with representatives from the FAO, the United States Center for Disease Control and Prevention (US CDC), the World Organisation for Animal Health (OIE), the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVE) and others (Joannis *et al*., 2008). Teams were regularly dispatched to suspected farms nationwide to collect samples and identify infected birds, advise on compensations and carry out cullings (Joannis *et al*., 2008). It is on record that a total of 1525 suspected cases were officially reported to the Federal Government of Nigeria from 97 Local Government Areas in 32 States and the Federal Capital Territory (FCT), of which 299 cases from 25 States and the FCT tested positive (Joannis *et al*., 2008). The only human case in Nigeria, which was officially reported by the World Health Organization on 3rd of February 2007, was diagnosed following a thorough investigation of a fever complicated by respiratory distress which finally led to the death of an average aged woman (Joannis *et al*., 2008) in Lagos. By the last quarter of 2007, outbreaks of HPAI in Nigeria appeared to have been successfully controlled by measures such as "stamping out with compensation," restrictions on movement of poultry, and enhanced surveillance (Fusaro *et al.,* 2010). But the detection of new cases of HPAIV in farms from Kano and Katsina States and in apparently healthy ducks in live bird markets in Gombe and Kebbi States (OIE, 2008, Fusaro *et al*., 2009, 2010) proved otherwise. Phylogeographic analysis conducted by Fusaro and collegues in 2009, identifying the north-central and southwest regions as the two major sources for the HPAIV in Nigeria (Fusaro *et al.,* 2009) may support the hypothesis that the introduction of the virus into Nigeria may be by wild birds and trade in poultry and its products. It is strongly believed that this may be the case because the north-central Nigerian states of Jigawa and Yobe are home to the Hadejia- Nguru wetlands and are said to be characterized by permanent and

seasonal lakes and numerous population of migratory and residential waterfowl (Fusaro *et al.,* 2009). It is also known that this area sustains a large backyard poultry population and the highest concentration of domestic ducks, reared under free-range conditions, providing opportunities for contact between wild birds and backyard poultry (Cecchi *et al*., 2008). On the other hand, the southwestern region of Nigeria, particularly the states surrounding the city of Lagos (Ogun, Oyo, Osun, and Ondo), holds much of Nigeria's poultry industry, estimated to be over 65% of the country's commercial poultry (FAO, 2008). Several investigations have been carried out and are available to the public on the HPAI H5N1 outbreaks in Nigeria, including the virological identification and confirmation (Adene *et al*., 2006; Joannis *et al*., 2006, 2008; Maina, 2006, 2008; Fasina *et al*., 2007, 2008a; Salsberg *et al*., 2007; and Aiki-Raji *et al*., 2008), epidemiology and pathology of early outbreaks (Kumbish *et al*., 2006a, 2006b; and Akanbi *et al*., 2007); molecular characterization (Ducatez *et al*., 2006, 2007; Fusaro *et al*., 2008; and Owoade *et al*., 2008), including regional mortality and morbidity characteristics (Saidu *et al*., 2008). There is no doubt that a great deal of research has been conducted in the area of the Nigerian HPAIV molecular epidemiology and virology but little or no work has been done on the pathology and pathogenesis of the disease. In itself epidemiological and virological research are not sufficient study for the effective diagnosis and control of HPAI in Nigeria. In view of the disease and cost burden of HPAI H5N1 in both commercial and local poultry populations where it had a devastating effect, coupled with persistence or detection of new cases as seen in farms in Kano and Katsina States and also in apparently healthy ducks in live bird markets in Gombe and Kebbi States, it is very pertinent to understand by way of research, the factors responsible for the persistence and/ or resurgence of the virus. It is also important for control efforts, to know the role of domestic ducks in the transmission of the virus. A detailed experiment using a reservoir host (duck) and host (chicken) will give a better understanding of the pathogenesis and pathology that may help surmount the diagnostic challenge faced in the maiden and subsequently, outbreaks in indigenous breeds of poultry, chicken and duck inclusive. A detailed study of the outbreaks, the pathologies seen and the molecular pathology of HPAI in chicken in Nigeria will in no doubt unfold new knowledge and understanding.

1.1 JUSTIFICATION

Several reports have been published on the HPAI outbreaks in poultry in Nigeria, but little is known about the use of immunochemical methods on archival FFPE in investigating epidemiology and pathology. Also, it is known that several factors influences mortality in HPAI-H5N1including mixed species backyard poultry rearing (Bavinck *et al*., 2009). But this has not been established in backyard poultry in Nigeria. For effective diagnosis and control measures against HPAI, a good understanding of the pathogenesis, pathology and immunopathology is important, and this can only be achieved through conducting animal experiments. Therefore a duck and chicken model is proposed for this. This study was undertaken in view of the above gap in research.

1.2 OBJECTIVES

1. To study the epidemiology and pathology of HPAI-H5N1 outbreaks in commercial and backyard poultry between 2006 and 2008 in Nigeria.

2. To investigate the influence of mixed species poultry farming on the mortalities of HPAI-H5N1 outbreaks in backyard poultry between 2006 and 2008 in Nigeria.

3. To determine if archival FFPE tissues from chickens naturally infected with HPAI H5N1 virus in Nigeria are useful for retrospective studies by the use of immunohistochemical methods to detect HPAI-H5N1 antigen and RT-PCR assays for amplifying RNA.

4. To determine the susceptibility, transmission, pathogenesis and pathology in three ducks species (Muscovy, Mallard and Pekin) infected with clade 1 H5N1 virus- Influenza A/duck/Vietnam/TG24-01/05 and clade 2.2 H5N1 virus- Influenza A/Cygnus cygnus/Germany/R65/2006 (homologous to Nigerian HPAI) isolates and their in-contact chickens and ducks

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF AVIAN INFLUENZA

From the late 1870s to 1981, Highly pathogenic avian influenza (HPAI) was known by various names including fowl plague (most common), fowl pest, peste aviaire, Geflugelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe (Stubbs, 1926 and 1948). HPAI was already recognized as an infectious disease in poultry in Italy in 1878 (Perroncito, 1878). Initially, HPAI was confused with the acute septicaemic form of fowl cholera until 1880 when Rivolto and Delprato differentiated the two based on clinical and pathological features. In 1894, a severe outbreak of HPAI occurred in northern Italy. This outbreak was spread by transport of chickens to eastern Austria, Germany, Belgium, and France (McFadyean, 1908; Stubbs, 1926). HPAI was spread throughout Germany as a result of the 1901 Brunswick Fowl Exposition (Stubbs, 1926). In 1901, Centanni and Savonuzzi determined the cause was a filterable agent, but the virus was not identified nor classified as an influenza virus until 1955 (Stubbs, 1948; Schafer 1955; Schafer *et al*., 1993). Early in the twentieth century, HPAI was reported in Switzerland, Romania, Russia, Netherlands, Hungary, Great Britain, Egypt, China, Japan, Brazil, and Argentina (Krohn, 1925; Mohler, 1926; Stubbs, 1948). By the mid-twentieth century, HPAI had been confirmed in most of Europe, Russia, North Africa, Middle East, Asia, South America, and North America (Easterday *et al*., 1972). HPAI was reported in the United States in 1924–1925 and 1929 (Stubbs, 1948). Following the characterization of the filterable agent as influenza A virus (Schafer, 1955), an outbreak during 1959 in chickens of Scotland and during 1961 in common terns (*Sterna hirundo*) of South Africa involved new subtypes of AI viruses, H5N1 and H5N3, respectively (Swayne and Suarez, 2000). This was different from HPAI outbreaks between 1901 and the mid-1950s that involved isolates that today have been classified as H7N1 and H7N7 subtypes (Easterday *et al*., 1997). The terminology "highly pathogenic avian influenza," was adopted as the official designation for the highly virulent form of AI at the first international symposium on avian influenza in 1981(Bankowski, 1981). Since 1959 until 2006 only 24 primary outbreaks of subtypes H5 and H7 were reported world-wide (Werner and Harder, 2006) with the majority occurring in Europe and the Americas. Recent figures stand at 26 primary outbreaks of H5 and H7 (Swayne and Halvorson, 2008). The evolution of H5N1 in geese in

Hong Kong in 1996 and its spread worldwide in Asia, Europe, and Africa with interspecies transmission and many human deaths (Swayne and Halvorson, 2003; WHO, 2006) raised public health concern worldwide. Also, the HPAI H5N1 Asia that arose before 1997 in Southern China subsequently caused numerous outbreaks in poultry farms as well as in live bird markets in Hong Kong. Additionally, human infections with HPAIV H5N1 occurred and six out of eighteen clinically affected people died— marking for the first time ever reported casualties after direct infection with a HPAIV from an avian source (Kalthoff *et al*., 2009). None of the previous outbreaks has reached the size of the ongoing HPAI H5N1 Asia epizootic spreading to numerous countries in East- and Middle-Asia, Europe and Africa since 2004, causing devastating losses to the poultry population. Besides, different HPAI H5 and H7 outbreaks were additionally reported in poultry from various countries all around the world since 2004. So far, predominantly the HPAI H5N1 Asia and also HPAI of subtype H7 have been responsible for the majority of human infections with avian influenza viruses (Kalthoff *et al*., 2009). From late 2003 until 2004 HPAIV H5N1 Asia spread across South-East Asia, including the Republic of Korea, Thailand, Indonesia, Viet Nam, Japan, Hong Kong, Cambodia, Lao People's Democratic Republic, People's Republic of China and Malaysia. HPAIV H5N1 was detected from poultry and wild birds, and for the first time from fatally diseased tigers and leopards (Kalthoff *et al*., 2009). Furthermore, human infections in this period were reported from Thailand and Viet Nam. In April 2005 HPAIV H5N1 Asia caused a mass die-off in wild birds at Qinghai Lake in North Western China. Subsequently, Kazakhstan and Russia confirmed outbreaks at poultry farms with dead wild birds in the vicinity of outbreaks in July 2005 (Kalthoff *et al*., 2009). By October 2005 poultry affected outbreaks were reported from Turkey and Romania. In addition, Croatia found wild birds positive for HPAIV H5N1. Subsequently, in early 2006 twenty European countries (Ukraine, Bulgaria, Greece, Italy, Slovenia, Germany, France, Austria, Bosnia-Herzegovina, Slovakia, Hungary, Serbia-Montenegro, Switzerland, Poland, Albania, Denmark, Sweden, Czech Republic, United Kingdom and Spain), twelve Middle-Eastern countries (Kuwait, Israel, West Bank/Gaza Strip, Iran, Iraq, Egypt, Afghanistan, Jordan, Pakistan, India, Azerbaijan and Georgia), and seven African countries (Nigeria, Niger, Cameroon, Burkina Faso, Sudan, Cote d'Ivoire and Djibouti) detected HPAIV H5N1 Asia from samples of dead poultry or dead wild birds (Kalthoff *et al*., 2009). Meanwhile HPAI H5N1 became endemic in most South-East Asian countries with the worst situation in Indonesia. In 2007, 2008 and 2009 countries from East-Asia, Europe, Middle East and Africa accounted for outbreaks in poultry and wild birds. Egypt declared H5N1 to be endemic in 2008 (Kalthoff *et al*., 2009). Until the end of 2006,

human infection caused by H5N1 virus was confirmed in 263 cases of which 158 were fatal. By 2009, 438 human infections with 262 fatalities were reportedly caused by HPAIV H5N1, the highest number of human fatal infections occurring in Indonesia (115), Viet Nam (56), Egypt (27), China (25) and Thailand (17) (WHO timeline of major events; Cumulative number of confirmed human cases of avian influenza A/ (H5N1) reported to WHO (Kalthoff *et al*., 2009). And now, the death toll figure is up to 295 of 499 known infected individuals in 15 countries from 2003 to 2010 (WHO, 2010). Besides, different HPAIV H5 and H7 outbreaks were additionally reported in poultry from various countries all around the world since 2004. So far, predominantly the HPAIV H5N1 Asia and also HPAIV of subtype H7 have been responsible for the majority of human infections with avian influenza viruses (Kalthoff *et al*., 2009). The most devastating influenza pandemic known to date and designated as 'Spanish flu' (H1N1, 1918) appeared to be caused by a virus that was entirely derived from avian origin (Belshe, 2005). But the pandemic influenza A viruses of 1957 (H2N2) and 1968 (H3N2) arose through reassortment between human and avian viruses.

2.2 SUSCEPTIBLE HOSTS

Avian influenza viruses naturally infect a wide variety of wild and domestic birds, most importantly free-living birds occupying aquatic habitats. AI infections have involved wild terrestrial birds, but it is reported that these birds do not represent a major source or reservoir of AI viruses (Stallknecht 1998). In summary, AI viruses have been isolated from more than 90 species of free-living birds representing 13 different orders: Anseriformes (ducks, geese, and swans), Charadriiformes (e.g., shorebirds [turnstones and sandpipers], gulls, terns, puffins, and guillemots), Ciconiiformes (herons and ibis), Columbiformes (doves), Falconiformes (raptors), Galliformes (partridge and pheasant), Gaviiformes (loons), Gruiformes (coots and moorhen), Passeriformes (perching birds—e.g., mynahs, finches, and weaverbirds), Pelecaniformes (cormorant), Piciformes (woodpecker), Podicipediformes (grebe), and Procellariiformes (shearwater) (Alexander, 1982; Alexander and Gough 1986; Alexander, 1993;Manvell *et al*., 2000; Stallknecht and Shane1988). This represents 61% of known avian species, but the actual number of naturally infected species is most likely much greater (Alexander, 1993). In man-made ecosystems (agriculture, caged, hobby flocks, and exhibition systems), infections have been reported in Psittaciformes (parrots, cockatoos, and parakeets), Casuariiformes (emu), Struthioniformes (ostrich), Rheiformes (rhea), and most domesticated Galliformes and Anseriformes. The latter two groups include chickens, turkeys, Japanese quail

(*Coturnix japonica*), helmeted guineafowl (*Numida meleagris*), bobwhite quail (*Colinus virginianus*), pheasants (various species), chukar partridges (*Alectoris chukar*), geese (*Anser anser domesticus*) and ducks (mallards [*Anas platyrhynchos domesticus*] and Muscovy [*Cairina moschata domesticus*]) (Easterday *et al*., 1997). Some infections of free-living Passeriformes (perching birds—starlings and sparrows) have been attributed with outbreaks on poultry farms where they may have acquired infections from close contact with poultry (Lipkind *et al*., 1982; Morgan and Kelly 1990). Low pathogenicity avian influenza viruses have caused epidemics of respiratory disease in mink, seals, and whales (Callan *et al*., 1995; Englund *et al*., 1986; Geraci *et al*., 1982; Hinhaw *et al*., 1986; Lang *et al*., 1981; Lvov *et al*., 1978; Webster *et al*., 1981). The H5N1 HPAI virus has caused sporadic infections in tigers, leopards, house cats, Owston's palm civets, a stone martin and pigs (FAO, 2006). Most of these cases involved close contact or consumption of infected birds. A few cases of natural infections by AI viruses in humans have been reported. In experimental studies, AI viruses have been shown to infect pigs, ferrets, rats, rabbits, guinea pigs, mice, cats, mink, nonhuman primates, and humans (Beare and Webster, 1991; Easterday *et al*., 1997; Easterday and Tumova, 1972; Hinshaw *et al*., 1981;Kilbourne, 1987;Shortridge *et al*., 1998).

2.3 ETIOLOGY OF AVIAN INFLUENZA VIRUS

Influenza viruses are segmented negative sense single stranded RNA, belonging to the family Orthomyxoviridae (Aly *et al*., 2008). This family is classified into influenza viruses' type A, type B, type C, Thogotovirus, and Isavirus (Buechen-Osmond and Dallwitz, 1996). Only influenza type A viruses infect poultry (Aly *et al*., 2008) and avian influenza viruses (AIVs) are known to be a diverse group of viruses in the *Orthomyxoviridae* family, genus Influenzavirus A and has been categorized into subtypes based on the two surface glycoproteins, the hemagglutinin (H) and neuraminidase (N) (Swayne and Halvorson, 2003). Sixteen (16) different hemagglutinin (H1–H16) and nine (9) different neuraminidase (N1–N9) subtypes (Alexander, 1995), which make 144 possible combinations of H and N subtypes. Influenza A viruses consist of eight segmented, single stranded RNA genomes of negative polarity. The spherically or longitudinally shaped virus particles possess a host cell-derived lipid envelope. The trans-membrane proteins hemagglutinin (HA) and neuraminidase (NA) as well as the integral M2 protein (fig.1), which functions as an ion channel (Lamb and Krug, 2006) are embedded in the virus envelope (Kalthoff *et al*., 2009). Along the inside, the envelope is coated by the matrix protein (M1), surrounding the eight ribonucleoprotein (RNP) complexes (fig.1).

Each RNP complex contains one single RNA segment, encapsulated by nucleoprotein (NP) molecules and the three polymerase proteins PB1, PB2 and PA (Noda *et al*., 2006) This is depicted by the schematic structure shown in fig.2 (Digard, 2009). Trimerized HA proteins serve as viral receptor-binding protein recognizing distinct terminal sialic acid species present at the cell surface. Avian influenza viruses particularly bind to a-2, 3-linked sialic acid, whereas Human influenza A viruses preferably bind to a-2, 6-linked sialic acids. After successful attachment the virus particles are internalized into an endosome and viral RNP complexes are released into the cytoplasm after HA facilitated fusion of viral envelopment and cellular endosomal membrane (Skehel and Wiley, 2000). The fusogenic activity of HA is restricted to mature HA after endo-proteolytical cleavage through tissue specific proteases. The cleavage site of the HA protein of the majority of all avian influenza viruses is composed of only one to two basic amino acids at distinct positions, for example, -1/-4 for H5 and -1/-3 for H7 subtypes (Wood *et al*., 1993). Trypsin-like enzymes preferentially expressed at the surface of respiratory and gastrointestinal epithelia recognize this monobasic cleavage motif (Kalthoff *et al*., 2009). Therefore efficient replication of AIV with a monobasic HA cleavage site is restricted to these tissues leading to only mild disease in poultry. Those viruses are of low pathogenicity (LPAIV). On the contrarily, avian influenza viruses that exhibit a multibasic cleavage motif (minimal consensus sequence of -R-X-K/R-R-) are recognised by subtilisin-like endoproteases that are virtually present in every tissue. Hence, those viruses are capable of replicating in multiple tissues (Horimoto *et al*., 1994, Rott *et al*., 1995) leading to systemic infection and almost 100% mortality in galliforme species. Those viruses with a multibasic HA cleavage site are called highly pathogenic avian influenza viruses (HPAIV), and so far have arisen only from virus subtypes H5 and H7. After release of the RNP complexes into the cytoplasm, they are transported into the nucleus, where viral transcription and RNA replication takes place (Whittaker *et al*., 1996). Replication cycles are completed by assembly of nucleocapsids harbouring replicated genomic RNA and budding at the cellular membrane, into which the viral glycoproteins have previously been inserted. The NA protein is responsible for the cleavage of budding virions from sialic acid residues, which results in infectious viral progeny (Varghese *et al*., 1983). Non-sterile immunity, suboptimal receptor-binding or antiviral drugs cause selective pressure that favour virus mutants with corresponding selective advantages. The pool of genetic variants generated during productive influenza virus infection [the viral RNA-dependent RNA polymerase lack proof reading activity, resulting in one point mutation per 1.5×10^5 nucleotides (Buonagurio *et al*., 1986)], may harbour mutants with selective advantages which may

become dominant under a respective pressure. If driven by immunological pressures, this process is referred to as antigenic drift (Ferguson *et al*., 2003). Exchange of whole gene segments between two viruses, by chance occurring if a single cell is infected by different virus subtypes, may lead to profound change of antigenic determinants, and is termed 'antigenic shift'. Providing that the combination of segments results in replication competent viral progeny, 'reassortants' carrying genetic information from two different parental viruses occur (Webster and Hulse, 2004). Avian influenza viruses have also been further classified into two different pathotypes (low [LP] and high pathogenicity [HP]), based on the ability to produce disease and death in the major domestic poultry species, the chicken (Gallus domesticus) (Swayne and Suarez*,* 2000). Only the viruses of H5 and H7 subtypes, which contain multiple basic amino acids at the cleavage site of the hemagglutinin molecule, have been shown to cause HPAI in susceptible species (European Community, 2005). However, not all H5 and H7 viruses are highly pathogenic. It has been proven that HPAI viruses emerge in domestic poultry from LPAI progenitors of the H5 and H7 subtypes (Garcia *et al*., 1996, Senne *et al*., 1996, Perdue *et al*., 1997).

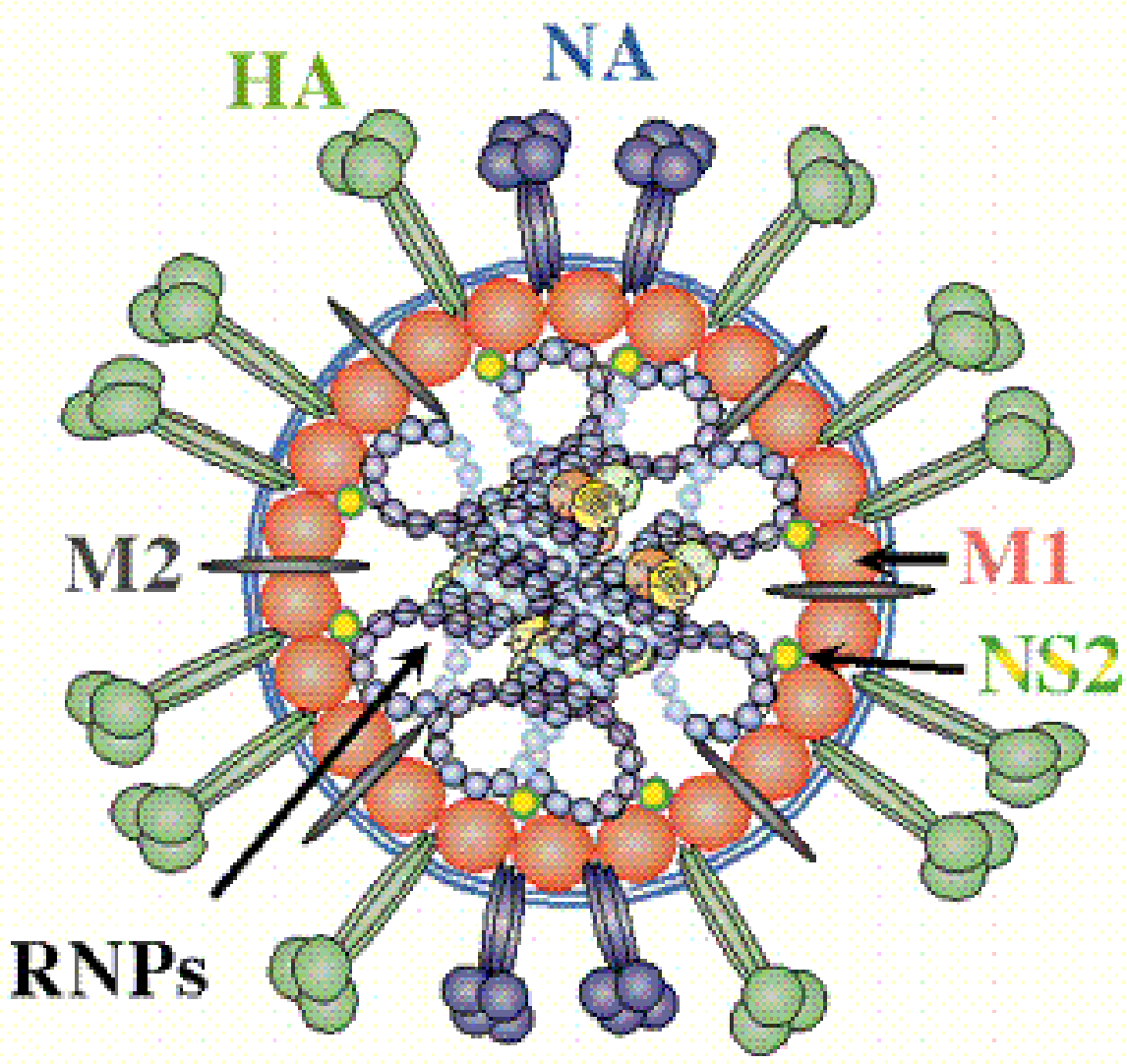

Figure 2.1. Schmatic Structure of Influenza A virus Trans-membrane proteins HA and NA, integral M2 protein and matrix protein (M1), surrounding the eight ribonucleoprotein (RNP) complexes.

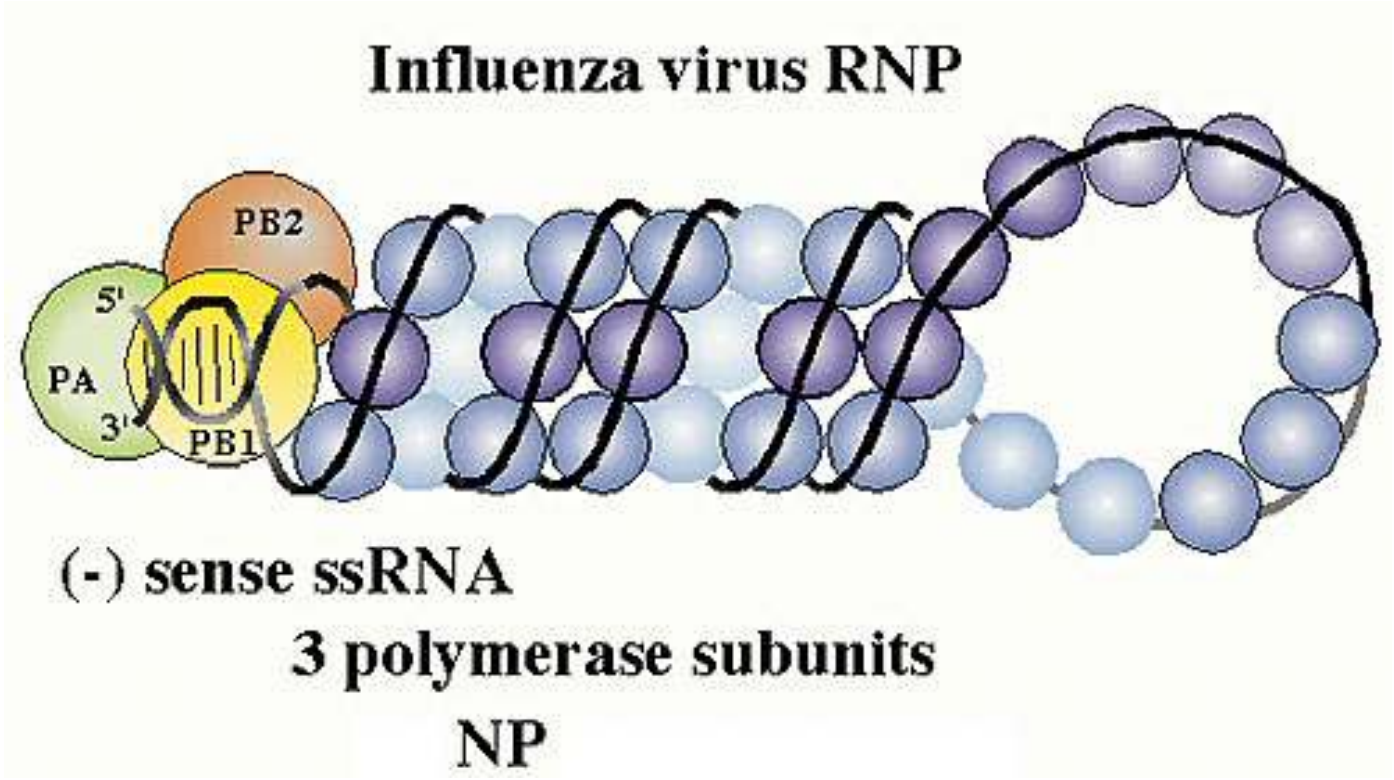

Figure 2.2. Schmatic Structure of Influenza A virus Ribonuclear Protiens (RNP) PB1, PB2, PA

Two of the subtypes of AI viruses, H5 and H7 have been associated with the highly pathogenic form of AI (HPAI). HPAI is caused by AIVs that are extremely virulent, causing up to 100% mortality in domestic chickens (Muzaffar *et al.,* 2010). Their virulence and ability to cause systemic infection has been attributed to the multibasic cleavage motif (minimal consensus sequence of -R-X-K/R-R-) in their hemagglutinin molecule (European Community, 2005), which are recognised by subtilisin-like endoproteases that are virtually present in every tissue (Horimoto *et al*., 1994, Rott *et al*., 1995), making them capable of replicating in multiple tissue. Those viruses with a multibasic HA cleavage site are called HPAIV, and so far have arisen only from virus subtypes H5 and H7. To date, all outbreaks of the highly pathogenic form have been caused by influenza A viruses of the subtypes H5 and H7. Over time, the HPAI H5N1 virus has diversified into multiple phylogenetically distinct lineages, classified as clades 0 to 9 according to the unified nomenclature system (WHO/OIE/FAO, 2008), and since 2006 HPAI H5N1 viruses belonging to clade 2.2 have disseminated across multiple countries in western, eastern, and northern Africa: Egypt, Niger, Cameroon, Sudan, Burkina Faso, Djibouti, Ivory Coast, Ghana, Togo, Benin, and Nigeria (Cattoli *et al*., 2009).

2.4 EPIDEMIOLOGY AND TRANSMISSION

The circulation and re-emergence of HPAIV H5N1 are considered as major concerns of public health and poultry industries (Capua and Alexander, 2010). Tens of millions of birds have died of HPAIV-H5N1, and hundreds of millions of poultry have been slaughtered to control the spread of the virus (Iwami *et al*., 2009). Cases of human HPAIV-H5N1 infection have been reported, and the death toll is up to 295 of 499 known infected individuals in 15 countries from 2003 to 2010 (WHO, 2010). As an emerging infectious disease, HPAIV-H5N1 has a short history (Xin *et al*., 2011). In 1996, HPAIV-H5N1 virus caused a moderate number of deaths in geese in Guangdong province, China (Webster *et al*., 2006). In 1997, this strain of HPAIV-H5N1 infected 18 humans in Hong Kong and caused the death of six people (WHO, 2005). As a result, millions of chickens were slaughtered by the Hong Kong Government (Anonymous, 2006). Six years later, HPAIV-H5N1 re-emerged in East and Southeast Asia (Gilbert *et al*., 2008) and then rapidly spread throughout the Eurasian and African continents from 2004 to 2006 (Enserink, 2006). From 2006 to 2009, HPAIV-H5N1 re-occurred in Asia, Europe, and Africa (Brown, 2010), without further expansion to Australia and America (Dusek *et al*., 2009, Haynes, 2009). HPAIV-H5N1 has threatened the global poultry industry, survival of wild avian species, and human health. In 2005, the virus was isolated during an outbreak among migratory

birds in Qinghai Lake, China, causing an estimated 10% decrease of the global population of bar-headed geese (*Anser indicus*) (Olsen *et al*., 2006). Surveillance for HPAIV H5N1 indicated wild birds in China had 149 positive samples in 14,472 samples (Kou *et al*., 2009), whereas no positive samples of wild birds were reported in the United States (Dusek *et al*., 2009), Australia (Haynes, 2009) and Nigeria (Maina, 2006). Many countries and regions have successfully controlled the virus after its occurrence, whereas some other countries face occasional re-occurrences despite intensive control efforts (Gilbert *et al*., 2008). Influenza A viruses circulate in their natural hosts, wild aquatic birds predominantly of the Orders *Anseriformes* (ducks, geese and swans) and Charadriiformes (gulls, waders and terns). These wild ducks are now know to be natural reservoirs of avian influenza (AI) viruses (Kawaoka *et al.,* 1988;Swayne and Halvorson, 2003) and epidemiologic evidence and experimental infections show that domestic ducks are also susceptible to AI viruses (Cooley *et al.,* 1989; Shortridge *et al.,* 1998; Chen *et al.,* 2004;). Reported evidence from the field and experimental data show that an introduction and subsequent spread of HPAIV (H5N1) in duck flocks is likely clinically silent (Smith *et al.,* 2006) and also in the cases reported, hardly was there any clinical signs suggestive of HPAIV infection, in particular, neurologic manifestations. Transmission occurs primarily by faecal–oral pathways through direct contact or indirect contact with contaminated surface water (Webster *et al.,* 1992). The H5N1 lineage currently circulating in Central Asia, the Middle East, Europe, and Africa is referred to as clade 2.2 (WHO/OIE/FAO, 2008) and has also been described as "EMA" or Qinghai-like in previous publications (Liu *et al.,* 2005;Salsberg *et al.,* 2007). This clade originated in April 2005 during a large outbreak of a phylogenetically distinct H5N1 virus among wild bird populations at Qinghai Lake in western China (Chen *et al.,* 2005; Liu *et al.,* 2005). It rapidly spread west through Central Asia and Europe, eventually reaching Africa in 2006 (Salsberg *et al.,* 2007). In Nigeria, in response to the outbreak of HPAI (H5N1) influenza in the poultry population in 2006, the inter-ministerial committee (Joannis *et al*., 2008), saddled with tackling the HPAIV outbreaks in the country, in a bid to controlling the virus and in order to identify the source of infection continued several routine surveillance efforts. Researchers and other investigators were also working tirelessly, regularly dispatching investigators to suspected farms nationwide to collect samples and identify source of infection and infected birds, advice on compensations and carry out cullings (Joannis *et al*., 2008). It is believed that since the West African sub-region operates as a free trade zone with poor quarantine and border controls, It is highly likely that trans-border movements of humans along with trade of poultry and poultry products and weak biosecurity played a significant role

in the spread of viruses in sublineage D, which contained a large cluster of geographically diverse viruses from infected African nations (Fasina *et al*., 2008a). Sublineages A and D were some of the earliest HPAI H5N1 viruses to affect the continent and the groups of outbreaks caused by these viruses were restricted to the commercial poultry in Nigeria and were similarly found early in some other West African countries and Sudan (between 1 March and 1 April, 2006). It is believed and this strongly suggests that imported infected commercial stock may have been the source of outbreak (Fasina *et al*., 2008a). The viruses in sublineage H which were isolated from vultures, pigeon, guinea fowl, free-range chicken and other birds from a wildlife park were observed to be geographically and chronologically dispersed in Nigeria following infection of the live bird markets (LBMs). Thus the importance of the LBM in the spread of the virus in West Africa is particularly evident (Fasina *et al*., 2008a). Inappropriate disposal methods and isolation of the virus in the LBMs and hatcheries were also considered as evidential for implicating these sources as important source of infection and spread of the virus (Fasina *et al*., 2008a). Previous studies have similarly confirmed the role of wet markets, LBMs and movement of poultry and poultry products without recourse to biosecurity in the viral ecology and spread of avian influenza and other viruses (Guan *et al*., 2000; Henzler *et al*., 2003; Webster, 2004). Poultry movement practices in Nigeria has been known to follow a particular trend: local guinea fowl, ducks, turkeys, local free range chickens and spent hens are usually moved towards the south of the country (Fasina *et al*., 2008a) especially around the festive period (Christmas, Easter, Eid-el-Fitri and Eid-el-Maulud). While Day-old-chicks and input supplies are the major poultry-related products moved up north (Fasina *et al*., 2008a). These movements and trend lines of dates of occurrences of outbreaks have been shown to explain the wide geographical diversities in outbreaks caused by the sublineages originating from northern Nigeria (Fasina *et al*., 2008a) and the highly restricted spread of those from South- West Nigeria (sublineages A and E). Also, it was reported that Nigeria continued to import poultry and poultry products from contaminated area of the world in 2005 despite the ban on such importation and these imports are distributed throughout the country (Gauthier-Clerc et al., 2007). It was also hypothesized that vultures probably contracted infection from disposed carcasses and viscera while infection of pigeons may have occurred due to their co-habitation with infected free-range chickens, turkeys and ducks, as suggested by the timeline of infections and the phylogram (Fasina *et al*., 2008a). The infection of wildlife park birds which occurred in early April 2006, coinciding with Easter and Eid-el-Kabir festive period and the increasing human movement to the park during this period coupled with the location of the park in the epicenter of the northern

Nigeria outbreaks, with no history of previous outbreak, is presumed to have been caused by human introductions. Inadvertent human introduction or an infected source of feed for the meat eating animals within the park is implicated as responsible for such introduction (Fasina *et al*., 2008a). The hypothesis that wild water-birds or migratory fowls were primarily responsible for the outbreaks or spread of HPAI H5N1 in Nigeria and other parts of Africa have been refuted by other works (Feare, 2007; Gaidet *et al*., 2007; Gauthier-Clerc *et al*., 2007 and Fasina *et al*., 2008a). Although, other regions of the world have been infected through migratory birds and legal and illegal importations (Sims *et al*., 2005 and Van Borm *et al*., 2005) and the FAO postulated that backyard farms and free-range village birds are at higher risk of infection (FAO, 2004). Fasina *et al*. (2008) found sectors 2 and 3 classification of the poultry industry by FAO (FAO, 2008), which are closely associated with the large-scale importers and hatcheries/distributors of day-old chicks and operate with minimum to no biosecurity, more widely affected (Fasina *et al*., 2008a). Since its emergence in 2006 in Africa, avian influenza viruses of the H5N1 subtype have spread rapidly to poultry farms in several African countries (Monne *et al*., 2008).The first case of HPAI H5N1 virus in Nigeria occurred in January 2006 in Kaduna State and was confirmed in February 2006 (Adene *et al*., 2006; Joannis *et al*., 2006) to be in the sublineage II, and it disseminated widely across the country during 2006 and 2007 (Monne *et al*., 2008). In the recent past, infection has been recorded in 25 of the 36 Nigerian states and the Federal Capital Territory (Monne *et al*., 2008), and in February 2007, one case of avian influenza was reported in a woman from the southern state of Lagos (Monne *et al*., 2008). Thus, Monne *et al*. (2008) suggested that the extensive circulation of influenza virus (H5N1) in Nigeria raises concerns about human and animal health issues. The H5N1 lineage currently circulating in central Asia, the Middle East, Europe, and Africa is referred to as clade 2.2 (WHO/OIE/FAO, 2008) and has also been described as "EMA" or Qinghai-like in previous publications (Chen *et al*., 2004; Liu *et al*., 2005; Salsberg *et al*., 2007). A previous study by Salsberg et al. (2007) indicated that 2 sublineages (EMA1 and EMA2) were cocirculating in Nigeria in 2006. However 3 sublineages were identified in the study by Ducatez *et al.* (2007), namely sublineage A (corresponding to EMA2) and sublineages B and C (corresponding to EMA1). The 2007 study by Salzberg *et al*. (2007) also identified a virus showing a 4:4 reassortment between genes of sublineages EMA1 and EMA2 (Salsberg *et al*., 2007). Clade 2.2 has further diversified, forming the genetic third-order clade 2.2.1 (WHO/OIE/FAO, 2009) and three genetically distinct sublineages (I, II, and III) (Monne *et al*., 2008; Starick *et al*., 2008; Cattoli *et al*., 2009). All are found in Africa. Clade 2.2.1, which has been prevalent in Egypt, Israel, and the Gaza

Strip from 2006 to 2008, was also detected in Nigeria in 2006 (Ducatez *et al.,* 2007). In July 2008 new cases of HPAI H5N1 from a sublineage never previously detected in Africa (sublineage III) were found in farms in Kano, Katsina; Gombe and Kebbi States live birds markets (OIE*,* 2008*;* Fusaro *et al.,* 2009). Making Nigeria the only African country where viruses belonging to clade 2.2.1 and to three different sublineages (I, II, and III) of clade 2.2 have all been detected (Fusaro *et al.,* 2010). At least three different reassortment events between sublineages have been documented in Nigeria (Fusaro *et al.,* 2010). Salzberg *et al*. (2007) identified the first reassortant strain (referred to as "R1"), in which four genome segments (hemagglutinin [HA], NP, NS, and PB1) belong to sublineage I and the other four segments (NA, MP, PA, and PB2) are derived from sublineage II (Salsberg *et al*., 2007). Subsequently, phylogenetic analysis showed that a 2007 reassortant strain (referred to as "R3") contained the HA and NS segments from sublineage I and the other six segments from sublineage II (Monne *et al*., 2008; Owoade *et al*., 2008). Another reassortant virus (which was referred to as "R5") contained only the NS gene segment from sublineage I, while the other seven segments were derived from sublineage II (Owoade *et al*., 2008). Although the genetic diversity of the Nigerian HPAI H5N1 virus population has been well characterized, including multiple introductions of the virus into Nigeria and several reassortment events, little is known about the evolutionary and population growth dynamics of the virus within Nigeria (Salsberg *et al*., 2007). Several reports concerning the introductions into European countries of distinct sublineages of clade 2.2 HPAI H5N1 viruses through wild birds, for example, in Germany, Denmark, Hungary, France, and Italy (Bragstad *et al*., 2007; Salsberg *et al*., 2007; Starick *et al*., 2008 and Szeleczky *et al*., 2009) have been documented. Co-circulation of multiple genetically distinct sublineages has also been reported in others countries, such as China (Vijaykrishna *et al.,* 2008), Indonesia (Lam *et al.,* 2008), Thailand (Suwannakarn *et al.,* 2009) and Vietnam (Pfeiffer *et al.,* 2009). Ducatez and co-workers (Ducatez *et al.,* 2006) previously identified three HPAI H5N1 virus strains circulating in Nigeria. They had close genetic relationships with viruses from Kurgan, Astrakhan and Egypt. Salzberg et al. (2007) demonstrated that African isolates have common ancestry with viruses from Euroape and Asia.

2.5 INCUBATION PERIOD

The incubation periods for the various diseases caused by avian influenza viruses ranged from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds and up to 14 days in a flock (Easterday *et al*., 1997). In timed studies, intranasally inoculated

chickens with H5N1 HPAI virus from Mongolia produced clinical signs within 24 hrs. The incubation period is dependent on the dose of virus, the route of exposure, the species exposed, and the ability to detect clinical signs (Easterday et al., 1997). However, for international regulatory purposes, OIE recognizes 21 days as the incubation period (OIE, 2006) where incubation period is defined as: the time from exposure to the onset of clinical signs, however it is opined this criterion may not be applicable to all AI viruses, especially the LPAI viruses. Because, many infections by LPAI viruses do not cause clinical disease in all ages and all species of birds. It was suggested that "Infectious period", as defined as the time from exposure or detection of the virus to when the virus is no longer detected, may be more applicable for control and eradication purposes, especially in dealing with LPNAI viruses (Swayne and Halvorson, 2008).

2.6 CLINICAL SIGNS AND MORTALITY OF HPAI

It has been said that exposure, which is access to the virus, is critical in beginning the process of avian influenza infection and that with some AI virus strains and some hosts, exposure to virus may not result in infections, especially if the route of exposure is inappropriate, the exposure dose is below the infection threshold, immunity against the virus strain is present, the virus strain is not adapted to the specific host species, or a combination (Swayne, 2007). Also, important to infection by AI virus is host adaptation, which is the result of progressive genetic changes in a virus, resulting in increasing efficiencies of binding, replication, and release of the virus from a specific host species (Swayne, 2007). Viruses with low adaptation fail to replicate in the host species unless there is high exposure dose or secondary factors that increase host susceptibility e.g lowered immunity. By contrast, with a high degree of adaptation to the host species, low doses of the virus strain are needed to produce infection and host adaptation is maximal for a single host species (Swayne, 2007). Although in evolutionarily closely related species, the virus strain may show some, but a lesser degree of adaptation than the optimal host species. Infectivity, which is the ability of the virus strain to bind to cells of a specific host, replicate, and release infectious virus, is critical to AI virus infection (Swayne, 2007). Three potential clinical outcomes have been observed with AI infection in birds: no clinical signs, mild disease, and severe disease with death (Swayne, 2007). Pathobiological changes are abnormal physiological and anatomic changes that occur as a result of virus replication within the cell, tissue, organ, or a combination. In general, as virus replication titers increase, so do the severity of pathobiological changes such as gross and microscopic lesions with the most pathogenic virus strains

causing major cell damage and death if it is sufficiently severe to affect critical organs. Finally transmissibility, which is the natural host-to-host spread, is dependent upon an adapted virus; exposure to the virus through infected animals or fomites; and a naıve, susceptible host (Swayne, 2007). In gallinaceous domestic poultry, infection with HPAI viruses produce severe depression, severe decrease in feed and water consumption, high morbidity and mortality rates, sudden death, and occasionally nervous signs if they survive the peracute syndrome. However, the frequency of clinical signs and gross lesions varies with virus and species of bird and are not consistent in all birds (Swayne, 2007). HPAI infections in gallinaceous birds results in mortalities of up to 100% within 48 hours (Swayne and Suarez, 2000; Pekins and Swayne, 2001). Individual birds are listless, exhibit oedema, cyanosis of the comb, wattles and legs besides diarrhoea. Sudden deaths without any symptoms may also occur. Less vulnerable poultry species such as ducks, geese, ratites, and pigeons typically exhibit nervous symptoms including ataxia, torticollis and seizures (Kwon *et al*., 2005; Werner *et al*., 2007). Some duck species even show no or limited virus replication and few clinical signs (Alexander *et al*., 1978, 1986; Perkins and Swayne, 2002). HPAI (H5N1) has been detected from tracheal swabs collected from apparently healthy domestic ducks during surveillance activities at live bird markets (Fusaro *et al*., 2009). Age-related susceptibility has been reported in turkeys and ostriches infected with low pathogenic AI (LPAI) viruses (Capua *et al*., 1999, 2000). The severity of the clinical signs and the capacity of recovery were related to the age of the bird. However, HPAI viruses produce high morbidity and mortality in gallinaceous domestic poultry regardless of age (Swayne and Halvorson, 2003; Swayne and Pantin-Jackwood, 2006). In avian hosts, the clinical picture of an HPAIV infection depends, among other factors, on the species affected (Alexander, 2000). In particular, domestic waterfowl showed substantial variations in clinical features resulting from infection with strains of HPAIV (H5N1) of Asian origin (Sturm-Ramirez *et al*., 2005). Factors influencing the clinical course relate to species, age of animals, and the virus strain (Pantin-Jackwood and Swayne, 2007; Pantin-Jackwood *et al*., 2007). It has been observed that through silently but productively infected ducks, an endemic status of HPAIV (H5N1) infection can be established and perpetuated (Songserm *et al*., 2006). Strains isolated from such endemic infections induce no clinical symptoms in ducks but retain high pathogenicity for chickens and turkeys (Harder *et al*., 2009). In intranasal and oral administration of HPAIV *A/ Duck/Vietnam/12/05* (H5N1) to ducks, clinical signs were observed to have started at 2 days post infection (dpi), with conjunctivitis and slight depression, progressing over a period of 1–3 days to severe neurologic signs consisting of torticollis,

incoordination, tremors, and seizures. Survival times varied from 3 to 7 dpi. General non-specific signs like weakness, somnolence and sternal or lateral recumbency were observed in chickens infected with HPAI H5N1 virus in the maiden case in Nigeria (Adene *et al*., 2006). Also, changes in fecal colouration e.g brownish, yellowish-white or greenish diarrhea were seen and were associated with anorexia. There was dyspnoea, moist rales, sneezing and coughing, while stretching of the neck, convulsion and torticollis were seen in 2-9 weeks old chickens (Adene *et al*., 2006). Majority of the mature chickens presented oedema of the head and facial region with extensive cyanosis or hemorrhagic patches of the combs and wattles, the shanks and toes were congested and dark red in some chickens (Adene *et al*., 2006; Joannis *et al*., 2006). Our research team in NVRI observed variable clinical signs from the index case of HPAI in Nigeria, and these were associated with age of bird, sex and concurrent diseases (Kumbish *et al*., 2006a). The clinical signs include depression, somnolence, and sneezing, mucous discharges from the oral and nasal cavity, cyanotic combs and wattles. Others are subcutaneous haemorrhages of the cervical and abdominal regions. In addition, shanks and digits were hyperemic with oedema of metatarsal pads, torticollis and unsteady gait, yellowish-green to whitish diarrhea and soft shelled eggs to shelless eggs were consistently observed (Kumbish *et al*., 2006a).

2.7 PATHOGENESIS AND PATHOLOGY OF HPAI

Avian influenza is an important disease of zoonotic origin that has caused morbidity and mortality in domestic animals, wildlife and humans (Muzaffar *et al*., 2006; Olsen *et al*., 2006; Gauthier-Clerc *et al*., 2007). HPAI is an acute, generalized, fatal disease characterized by systemic infections in gallinaceous poultry, water fowl, and other avian and mammalian hosts including humans and can cause outbreaks of severe notifiable disease of zoonotic importance (Swayne and Suarez, 2000). The H5N1 HPAI virus infection has been a great concern not only for the poultry industry but also for human health since 1997**.** The H5N1 HPAI viruses have been observed to vary in the lesions they produce depending on host species, host age, and strain of virus (Pantin-Jackwood and Swayne, 2007; Pantin-Jackwood *et al*., 2007). It has been observed that since 1997, the H5N1 HPAI virus has evolved into multiple strains that vary in pathogenicity for different bird species (Swayne, 2007). Many of the strains isolated during 1999–2002 from ducks in China when experimentally inoculated into domestic ducks have been seen to cause infection with shedding from oropharynx and cloaca, but they did not cause illness or death (Chen *et al*., 2004). For domestic ducks, using the 2-wk-old duck

model and the same intranasal dose of virus (10^6 50% EID_{50}), it was observed that new strains have evolved with the ability to produce illness and death along with replicating in internal organs such as the brain (Swayne and Pantin-Jackwood, 2006). Initially between, 1997–2000, the viruses caused local virus replication in the respiratory tract with associated mild respiratory lesions. However, in 2001, a strain was isolated from frozen duck meat imported from China to South Korea that was able to replicate and spread systemically in domestic ducks without producing clinical signs or death, but virus was isolated from and visualized by immunohistochemistry in meat and brain (Tumpey *et al*., 2002). The pathobiology of a prototype H5N1 HPAI virus A/chicken/Hong Kong/220/1997, were divided into four groups (Perkins and Swayne, 2002). In group 1, the virus produced a severe systemic disease in gallinaceous birds; chickens [*Gallus domesticus*], turkeys [*Meleagris gallopavo*], Japanese quail [*Coturnix coturnix japonicus*], Bobwhite quail [*Colinus virginianus*], pearl guineafowl [*Numida meleagris*], ring-neck pheasant [*Phasianus colchicus*], and chukar partridges [*Alectoris chukar*]) and zebra finches (*Taeniopygia guttata*), with 100% morbidity and >75% mortality. Typically, the birds exhibited severe listlessness before death, and some had neurological dysfunction. Some birds may die peracutely without exhibiting clinical signs. The virus was observed to replicate in vascular endothelium and phagocytic leucocytes (chickens and turkeys) or in parenchymal cells such as in the heart, adrenal, pancreas, and brain (other gallinaceous birds and zebra finches) with accompanying necrotic and inflammatory lesions. In group 2, the virus produced severe lesions in two to three critical organs of domestic geese (*Anser anser domesticus*), emus (*Dromaius novaehollandiae*), house finches (*Carpodacus mexicanus*), and budgerigars (*Melopsittacus undulatus*). The morbidity was delayed compared with group 1, and mortality ranged from 0 to 75%. Neurological signs were common because the virus has a strong tropism for the central nervous system, although heart and pancreas also were frequently affected. In group 3, the virus produced in Pekin ducks (*Anas platyrhyncos*), house sparrows (*Passer domesticus*), and laughing gulls (*Larus atricilla*) minimal clinical signs and no mortality. Lesions were limited to predominantly the respiratory tract, but to a lesser extent the heart and gonads. Virus titers in tissues with lesions were usually low. In group 4 (pigeons [*Columba livia*] and European starlings [*Sturnus vulgaris*]), the virus failed to produce evidence of infection or infection was infrequent, and the titers were minimal and without pathological consequence. In 2002–2003, new strains were identified that caused mortality in experimentally inoculated domestic ducks (Chen *et al*., 2004; Ellis *et al*., 2004; Sturm-Ramirez *et al*., 2004; Lee *et al*., 2005). The mortality was the result of systemic infections and was associated with increasing replication titers within respiratory

tract (oropharyngeal swabs) and brain compared with nonlethal infections (Chen *et al*., 2004; Ellis *et al*., 2004; Sturm-Ramirez *et al*., 2004; Lee *et al*., 2005). Pathobiologically, the viruses have changed from being in groups 3 and 4 with increasing severity of lesions in respiratory tract (group 3) to severe lesions in nervous and cardiovascular systems (group 2) to some newer strains producing severe lesions in multiple organs and tissues (group 1) (Perkins and Swayne, 2002). In group 1, the lesions and pathogenesis of the infection and disease are identical between chickens and 2-wk-old ducklings. However, this lethality is age dependent with some strains only causing high mortality in 2- and not 5-wk-old domestic ducks (Swayne and Pantin-Jackwood, 2006). In chickens, the most frequently infected species with HPAI viruses, common lesions include edema to necrosis of comb and wattle, edema of the head and legs, subcutaneous hemorrhage of legs, lungs that are filled with fluid and blood, and small hemorrhages on internal organs such as coronary fat. All these lesions point to alternations in the cardiovascular system, principally affecting vascular endothelium and the resulting viremia. The pathogenesis of infections by HPAI viruses has been extended by study of histological lesions and immunohistochemical localization for AI viral antigens (Swayne, 2007). In commercial layers, the systems affected included mainly circulatory, respiratory and the gastrointestinal (GIT) as observed by earlier investigators from the index case of HPAI in Nigeria (Adene *et al*., 2006, Joannis *et al*., 2006). It was observed that though the chicken carcasses were in good bodily conditions, most of the external appendages and the viscera had gross lesions (Adene *et al*., 2006). The combs and wattles were either generally cyanosed and / or haemorrhagic in some, while the shanks and digits were congested. Catarrhal tracheitis was observed in all carcasses, with cloudy and diphtheritic airsacculitis while there was no visible lesion in the lungs of the chicken in the first two days. The liver, spleen and kidneys in each carcass were enlarged, congested or diffusely necrotic. Ecchymotic haemorrhages were seen on the serosa of the intestines (Joannis *et al*., 2006, Adene *et al*., 2006). There was catarrhal enteritis, especially in the duodenum; while the caecal tonsils were thickened and haemorrhagic (Adene *et al*., 2006). A report of the presence of nervous signs and lesions with no particular reference to age (Joannis *et al*., 2006) and with reference to adult birds (De Benedictis *et al*., 2007) was observed by the two authors. In addition, Adene *et al*., 2006, described the clinico-pathological and husbandry features associated with the maiden diagnosis of AI in Nigeria which included lesions in circulatory, respiratory and in the GIT and nervous system signs observed only in young birds. Also the practices of rearing birds of different ages and with other species were highlighted. Other reports were limited to cases seen in 2006 only (Kumbish *et al*., 2006a and De

Benedictis *et al*., 2007), and included congestion and haemorrhages of nasal sinuses with marked serosal and mucosal congestion of trachea, cloudy airsacs and frothy lungs. Petechiation and ecchymoses were seen on the epicardial sufaces and within the rib cages, and on abdominal fat depot, serosa of duodenum, jejunum, mucosa of proventriculus and on ovarian follicles. Peritonitis of varying degrees characterized by mild to moderate amount of fibrinous exudates and relatively large amount of yellowish abdominal fluid (Kumbish *et al*., 2006a). Histologic lesions were observed in multiple organs and were characterized by exudation, haemorrhage, necrosis and inflammation or a combination of these (Akanbi *et al*., 2007). In particular was necrosis of pancreatic acinar cells and hepatocytes (Akanbi *et al*., 2007). During the HPAI outbreaks in Nigeria in affected backyard flocks, acute mortalities were experienced but with little or no premonitory signs and with or without gross lesions of petechiation of coronary fat as we have earlier observed (Akanbi *et al*., 2007). The pathology of natural infection of Clade 2.2 Influenza A/Cygnus cygnus/Germany/R65/2006 in both adult mute and whooper swan showed no external gross pathologic lesion in most of the swans (Teifke *et al*., 2007, Weber *et al*., 2007). Only juvenile swans at necropsy, showed the most consistent and predominant lesions which were multifocal, sharply demarcated, and partly coalescent hemorrhages with necrosis in the pancreas (Teifke *et al*., 2007). Severe congestion of the lungs with pronounced alveolar and bronchiolar edema was characteristic, but this was not present in all infected birds. Subepicardial and scattered intramyocardial hemorrhages or petechiation in adipose tissue were additional but rather inconsistent findings. In many cases, liver and spleen were moderately enlarged and congested (Teifke *et al*., 2007). Major microscopic lesions and the incidence of viral antigen detection in stained tissue samples were found in the brain, pancreas, and liver. Almost all animals displayed multifocal and severe lymphoplasmacytic encephalitis in the cerebrum and severe necrosis and loss of Purkinje cells in the cerebellum. Within the neuropil there was vacuolation of the white and gray matter (edema), especially around Virchow-Robins spaces, which were expanded by marked cuffs, composed of lymphocytes, histiocytes, and plasma cells. Randomly distributed foci of neuronal necrosis and neuronophagia with increased numbers of partly swollen glial cells were scattered throughout the neuropil. Focal hyperemia and hemorrhage were present inconsistently in the gray matter. Rarely, spinal cord ependymal cells were hypereosinophilic, necrotic, and sloughed (Teifke *et al*., 2007). The liver showed randomly distributed and partly coalescent coagulative necrosis surrounded by various numbers of heterophils and lymphocytes. In the pancreas multifocal necrosis of the acini was present constantly. The inflammatory reaction was only mild and preferentially confined

to the necrotic exocrine pancreatic acini but not affecting the islets of Langerhans. Adrenals displayed multifocal, well-circumscribed areas of cortical and medullary cell necrosis that were infiltrated by few heterophils. Sections of the spleen and Peyer's patches showed multifocal, mild lymphocyte necrosis with replacement by cellular debris and fibrin. In the lungs there was marked congestion, edema, and hemorrhage with slight endothelial cell swelling. Scattered throughout the myocardium was mild hemorrhage. Nasal mucosa, trachea, gastrointestinal tract, cloaca, bone marrow, kidneys, gonads, thyroids, and skin had no lesions (Teifke *et al*., 2007). Immunohistochemical demonstration of influenza virus antigen in pancreas, adrenals, liver, and brain was said to be strongly consistent with histological lesions. In the cerebrum a large number of viable and degenerating neurons and glial cells showed strong intranuclear and intracytoplasmic staining for NP antigen. Purkinje cells and cells of the granular layer of the cerebellum also reacted positively for viral antigen (Teifke *et al*., 2007). Furthermore, ependymal cells lining the central canal of the spinal cord were strongly positive but only in a few animals. Areas of coagulative necrosis in the liver, pancreas, and adrenals were surrounded by rims of numerous antigen-positive cells. Within spleen, bone marrow, and GALT, few mononuclear cells, arteriolar endothelium, and rarely lymphocytes were infected. In the lungs, NP antigen could be identified in a few capillary and arteriolar endothelial cells. Individual birds displayed antigen-positive cells in the heart (cardiomyocytes), kidney (tubular epithelium), proventriculus (mucosa), thyroid (follicular epithelium), and trachea (mucosal epithelium). Chickens, regarded as the most frequently infected species with HPAI viruses, common lesions are said to include edema to necrosis of comb and wattle, edema of the head and legs, subcutaneous hemorrhage of legs, lungs that fill with fluid and blood, and small hemorrhages on internal organs such as coronary fat (Swayne, 2007). All of these lesions are said to point to alternations in the cardiovascular system, principally affecting vascular endothelium and the resulting viremia (Swayne, 2007). In commercial layers, the systems affected included mainly circulatory, respiratory and the GIT as observed by earlier investigators from the index case of HPAI in Nigeria (Adene *et al*., 2006; Joannis *et al*., 2006). Neurologic signs and lesions were observed in the index case in young stocks (Adene *et al*., 2006) and in adult birds (Joannis *et al*., 2006; De Benedictis *et al*., 2007). During the HPAI outbreaks in Nigeria in affected backyard flocks, acute mortalities were experienced but with little or no premonitory signs and with or without gross lesions (Akanbi *et al*., 2007). Gross lesions included oedema and necrosis of comb and wattles, subcutaneous oedema of the head and legs, cutaneous haemorrhages of legs, lungs filled with fluid and blood, and small haemorrhages on internal organs such as coronary fat, severe

vascular lesions, pancreatic and hepatic necrosis. Histological lesions were observed in multiple organs and were characterized by exudation, haemorrhage, necrosis, inflammation, or a combination of these. In particular, the lung, heart, brain, spleen, pancreas, kidneys, combs and wattles were the most consistently affected, which was consistent with the high amount of viral antigens in the parenchyma of these organs. In addition, immunohistochemistry (IHC) was useful in detecting virus antigen in Formalin-fixed and paraffin-embedded (FFPE) tissues (Akanbi *et al*., 2007). Influenza virus antigens were observed by immunohistochemistry mainly in the nucleus of parenchymal and endothelial cells. Massive viral antigen was distributed in the endothelium of sinusoids and arteries and necrotic foci in the livers, endothelium and necrotic foci in the spleen, and in cardiomyocytes and endocardium, necrotic foci of lymphoid tissues in the lamina propria of the intestine, and occasional in intestinal epithelial cells, vascular endothelium in the lamina propria of proventriculus, the surface epithelium of the gizzard, the theca interna of ovary and the epithelial cells in the oviduct. Virus antigens were also seen in the tubular epithelium and endothelial glomerular tufts in the kidneys. It was possible to demonstrate HPAIV antigen e.g. in neurons of the cerebrum and in interstitial cells and in the endothelium of subcutaneous blood vessels of the comb (Akanbi *et al*., 2007).

2.8 DIAGNOSIS OF AVIAN INFLUENZA

A definitive diagnosis of AI is established by 1) direct detection of AI viral proteins or genes in specimens such as tissues, swabs, cell cultures, or embryonating eggs; or 2) isolation and identification of AI virus. A presumptive diagnosis can be made by detecting antibodies to AI virus. During outbreaks of HPAI, mortality rates, clinical signs and lesions may be useful as part of the case definition in deciding which farms to quarantine and possibly for depopulation of birds for eradication purposes (Swayne and Halvorson, 2008). Avian influenza viruses have been recovered from the tracheal, oropharyngeal or cloacal swabs of either live or dead birds. This is because most HP and LPAI viruses replicate in the respiratory and intestinal tracts. The swabs are placed in a sterile transport medium containing high levels of antibiotics to reduce bacterial replication (Swayne *et al*., 1998). Tissues, secretions, or excretions from these tracts are appropriate for virus isolation or detection. Tissues can be collected and placed into sterile plastic tubes or bags. In the case of systemic infections produced by HPAI viruses, virtually every organ can yield virus because of the high levels of viremia or replication in parenchymal cells. Samples are stored at 4°C for virus

detection test within 48 hours or stored at –70°C, if the samples must be held for additional time (Swayne and Halvorson, 2008).

2.8.1 Direct Detection of AI Viral Proteins or Nucleic Acids

Demonstrating influenza virus RNA or viral proteins in samples from animals is routinely used as a diagnostic screening test (Swayne and Halvorson, 2008). Several commercial and laboratory specific antigen detection kits are available for detection of influenza A nucleoprotein. These have been used to detect influenza viral antigen in avian samples and allantoic fluid of inoculated embryonating chicken eggs (Davidson *et al*., 1998, Kodihalli *et al*., 1993, Slemons and Brugh 1998; Woolcock and Cardona, 2005). These antigen capture immunoassays vary in sensitivity with the best tests being 3–4 logs less sensitive than virus isolation (Woolcock and Cardona, 2005). Also, polyclonal and monoclonal antibodies are useful for localizing viral antigen in tissues by immunofluorescent or immunoperoxidase staining methods (Skeeles *et al*., 1984;Slemons and Swayne 1990; Van Campen *et al*., 1989a), and radiolabeled gene probes for *in situ* hybridization can locate cells involved in viral replication in tissues of infected birds (Van Campen *et al*., 1989b). Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (RRT-PCR) methods are in used in some laboratories for experimental studies and field case diagnosis of AI (Akey, 2003; Spackman *et al*., 2002; Xie *et al*., 2005). The RRT-PCR has a 3 hour test time and a sensitivity and specificity comparable to virus isolation procedures (Fouchier *et al*., 2000). Screening of tracheal or oropharyngeal samples can be done using a matrix gene RRT-PCR test and if positive, the samples are tested with H5 and H7 subtype specific RRT-PCR tests (Spackman *et al*., 2002).

2.8.2 Virus Isolation

Methods for the isolation and identification of influenza viruses have been described in detail (CDC, 1982, Easterday *et al*., 1997; Swayne *et al*., 1998). Chicken embryos, 9–11 days old, are inoculated via the allantoic cavity with approximately 0.2 mL of sample (Woolcock *et al*., 2001). A few viruses may grow rapidly and kill the embryos by 48 hours; however, in most cases the embryos will not die before this time. After 72 hours, or at death, the eggs should be removed from the incubator, chilled, and allantoic fluids collected (Swayne and Halvorson, 2008). The presence of virus is demonstrated by hemagglutinating activity using chicken erythrocytes; however the presence of NDV must be excluded (Swayne and Halvorson, 2008). Generally, if virus is present in a sample, there will be sufficient growth in the first passage to result in hemagglutination, and repeated passage

is unnecessary. Repeated passage of samples increases the risk of cross-contamination in the laboratory (Swayne and Halvorson, 2008).

2.8.3 Virus Identification

Standardized methods for testing the egg fluids for the presence of hemagglutinating activity using chicken erythrocytes by macro- or micro-techniques are employed (CDC, 1982; Easterday *et al*., 1997; Swayne *et al*., 1998). Allantoic fluid positive for hemagglutination is used for virus identification. It is important to distinguish between the hemagglutinating activity detected in the allantoic fluid due to influenza virus or other hemagglutinating viruses, such as paramyxoviruses like Newcastle disease virus (NDV). Therefore, the isolate should be tested in HI assays against Newcastle disease and other antisera. If negative, the virus then is tested for the presence of the type A specific antigen to establish that an influenza A virus is present. The type-specific NP (nucleoprotein) or matrix protein may be detected by the double immunodiffusion test (Beard, 1970; Dowdle and Schild, 1975), the single radial-hemolysis test (Dowdle and Schild, 1975) or commercial antigen capture immunoassay. Monoclonal antibodies that react with the nucleoprotein or matrix proteins have proven useful in identifying these antigens in ELISA (Walls *et al*., 1986). The next step in the identification procedure is to determine the antigenic subtype of the surface antigens, HA and NA. The NA subtype is identified by a micro-NI assay with antisera prepared against the nine known NAs (Palmer *et al*., 1975; Swayne *et al*., 1998; Van Deusen *et al*., 1983). The HA is identified in the HI test (Swayne *et al*., 1998) using a panel of polyclonal antisera prepared against whole virus representing the 16 distinct HA subtypes. Subtyping is facilitated by using antisera against the HA alone (i.e not the whole virus) or against reassortant viruses with heterologous NAs; this helps avoid steric inhibition due to antibodies against the NA (Kawaoka *et al*., 1990; Kendal, 1982). However, an influenza virus with a new HA would not be detected in tests using antisera to the known HA subtypes. It is therefore essential to confirm that the unknown hemagglutinating agent is an influenza virus using the type-specific test. Final identification is most commonly accomplished by state, federal, or OIE influenza reference laboratories.

2.8.4 Serology

Serologic tests can be used to demonstrate the presence of AI specific antibodies, which may be detected as early as seven days after infection. Several techniques are used for serologic surveillance and diagnosis. In serologic surveillance programs, a double immunodiffusion test (agar gel immunodiffusion or AGID) for the detection of anti-NP antibody is frequently used, because this

detects antibodies to type A-specific antigens shared by all influenza A viruses. Enzyme Linked Immuno-Sorbent Assay (ELISA) assays have been developed to detect antibodies to AI viruses (Snyder *et al*., 1985, Zhou *et al*., 1998). ELISAs are commercially available for detecting antibody to influenza from chickens and turkeys. Once influenza is detected by immunodiffusion or ELISA, HI tests can be used to determine the HA subtype. In serologic assays, be aware that there is considerable variation in the immune response among the various avian species. For example, antibodies to the NP are generally prominent in turkeys and pheasants but may be undetectable in ducks known to have been infected (Slemons and Easterday 1972). In addition, antibodies may be induced in ducks, as well as other species, which fail to be detected in conventional HI tests performed with intact virus (Kida *et al*., 1980; Lu *et al*., 1982). The sera of many species contain nonspecific inhibitors that may interfere with the specificity of the HI and other tests. Because these inhibitors are especially active against certain viruses, they present a very practical problem in serologic testing and the identification of viruses. Therefore, sera should be treated to reduce or destroy such activity, although it should be recognized that some treatments may lower specific antibody levels. The two most commonly used treatments for these inhibitors have been receptor destroying enzyme (RDE) and potassium periodate (CDC, 1982; Dowdle and Schild, 1975). In addition to the non-specific inhibitors of hemagglutination, sera from other bird species, such as turkey and goose, may cause non-specific agglutination of the chicken erythrocytes used in the HI test. This may mask low levels of HI activity. Such hemagglutinating activity can be removed by pretreatment of the serum with chicken erythrocytes (Nakamura and Easterday, 1967). This problem may sometimes be avoided by using erythrocytes in the HI test of the same species as the serum being tested.

2.8.5 Differential Diagnosis

Because of the broad spectrum of signs and lesions reported with infections by AI viruses in several species, a definitive diagnosis must be made by virologic and serologic methods (Swayne and Halvorson, 2008). For HPAI viruses, other causes of high mortality must be excluded such as Newcastle disease, septicemic fowl cholera, heat exhaustion, water deprivation and some toxins. For LPAI viruses, other causes of respiratory disease and drops in egg production must be investigated such as lentogenic Newcastle disease virus, avian pneumovirus and other paramyxoviruses, infectious laryngotracheitis, infectious bronchitis, chlamydia, mycoplasma, and various bacteria. Concurrent infections with other viruses or other bacteria have been commonly observed (Easterday *et al*., 1997).

2.9 CONTROL

Three different goals in the control of AI have been identified (Swayne and Halvorson, 2008), and these are 1) prevention, 2) management, 3) eradication. These control measures are achieved based on strategies using combinations of five specific components: 1) education, 2) biosecurity, 3) diagnostics and surveillance, 4) elimination of infected poultry, and 5) decreasing host susceptibility. The goals for individual LPAI and HPAI control strategies may vary depending on the country, subtype of the virus, economic situation and risk to public health (Swayne and Halvorson, 2008). There is no single feasible control strategy for AI. In most developed countries, HPAI outbreaks have been eradicated within six months to a year by traditional stamping-out programs, but in some developing countries, the lack of indemnities, poor veterinary infrastructure and high level of poultry production at the village or rural level, have made immediate eradication unachievable (Swayne and Halvorson, 2008). Although in Nigeria, the stamping out program with indemnities to poultry farmers has paid of, as there has been no report of HPAI outbreak in more than 2 years. In these situations, management of the disease to a low infection rate had been a realistic option. The designation of H5 and H7 LPAI as LPNAI has increased the use of stamping out programs in dealing with these two AI subtypes as a means to prevent emergence of HPAI viruses. Historically HPAI viruses are known to have emerged after LPAI H5 or H7 viruses circulated widely in susceptible poultry for several months (Swayne and Halvorson, 2008). Education is one critical aspect in the control of AI.The education of all poultry and allied industry personnel regarding how the viruses are introduced, how they spread, and how such events can be prevented. An individual's control of risky behaviors greatly reduces the spread of AI virus by controlling fomite or aerosol movement of the virus thus preventing AI virus movement on the farm and between farms. Biosecurity has been identified as the first line of defense and is practiced as inclusion biosecurity, such as quarantine, to keep the virus on infected premises and exclusion biosecurity to keep the virus off of virus free premises (Swayne and Halvorson, 2008). The most likely source of virus for poultry is other infected birds; therefore it is important to separate susceptible birds from infected birds and their secretions and excretions. Transmission can occur when susceptible and infected birds are in close contact or when infectious material from infected birds is introduced into the environment of susceptible birds. Such introductions are associated with the movement of cages, equipment, footwear and clothing, vehicles, insemination equipment, etc. The presence of virus in fecal material and respiratory secretions is a likely means for movement of the virus either by

ingestion, contact with mucous membranes, or inhalation. Contaminated poultry manure has been identified as a high risk source for virus transmission between flocks. Poultry raised outdoors or that have outdoor access have been infected following exposure to wild birds, primarily to infected ducks and shorebirds. In some countries, live bird market (LBM) and village poultry are an important reservoir of influenza virus and pose a risk for introduction to commercial poultry if adequate biosecurity is not practiced (Swayne and Halvorson, 2008). Swine may serve as a source of H1 and H3 swine influenza viruses to turkeys where the virus is transmitted mechanically or by infected pigs (Easterday *et al*., 1997). Persons who have direct contact with birds or their manure have been identified as the cause of most virus transmission events between houses or premises, but airborne transmission has served as a source to some farms in association with certain depopulation and cleaning activities during the peak of infection (Bowes *et al.*, 2004; Davison *et al*., 2003; Selleck *et al*., 2003). Direct contact of birds with their manure or equipment should be avoided and movement of equipment from farm to farm without adequate cleaning and disinfection should be discouraged. It is important to keep the traffic area near the poultry house free from contamination by manure. Visitors on farms should not be allowed or should be strictly controlled with mandatory disinfection of footwear and cleaning of clothing (Swayne and Halvorson, 2008). Efficient diagnostics and surveillance system that ensure accurate and rapid diagnosis of AI is a prerequisite to early and successful AI control. The pace with which AI is controlled is largely dependent upon how rapid the first case or cases are detected, the existing biosecurity, and how quickly control strategies are implemented, especially if eradication is the goal (Swayne and Halvorson, 2008). Passive surveillance is critical to differentiate LPAI virus as the cause of respiratory disease or drops in egg production from causes of endemic diseases with similar signs. It is also important to differentiate HPAI virus from other causes of high mortality events. Active surveillance is essential to determine where the virus is located within a country, zone, or compartment and can best be accomplished through either serological testing of birds for antibodies and/or random testing of daily mortality for the presence of AI virus. Surveillance is also crucial for on-going evaluation of the success of control strategies and for use in decision making as a prelude to improving control strategies (Swayne and Halvorson, 2008). Serological testing has been used to declare a country, zone, or compartment as AI free. Also, Serological testing can be used during an AI outbreak to determine the extent of the infected zone for quarantine purposes (Swayne and Halvorson, 2008). Elimination of infected poultry, infected flocks, their eggs and manure are essential to preventing future transmission. For HPAI, this has been

typically accomplished through depopulation and disposal of carcasses, eggs, and manure by an environmentally sound method such as composting, incineration, rendering, or landfill burial (Swayne and Halvorson, 2008). For LPAI, orderly marketing of birds after recovery from infection has been an acceptable means for elimination, and eggs have been marketed if properly cleaned. It is known that most influenza virus shedding occurs during the first 2 weeks of infection and usually by 4 weeks after the initiation of the infection; virus cannot be detected by sampling (Swayne and Halvorson, 2008). Sero-positive flocks have not been associated with a high risk of transmission if maintained under biosecure practices. However, it is said that there should be no contact with recovered flocks because the length of time birds within a population shed virus is not clearly defined. Because the economic losses due to influenza may be severe, the control program should not unnecessarily penalize the growers (Swayne and Halvorson, 2008). Decreasing host susceptibility if poultry are at risk to AI virus exposure by increasing the resistance of birds to infection may be necessary to break the infection cycle. In theory, this can be achieved by genetic selection for resistant bird strains or breeds, but to date, only minor chicken breed resistant to LPAI virus has been identified and scientifically verified (Swayne *et al*., 1994). Active or passive immunity to the AI viral hemagglutinin or neuraminidase proteins is another method to increase resistance. This is predominantly done through vaccination, but antibody and immune cell transfer can be protective (Swayne and Halvorson, 2008).

Vaccination option has brought about various vaccine technologies. Vaccine technologies have been developed in the laboratory and have shown efficacy in experimental studies, in mostly chickens and turkeys, to provide protection from LPAI and HPAI viruses (Swayne, 2004). Inactivated whole AI virus vaccines are the most frequently licensed AI vaccine technology, typically made using LPAI field outbreak strains, and more recently reverse genetic generated AI vaccine strains, followed by chemical inactivation and oil emulsification (Swayne, 2006). These vaccines have been used in a variety of poultry and other avian species, and their effectiveness in preventing clinical signs and mortality is well documented. But protection is virus-subtype specific. Birds are susceptible to infection with influenza viruses belonging to any of the 16 hemagglutinin subtypes, and there is no way to predict their exposure to any particular subtype (Swayne and Halvorson, 2008). At present, there are no practical, specific treatments that exist for AI virus infections in commercial poultry. Amantadine has been shown experimentally to be effective in reducing mortality (Easterday *et al*., 1997; Van Deusen et al., 1983), but the drug is not approved for food animals, and its use rapidly gives rise to amantadine-resistant viruses. Supportive care and antibiotic treatment have been employed to

reduce the effects of concurrent bacterial infections. The use of human antiinfluenza drugs is strongly discouraged.

2.10 HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 IN COMMERCIAL POULTRY PRODUCTION SYSTEM

According to Caron *et al*. (2009), intensive poultry production units ideal for viral breeding due to: (a) genetic homogeneity, decreasing the number and variability of resistance genes, little variation between individuals and therefore high infection potential for viruses; (b) no reproduction for the majority of birds prevent any co-evolution with the pathogen providing the conditions for a highly lethal strain to be maintained; (c) artificial density of individual hosts increases spread to other individuals; (d) short life cycles prevent the host organism from developing immunity; (e) in a compartment with high density and short life spans contagiousness is not under selective pressure and a selection criterion on strains, allowing strains to be selected mainly on virulence i.e harm to the host; linked to host exploitation (Read and Taylor, 2001); (f) sometimes inadequate disinfection measures between batches allow the virus to survive in the environment. These suggest some direct inferences: the change in excretion of the H5N1 HPAI could be an adaptation of the virus to intensive production systems where infection would be enhanced by aerosol transmission, whereby a three dimensional space can be utilized for spread compared to faecal transmission which is constrained in a two dimensional space on the ground. This combination of variables will select for viruses with high virulence, as other life history traits are released from selective pressure (Read and Harvey, 1993). Therefore, a highly virulent mutation which would be selected against under natural conditions (since spread would be limited at low density) will thrive in these conditions. In intensive poultry production systems, HPAI strains, if introduced or created will be selected. HPAI H5N1 could be a product of such selective process (Caron *et al*., 2009). The index HPAI-H5N1 in Nigeria occurred at a commercial poultry farm in Kaduna State and by the end of the initial outbreak, over 46 000 poultry cases had been reported (NVRI, 2006; Adene *et al.,* 2006). Following the index **(**HPAI) H5N1 in Nigeria, several efforts have been directed to increased surveillance and diagnosis of the disease. These have lead to increased diagnostic capacity, reporting and research into the diverse genotype of H5N1 isolates from Nigeria. The episodes of mortality that characterized the first outbreak of HPAI in Nigeria (Joannis *et al.,* 2006; Adene *et al.,* 2006) in a farm that had predominantly commercial birds with small stocks that included turkeys, geese and ostriches (Joannis *et al.,* 2006, 2007; Adene *et al.,*

2006) may have preceded the national catastrophy of AI outbreaks. The disease devastated the poultry sector of the economy, especially in the north-central region of Nigeria (Fasina *et al*., 2008; Saidu *et al*., 2008).

2.11 HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 IN BACKYARD/RURAL POULTRY IN NIGERIA

Rural poultry are mainly kept in free-range, multispecies, multiage holdings that have low biosecurity levels and are thus exposed to many at-risk contacts. They could also act as the epidemiologic link between the wild reservoir of AI viruses and industrial poultry (Cecchinato *et al.,* 2011). This was evident in Nigeria as new cases of HPAI (H5N1) were detected during the surveillance activities at the live bird markets in July 2008 in Gombe city, Gombe State after a 9-month period of influenza virus detection (Fusaro *et al.,* 2009). The predominant species in the rural poultry sector of Africa is the scavenging indigenous domestic fowl (*Gallus domesticus*) (Kitalyi, 1998). In most African countries, the chickens have no regular health control programme, may or may not have shelter, and scavenge for most of their nutritional needs (Kitalyi, 1998). In fact, village chickens have been reported to act as potential reservoirs and carriers of infections to themselves and to the more susceptible exotic breeds in commercial poultry farms (Adu *et al.,* 1986). The first reported outbreak in backyard poultry flock was in chicken and duck in Potiskum and Jakusko towns in Yobe State on the 16th of February, 2006 (NVRI, 2006). Subsequently, HPAI was detected in Hadeija in Jigawa State between February and April of 2006 before been reported from Ibi and Wukari local government areas of Taraba State in June 2006 (NVRI, 2006). It was reported that the epidemic of AI might have spread from Jigawa State to Kano state (Saidu *et al*., 2008) because as at December 2005 to January 2006, history revealed that local poultry in Jigawa state have been dying as a result of a disease that was more devastating than the annual epidemic of Newcastle disease that is usually experienced during the cold, windy harmattan period (Saidu *et al.,* 2008). The course of HPAI H5N1 infection in free-ranged rural backyard chicken in Nigeria was generally more acute with high mortality and yielded fewer lesions (Kumbish *et al*., 2006b).

2.12 ECONOMIC IMPORTANCE OF HPAI

It is generally known that the most accurate reports on losses have resulted from HPAI eradication programs (Swayne and Halvorson, 2008). Direct losses in HPAI outbreaks have included depopulation and disposal costs, high morbidity and mortality losses, cleaning and disinfection, quarantine and surveillance costs, and indemnities paid for the birds (Swayne and Halvorson, 2008). However, indirect costs does exist, such as uncompensated losses to the poultry industry including temporary or permanent loss in poultry exports, income lost by farmers and communities during the production down time, increased consumer costs from reduced supply of poultry products, and losses from decreases in consumer purchases can easily escalate losses by 5–10 folds (Swayne and Halvorson, 2008). The economic costs for eradication of HPAI have varied greatly, but eradication costs have been very high and appear to be proportional to the number of birds that died and were culled. In Nigeria, the HPAIV outbreaks caused high mortalities in both commercial and local poultry populations. By mid-January, 2007, Nigeria lost 945,862 birds of various species (Fasina *et al.,* 2007) and as at December 2007, 15 states and Abuja had experienced outbreaks for a second time (Akanbi *et al.,* 2009). By the end of the outbreaks in poultry farms and flock, about 1,264,191 birds were depopulated, and compensation paid to farmers was about N631 million (US$5.43 million) (Maina, 2008). Several attempts have also been made to determine the losses in terms of number of poultry, monetary and social value as a result of the disease burden in Nigeria (Fasina *et al*., 2007). These attempts have been at some point in the course of the disease (Fasina *et al*., 2007) or aimed at some states of the country (Saidu *et al.,* 2008). The poultry sector is said to represent a major source of income in Nigeria and it contributed approximately 4.45 % of the total animal contribution to the agricultural gross domestic product (GDP) in 2004 (Central Bank of Nigeria, 2004). Nigeria is said to have over 140 million birds, composed of about 60 % backyard poultry stock and about 40 % commercial or semi-commercial birds (Adene and Oguntade 2006), of which, approximately 75 % of the commercial birds are layer stock and they are responsible for the production of eggs and poultry meat in Nigeria. Poultry is an important subsector to the rural poor since it is the most widespread form of livestock in Nigeria that the poor rural individuals can afford to keep as a source of income and assets. However, estimating the economics and other financial parameters in the Nigerian poultry industry is extremely difficult since data collection is often incomplete or fragmented (Fasina *et al*., 2008b). It is envisaged that the costs due to HPAI outbreaks is less significant than those associated

with the post outbreak effects on market, trade, enzootic potential, productivity, dwindling interest in poultry enterprises and the attendant zoonotic and food security risks (Fasina *et al*., 2008b). 75% (42 million) of the approximately 40 % (56 million) commercial or semi-commercial are layer stock and responsible for the majority of an annual production of over 476 000 metric tonnes of eggs (0.8 % of the world total) and 211 000 metric tonnes of meat (0.3 % of the world total) (FAO 2008). Laying hens contribute huge resources to the national poultry flock, emphasizing the importance of commercial layer flocks in Nigeria's economy. Laying hens not only have production value, but old birds (spent hens) also serve as a major source of eggs and meat for resource poor families (Fasina *et al*., 2008b). The poultry industry is said to rank second in importance to petroleum, the country's major source of income (Ducatez *et al*., 2006). In 2006, the World Bank estimated that the layer industry had lost about $60 million as a result of HPAI H5N1 between January and August, 2006. Nigeria's gross national income was $55.9 billion and the gross domestic product was $72.1 billion as at 2004 (World Bank, 2006). This figure is therefore considered a huge economic loss by any assessment (Fasina *et al*., 2008b). These losses are independent of losses associated with the downstream sectors and broiler industry. Economists have suggested that the price paid for livestock disease should not be assigned monetary values alone (Hanson & Hanson 1983, Howe, 1985, McInerney, 1988). The cost implication of the disease is, however, important as a starting point to assessing the true effect of the outbreak. Previous estimates of the cost of avian influenza outbreaks using direct costs grossly undervalue costs associated with HPAI (Fasina *et al*., 2008b). It is said that a point-of-lay commercial bird or a breeder chicken, although may cost less than a rooster/broiler or a turkey respectively at any point, is more valuable than the latter in terms of economic benefit since the laying hen or the breeder will bring economic benefit for at least a year. "Economic value is not simply prices" (McInerney, 1988). Apart from financial losses, the HPAI H5N1 outbreak also had severe impacts on trade and tourism, created scarcity/unavailability of animal protein due to public health misconceptions about consumption of poultry, led to higher prices for alternative and often lesser quality products, and increased the costs of livestock farming (Fasina *et al*., 2008b). There are concerns that HPAI may become enzootic in the sub-region or in the African continent, which may then become a source of infection or re-infection to other parts of the world (Fasina *et al*., 2008b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

GENERAL OBJECTIVES

3.1. To study the epidemiology and pathology of HPAI-H5N1 outbreaks in commercial and backyard poultry between 2006 and 2008 in Nigeria.

3.2. To investigate the influence of mixed species poultry farming on the mortalities of HPAI-H5N1 outbreaks in backyard poultry between 2006 and 2008 in Nigeria.

3.3. To determine if archival FFPE tissues from chickens naturally infected with HPAI H5N1 virus in Nigeria are useful for retrospective studies by the use of immunohistochemical methods to detect HPAI-H5N1 antigen and RT-PCR assays for amplifying RNA.

3.4. To determine the susceptibility, transmission, pathogenesis and pathology in three ducks species (Muscovy, Mallard and Pekin) infected with clade 1 H5N1 virus- Influenza A/duck/Vietnam/TG24-01/05 and clade 2.2 H5N1 virus- Influenza A/Cygnus cygnus/Germany/R65/2006 (homologous to Nigerian HPAI) isolates and their in-contact chickens and ducks

3.1 STUDY 1: THE EPIDEMIOLOGY AND PATHOLOGY OF HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 OUTBREAKS IN COMMERCIAL AND BACKYARD POULTRY BETWEEN 2006 AND 2008 IN NIGERIA.

Specific objectives

1. Epidemiology: to quantify and use geographic information system Arc-view software to map the location of HPAI outbreaks in Nigeria
2. To determine the association between outbreak location and geo-ecological features such as wetlands
3. To determine the pathology of HPAI in commercial and backyard poultry by the use of clinical, postmortem and histopathological examination.

3.1.1 Epidemiology

The data used for this study were pooled from poultry necropsy cases submitted for HPAI diagnosis and confirmation at the National Veterinary Research Institute. The coordinates of farms and live bird markets affected were collected using Personal Digital Assistant (PDA) running Global Positioning System (GPS). Non-spatial data were collected through case reports that accompanied the specimens for diagnosis. Spatial and non-spatial data gathered were added to the Arc view GIS software for mapping. A geoecological map that shows the 24 wetlands (Saidu *et al*., 2008) in Nigeria was also used to predict relationship between HPAI occurrence and geo-ecological feature

3.1.2 Carcasses

These carcasses were derived from routine diagnostic cases of the Central Diagnostic Laboratory, NVRI Vom Nigeria or from field cases during the Avian Influenza outbreak in Nigeria 2006-2008. The carcasses derived from routine diagnostic cases were submitted directly by clients for post-mortem examination and avian influenza diagnosis and were selected based on laboratory confirmation of HPAI H5N1 virus infection.

3.1.3 Clinical Signs, Gross and Histopathology

Carcasses of the commercial and backyard poultry that died after natural infection with HPAI were submitted for pathologic and microbiologic examination. Clinical signs observed by clients were given with case histories. Following postmortem examination of the carcasses, sections of liver, heart, spleen, kidney, lung, trachea, proventriculus, gizzard, duodenum, ileum, cecum, and brain were removed and fixed in 10% buffered formalin. All tissue samples were then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for histologic examination under the light microscope fitted with camera.

3.2 STUDY 2: THE INFLUENCE OF MIXED SPECIES POULTRY FARMING ON THE MORTALITIES OF HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 OUTBREAKS IN BACKYARD POULTRY BETWEEN 2006 AND 2008 IN NIGERIA.

Specific objectives

1. To perform statistical analyses on the data from cases submitted to NVRI from backyard poultry in Nigeria using Medicalc software (Medicalc Inc.2011) to determine the influence of mixed species poultry farming on HPAI mortalities

3.2.1 Study Design and Data Set

This study was conducted following the HPAI H5N1 outbreaks and detection in Nigeria from 2006-2008. All Poultry cases (carcasses) submitted for post-mortem examination and avian influenza diagnosis at the NVRI's Central Diagnostic Laboratory were grouped into commercial (layers, pullet, cockerels and broilers) and backyard poultry (local chicken, duck, turkey, guinea fowl and geese). In the commercial poultry group, a total of one hundred and fifty-three (153) farms in eighteen (18) states and FCT, Abuja submitted cases. While in the backyard poultry group, a total of eighty (80) farms in sixteen (16) states and the FCT, Abuja submitted cases. These cases, including data on State, location, farm flock size, morbidity and mortality records reported in this study were supplied directly by clients who submitted carcasses for post-mortem examination and avian influenza diagnosis and were selected based on laboratory confirmation of HPAI H5N1 virus infection (NVRI, 2006-2008).

Of the eighty (80) farms in the backyard poultry group, fifty-six (56) farms with full case histories (flock size and number dead) were included for further analysis. These were further sorted as Group A: Single species backyard flocks and group B: mixed species backyard flocks. Single species backyard flocks included: I) HPAI infected chicken only flocks; II) HPAI infected duck only flocks; III) HPAI infected turkey only flocks and IV) HPAI infected guinea fowl only flocks. Mixed species backyard flocks included, V) HPAI infected chicken and duck only flocks; VI) HPAI infected chicken, duck and turkey only flocks; VII) HPAI infected chicken, duck and guinea fowl only flocks.

3.2.2 Statistical Analyses

Flock size, number alive and number dead were expressed as percentage proportions. A Chi-square (X^2), test for the comparison of two proportions (from independent samples) expressed as percentage was performed and when the P-value was <0.05.The conclusion was that the two proportions were significantly different. The difference between these two proportions and a 95% confidence interval (C.I) for this difference were also calculated. An odd ratio analysis was also performed, and odds ratio (OR)> 1 indicated association, while OR< 1 was interpreted as lack of association (Medicalc Inc.2011).

3.3 Study 3: Determination of the Usefulness of Archival Formalin-fixed Paraffin-embedded (FFPE) Tissues from Chickens naturally infected with HPAI H5N1 virus for Retrospective Diagnosis of AI using Immunohistochemistry and RT-PCR.

Specific objectives

1. To determine the usefulness of FFPE tissue of chickens for retrospective HPAI diagnosis, by the use of immunohistochemistry, nucleic acid extraction, RT-PCR, sequencing and molecular analysis.

3.3.1 Experiment Design

Formalin fixed Paraffin-embedded (FFPE) tissue samples from ten outbreaks (A-J) of HPAI-H5N1 in commercial chickens in Nigeria, between 2006 and 2007 were obtained from the archive of the Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI), Vom-Jos Plateau State, Nigeria. Fresh samples from these cases had been previously characterized as H5N1 HPAI positive by virus isolation and RT-PCR. Gross and histopathology studies were also conducted on all cases previously. Tissue pools which included comb, wattle, brain, heart, trachea, lung, spleen, liver, kidney, proventriculus, intestine and pancreas were investigated by IHC, real-time RT-PCR (rRt-PCR) and the PCR products were further characterized by sequence analysis.

3.3.2 Immunohistochemistry on FFPE sections

Paraffin-wax sections (5µm) were dewaxed and stained with hematoxylin-eosin for histopathological investigations of tissue samples by standard method. For immunohistochemistry, paraffin-wax sections (5µm) were mounted on charged microscope slides (Menzel, Braunschweig, Germany), dewaxed and rehydrated. To detect influenza virus antigen, sections were incubated with an in-house rabbit antinucleoprotein serum (Starick *et al.,* 2006) in a dilution of 1:500 in Tris-buffered saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6) as earlier described by Klopfleisch et al. 2006. In brief, a biotinylated goat anti-rabbit IgG_1 (Vector, Burlingame, CA; diluted 1:200 in TBS) was used as linker-antibody for the avidin-biotin-complex (ABC) method. As negative control, the pre-immunization serum of the same rabbit was applied. Positive samples produce a bright red signal with an IHC kit (Vectastain Elite ABC Kit, Vector) and the substrate 3-amino-9-ethylcarbazole

(DAKO AEC substrate- chromogen system; Dako, Carpinteria, CA, USA). The sections were counterstained with Mayer's hematoxylin and sealed with aqueous medium (Aquatex; Merck, Darmstadt, Germany). Positive and negative control tissues of chicken originating from animal experiments were included for each immuno-histochemistry procedure.

3.3.3 RNA Extraction and Purification from FFPE

5-10 µm thick sections of HPAI positive FFPE chicken tissues from all ten outbreaks were cut and RNA was extracted and purified using the RNeasy FFPE kit (QIAGEN, Hilden, Germany),following the manufacturer's instructions. Briefly, samples were placed in 2ml microcentrifuge tubes and 1ml xylene was added. Following full speed (8000 r.p.m) centrifugation at 25^0C for 2 minutes, 1ml of 100% ethanol was added to the pellet to remove residual xylene. Following an additional centrifugation step residual ethanol was removed by incubation at room temperature for 10 minutes after the main part of the supernatant was pipetted. The pellet was resuspended in 150µl buffer protein kinase dehydrogenase (PKD) and was treated with proteinase K (in house). The remaining steps complied with the usual terms of matrix binding and elution of nucleic acids with a final volume of 14µl RNA (manufacturer).

3.3.4 RT-PCR, Sequencing and Molecular Analysis of FFPE

Ten RNA samples obtained from the investigated outbreaks representing 26 animals (chicken) were analyzed by modified TaqMan one-step real-time RT-PCR (rRT-PCR) assays which specifically amplified fragments of the AIV M and H5 (HA2 part) genes according to the European Commission (EC) decision 2006/437/EC (European Commission, 2006). In addition, an rRT-PCR assay using a probe specific for the cleavage site of the HA gene of H5N1 clade 2.2 viruses was used (Goelz *et al*., 1985). For the H5 rRT-PCR test targeting the HA2-part of the hemagglutinin, two independent assays with each twice tubes per sample was carried out and the resulting four PCR products per sample were directly sequenced using the same primers (in house) as for RT-PCR. In addition, conventional RT-PCR targeting four overlapping regions of the H5 gene were carried out. All primers and probes are summarized in table3.1. Sequencing was done as described previously (Starick *et al*., 2008). Sequence data were assembled and consensus sequences were generated with GCG Version 11.1 (Accelrys Inc.). Sequences of AIV isolates with the highest homology to these were chosen from the National Center

for Biotechnology Institute (NCBI), Basic Local Alignment Search Tool (BLAST) and sequences were aligned using Clustal W2 (Zang *et al*., 2000).

3.3.5 Primer Set

Table 3.1. Primers used for the detection and sequencing of the H5N1 HA and M gene segments of the Nigerian isolates using real-time and conventional RT-PCR assays

PCR Test	Gene segment	Primers for detection and sequencing of HA and M-gene
rRT-PCR	M gene	IVA-M, forward AGATGAGTCTTCTAACCGAGGTCG
		IVA-M, reverse TGCAAAAACATCTTCAAGTYTCTG
		IVA-M, probe FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1
rRT-PCR	H5(HA2)	H5LH1, forward ACATATGACTACCCACARTATTCAG
		H5RH1, reverse AGACCAGCTAYCATGATTGC
		A IV-H5-1,probe HEX-TCAACAGTGGCGAGTTCCCTAGCA-BHQ1
RT-PCR	H5(HA)	H5-1, forward AGCRAAAGCAGGGGT
		H5-1, reverse CTCTGRTTYAGTGTTGATGT
		H5-2, Forward GAGCAGAATAAAYCATTTTGAGA
		H5-2,reverse TGAGTGGATTCTTTGTCTGCAGC
		H5-3,forward ACATCAACACTRAAYCAGAG
		H5-3, reverse AAGTCTAGAGTTCTCTCATTTT
		H5-4, forward GACCAGTAGAAACAAGGGTGTTTT
		H5-4, reverse GCTGCAGACAAAGAATCCACTCA

3.4 STUDY 4: SUSCEPTIBILITY, TRANSMISSION, PATHOGENESIS AND PATHOLOGY IN MUSCOVY, PEKIN AND MALLARD DUCK AND THEIR IN-CONTACT CHICKENS AND DUCKS INFECTED WITH CLADE 1 H5N1 AND CLADE 2.2 H5N1 VIRUSES

Specific objectives

1. To conduct animal experiment using ducks (Muscovy, Pekin and Mallard) infected with clade 1 H5N1 and clade 2.2 H5N1 viruses and their in-contact chickens and ducks for the study of susceptibility, transmission, pathogenesis and pathology.

2. To observe and document clinical signs, gross and histopathological lesion and Immunohistochemical detection in tissues.

3. To use Real-time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) and tissue culture virus isolation to quantify virus shedding and tissue load during HPAI infection in chicken and ducks.

4. To use Medicalc software to compare means of RRT-PCR ct-values between two variables (virus type and duck species) in tissues and swabs (tracheal and cloacal) by employing graphs and Analysis of Variance (ANOVA).

3.4.1 Animals

Sixty-two day-old clinically healthy and serologically AIV-negative specific pathogen free (SPF) Pekin (Anas platyrhynchos), Muscovy (*Cairina moschata domesticus*) and Mallard (*Anas platyrhynchos domesticus*) ducks and twelve (12), day-old White Leghorn chickens (*Gallus domesticus*) (VALO SPF, Lohmann, Cuxhaven, Germany) were raised under hygienic conditions for 3 weeks. These sero-negative to avian influenza virus ducks and chickens as tested by a competitive ELISA (Starick *et al*., 2006), were transferred to the duck experiment facility within the high containment facilities for performing *in vivo* experiments with HPAIV (BioSecurity Level 3 human), of the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (FLI) Insel-Riems, Germany (Klopfleisch *et al*., 2006).

3.4.2 Viruses

The viruses (Clade 1 and Clade 2.2) stocks used in these experiments were isolated 1) Clade 1, from a domestic duck (*Anas domesticus*) suspected to be naturally infected with HPAI virus. The outbreak sample organs received from Vietnam by the national reference laboratory for avian influenza at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (FLI) Insel-Riems, Germany was screened for HPAI H5N1; 2) Clade 2.2, from an adult whooper swan (Cygnus cygnus) that was found dead and in good postmortem condition at the Baltic Sea coastal area along the northwestern shores of the Island of Ruegen in late February 2006 (Weber *et al.,* 2007). This area, which is part of a national park of Germany, an area characterized by open coastal landscape, shallows, and inlets of the Baltic Sea, which are a temporary home for thousands of migratory and resident aquatic birds (Weber *et al.,* 2007). These isolates were once propagated in the allantoic cavities of 10-day-old embryonated SPF chicken eggs. The allantoic fluid was harvested and stored at -70 ºC. The isolates were also taken and analyzed for HPAIV RNA by real time RT-PCR and nucleotide sequencing. RNA was isolated from the tracheal swabs and organs respectively using the QIAamp Viral RNA Mini Kit (Qiagen). Diagnosis and characterization of H5N1 HPAIV by RRT-PCR followed a cascade style recommended by the Diagnostic Manual issued by the European Commission: 1) Generic influenza PCR: Detection of an influenza A virus matrix (M) gene fragment by duplex RRT-PCR, including an externally added extraction and inhibition control (Spackman *et al.,* 2002); 2) Specific detection of subtypes H5 and/or H7: RRT duplex PCR for the detection of subtypes H5 and N1 and exclusion of subtype H7 by uniplex RRT-PCR (Spackman *et al.,* 2002); 3) Molecular pathotyping of H5/H7 subtypes. The virus isolates were designated clade 1 H5N1 virus- Influenza A/duck/Vietnam/TG24-01/05 (Starick, Per. Comm. 2009) and clade 2.2 H5N1 virus- Influenza A/Cygnus cygnus/Germany/R65/2006 (Weber *et al.,* 2007) respectively.

3.4.3 Experimental Design

The 62 ducks were divided into two (2) major groups based on virus type and negative control (table 3.2). Twenty-eight (28), 3-week-old Muscovy (10), Pekin (10) and mallard (8) ducks were housed in separate compartment in a duck experiment facility for the clade 1 H5N1 virus- Influenza A/duck/Vietnam/TG24-01/05 challenge group and fed *ad libitum.* Another twenty-eight (28), 3-week-old Muscovy (10), Pekin (10) and mallards (8) ducks were housed in separate compartment in another

duck experiment facility for the clade 2.2H5N1 virus- Influenza A/Cygnus cygnus/Germany/R65/2006 and fed *ad libitum.* The remaining six (6) ducks (Pekin) were used as contact control for the clade 2.2 virus groups; one with the Pekin, one with the Muscovy and one with the Mallard, while the remaining ducks serves as the negative control. The twelve (12) chickens were divided equally into two (2) major groups based on virus type and infected duck group. Six (6) chickens, designated as chicken 1-6, were housed in twos with Pekin (chicken 1&2), Muscovy (chicken 3&4) and mallard (chicken 5&6) infected ducks groups respectively in separate compartment in duck experiment facility for the clade 1 H5N1 virus- Influenza A/duck/Vietnam/TG24-01/05 (Starick, Per. Comm. *2009*) challenge duck groups and fed *ad libitum.* Another six (6) chickens, designated as chicken 7-12, were housed in twos with Pekin (chicken 7&8), Muscovy (chicken 9&10) and mallard (chicken 11&12) infected ducks groups respectively in separate compartment in another duck experiment facility for the clade 2.2 H5N1 virus- Influenza A/Cygnus cygnus/Germany/R65/2006 and fed *ad libitum.* For duck and chicken identification purpose and to monitor vital signs especially temperature, the ducks had a chip IPTT-300 that transmit to a transponder Plexx NL WRS-6007 (USA), inserted subcutaneously in the medial aspect of the right thigh. The ducks were inoculated oculo-oro-nasally with 0.5 ml allantoic fluid containing 10^6 50% egg-infective dose (EID_{50}) of Influenza A/duck/Vietnam/TG24-01/05 and Influenza A/Cygnus cygnus/Germany/R65/2006 of clade 2.2 subtype H5N1 virus. The in-contact chickens had direct contact with the infected ducks' faeces (solid ground, cleaned daily) and also, a common source of feed and drinking water for infected and in-contact chickens was used. The birds were monitored daily for a total of 6 days or until death, and clinical signs were recorded. Oropharyngeal and cloacal swabs were taken daily from each in-contact chicken for 8 days post-infection (dpi) of the ducks in the two virus groups. Using histopathology and immunohistochemistry the lesions and distribution of influenza virus nucleoprotein were investigated alongside gross changes. Also, Oropharyngeal and cloacal swabs were taken daily from each duck in the two virus groups until death or 8 dpi. Swabs were collected in 1 ml Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum (FBS) containing antibiotics (Enrofloxacin 1mg/ml, Gentamycin 0.05mg/ml and Lincomycin 1mg/ml) and stored at 27ºC until analysed. Ducks in the different groups were monitored daily for clinical signs of disease and mortality. Those that died were autopsied immediately or when observed, others were sacrificed humanely by cervical dislocation at day 4 and 8 post-infection (p.i). The following tissues were sampled: cerebrum, cerebellum, spinal cord, nasal cavity, trachea, lung, heart, oesophagus, proventriculus, gizzard,

duodenum, jejunum, ileum, caecum, rectum, pancreas, liver, spleen, bursa, thymus, brain, eye, harderian gland, pectin (duck), eyelid, kidney, bone marrow, adrenal, ovary or testis, skeletal muscle and skin. Realtime RT-PCR (rRT-PCR) was applied to quantify HPAIV load in tracheal and cloacal swabs and in tissue samples. The data obtained by rRT-PCR in the lung, the brain and terminal colon were compared to viral titers of calculated EID_{50} per ml sample. All experiments with HPAI virus subtype H5N1 were conducted under Biosaftey Level (BSL) 3+ conditions in the high containment facility at FLI, Insel Riems, Germany. The Animal Welfare Act (Tierschutzgesetz, TSchG, Tierschutzgesetz in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl. I S. 1206, 1313), zuletzt geändert durch Gesetz vom 15. Juli 2009 (BGBl. I S. 1950) based on EWGRL 628/91, EWGRL 630/91, EGRL 119/93, EWGRL 609/86, and EWGRL 35/93) of the Federal Republic of Germany as well as the Animal Diseases Act were consistently applied. All animal experiments were evaluated and approved by the Ethics Committee of the Federal State of Mecklenburg-Western Pommerania.

Table 3.2. Experiment design: Viruses and Animals

HPAI H5N1 Virus	Animals			
	Experiment; Duck	Exposure		Control
		White-leghorn chicken,	Duck	
Clade 1	Muscovy (10)	Chicken (2)		Pekin ducks (3)
	Pekin (10)	Chicken (2)		
	Mallard (8)	Chicken (2)		
Clade 2.2	Muscovy(10)	Chicken (2)	Pekin(1)	
	Pekin (10)	Chicken (2)	Pekin(1)	
	Mallard (8)	Chicken (2)	Pekin(1)	

3.4.4 Clinical signs, Pathogenesis and Gross Pathology

Infected ducks and in-contact chickens were observed daily from the day of inoculation until death or 8 dpi. The clinical signs were recorded and necropsies were carried out immediately after death or preserved at -4°C until necropsy. At necropsy, all carcasses were examined and gross morphological changes were recorded.

3.4.5 Histopathology and Immunohistochemistry on Fresh Samples

5µm Paraffin-wax sections were dewaxed and stained with hematoxylin-eosin for histopathological investigations of tissue samples. For immunohistochemistry, sections were mounted on charged microscope slides (Menzel, Braunschweig, Germany), dewaxed and rehydrated. To detect influenza virus antigen, sections were incubated with an in-house rabbit anti-nucleoprotein (NP) serum (Starick *et al*., 2006) in a dilution of 1:500 in Tris-buffered saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6) as earlier described (Klopfleisch *et al.,* 2006; Teifke *et al*., 2007). Other procedures were as described in section 3.2.2, above.

3.4.6 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) Detection

An rRT-PCR for the direct, fast, and highly sensitive analysis of the HPAIV H5N1/Qinghai-like HA cleavage site sequence representative of the AIV H5N1 strains was used (Hoffmann *et. al*., 2007). A set of primers (FliH5_1028F and FliH5_1190R) and two probes were designed for the amplification and detection of a fragment spanning the cleavage site sequence of the H5 HA gene. The hexachloro-6-carboxyfluorescein (HEX) - labeled probe (FliH5-1148-HEX) was designed to target to a sequence reasonably conserved among various H5 strains. The 6-carboxyfluorescein (FAM)-labeled probe (FliH5-CS-FAM) was specific for the cleavage site sequence of H5N1 isolates of the Qinghai lineage. A FAM fluorescent signal of the M gene confirms influenza A virus but not subtype H5N1 and this indicates absence of cross reactivity to other subtypes of influenza A virus. Viral RNA was extracted from tracheal or cloacal swabs or allantoic fluid by use of a viral RNA mini kit (QIAGEN). One-step rRT-PCR was accomplished with an ABI 7500 (Applied Biosystems) or an MX3000p (Stratagene) cycler by use of a QuantiTect probe RT-PCR kit (QIAGEN). To amplify the virus genome segments encoding the NP, M, HA, NA and NS proteins, a total of 5µl of RNA extract was amplified in a

volume of 25µl by use of an Eppendorf thermal cycler and employing the following temperature profile: 30 min at 50°C, 15 min at 95°C, and 42 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. FAM- and HEX-specific emission data were collected during the annealing step. Cycle threshold (CT) values of <40 were considered indicative of the presence of H5- specific RNA when all negative controls revealed CT values of ≥40.

3.4.7 Virus Re-isolation

All birds were necropsied and brain, lung, as well as intestine (terminal colon and cloaca) were collected for virus recovery and titration. Tissue homogenates were inoculated into 10-day-old embryonated eggs for virus isolation as recommended (OIE*,* 2008). Infectivity titres were calculated by the method of Reed and Muench (1938) and expressed as EID_{50} per gram of tissue. Tissue samples received a second egg passage when embryonic death, but no haemagglutination, was detected in the allantoic fluid. Additionally, the tissues were analysed by RT-PCR by amplifying a conserved part of the matrix protein gene as described elsewhere (Starick *et al*., 2000; Starick & Werner, 2003). Mean virus titre values lower or higher than the infectivity dose of EID_{50} log 10^6 in tissues of all duck species was indicative of successful re-isolation.

3.4.8. Statistical Analysis

The RRT-PCR mean threshold cycle (ct) values of tracheal, cloacal and tissues of all the ducks and tracheal and cloacal RRT-PCR mean ct-values of the chickens were inputed into Medicalc statistical software. This was used to test the null hypotheses; where there is no significant difference between the variables used eg (virus type vs duck type), null hypotheses is acceptable. In case there is a significant difference between the variables, the null hypotheses is rejected and the alternate hypotheses is accepted. This software was also used for the one-way and two-way analysis of variance (ANOVA) and used to plot a multiple clustered variable graphs for the variables versus days post infection. A Chi-square (X^2), test for the comparison of two proportions (from independent samples) expressed as percentage was performed and when the P-value was <0.05.The conclusion was that the two proportions were significantly different. The difference between these two proportions and a 95% confidence interval (C.I) for this difference were also calculated. (Medicalc Inc.2011).

CHAPTER FOUR

4.0 RESULTS

4.1 STUDY 1: THE EPIDEMIOLOGY AND PATHOLOGY OF HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 OUTBREAKS IN COMMERCIAL AND BACKYARD POULTRY BETWEEN 2006 AND 2008 IN NIGERIA.

4.1.1 Epidemiology

In commercial chickens, mainly layers together with pullets, cockerels and broilers, flocks in the south west and northwest with highest total flock size were the most affected (table 4.1) by the HPAI-H5N1 infection. The number dead before depopulation were highest in northwest (69,741), which was followed by southwest (table 4.1). Other values are shown in the same table. Also, in backyard flocks, the number dead before depopulation were highest in northwest (9,382) and the region had the highest flock size affected (table 4.2). There were fewer reporting in backyard poultry from the southwest and south-south regions while there was none from the south-east. Although layer farms were most affected (127 farms) with highest number dead (104,351) before depopulation, mortality rate was least (11.11%) in the layer flock (table 4.2). Broiler farms had the highest mortality rate (73.92%). The domestic duck had the highest mortality rate (87.1%) in the backyard poultry. This was followed by the domestic chicken (82.13%) and the turkey (80.59%) in the backyard poultry (table 4.4). In total, Two hundred and ninety-nine (299) positive HPAI cases were recorded in Nigeria from 2006-2008. This includes outbreak cases in commercial poultry which affected one hundred and fifty-three (153) including three (3) ostriches farms (Table 4.3) in eighteen (18) states and the Federal Capital Territory (FCT), Abuja and outbreak cases in backyard poultry which affected eighty (80) farms (Table 4.4) in sixteen (16) states and the FCT, Abuja. Also included were 66 positive cases (tissue samples) from live bird markets without detailed case histories, submitted for HPAI diagnosis or confirmation at the National Veterinary Research Institute. Other birds not included in commercial and backyard poultry are vulture, pheasant, water fowl, pigeon and canary; all totaling one hundred three birds (103).The bird losses (as a result of death or stamping out of infected poultry) from documented case histories obtained directly from farmers who reported to the Central Diagnostic Laboratory of NVRI was one

million, one hundred and six thousand, six hundred and ninety-three birds (1,106,693) including chicken (broiler, cockerel, pullet and layer), duck, turkey, geese and guinea fowl.

Table 4.1. Highly Pathogenic Avian Influenza in Nigerian states: mortalities in intensively reared commercial poultry types.

S/N	Zone	State	Flock size	Number dead	Chicken type
1	**North East**	Adamawa	55	36	Cockerel
2		Bauchi	28731	4637	layer, pullet, broiler
3		Bornu	800	207	layer,pullet,cockerel
	Regional subtotal		**29586**	**4880**	
1	**North West**	Jigawa	13786	3000	Layer
2		Kaduna	87435	51333	layer,pullet,broiler,cockerel
3		Kano	234998	7930	layer, cockerel
4		Katsina	95605	6778	Layer
5		Sokoto	6562	700	Layer
	Regional subtotal		**438386**	**69741**	
1	**North Central**	Abuja	236	165	layer, broiler
2		Plateau	24210	4712	layer,pullet,broiler,chick
3		Nasarawa	58	56	broiler,cockerel,mixed
4		Kwara	6685	1485	Layer
	Regional subtotal		**31189**	**6418**	
1	**South West**	Lagos	291780	21632	layer,pullet,broiler
2		Ogun	138536	642	layer, cockerel
3		Oyo	15004	4604	layer,broiler,cockerel
	Regional subtotal		**445320**	**26878**	
1	**South South**	Rivers	1200	200	Layer
2		Edo	6500	5109	layer,pullet,broiler
	Regional subtotal		**7700**	**5309**	
1	**South East**	Anambra	13352	1653	Layer
2		Enugu	50	50	Broiler
	Regional subtotal		**13402**	**1703**	
	Others	north central	**20**	**10**	Ostriches
	National Total		**965603**	**114939**	

Table 4.2. Highly Pathogenic Avian Influenza in Nigerian states: losses in backyard poultry in Nigeria, 2006-2007.

S/N	Region	State	flock size	no. dead	Village Poultry type
1	**North West**	Kano	22	13	Geese, turkey
2		Jigawa	9343	9062	Chicken, duck, turkey, guinea fowl
3		Kaduna	425	179	Chicken, duck, turkey, geese
4		Katsina	127	127	Chicken, duck, turkey, guinea fowl, geese
5		Zamfara	1	1	Chicken
	Regional subtotal		**9918**	**9382**	
1	**North East**	Adamawa	250	37	Chicken, guinea fowl, mixed
2		Bauchi	737	451	Chicken, duck, turkey, guinea fowl
3		Yobe	6	6	Chicken, duck
4		Bornu	50	29	Chicken
	Regional subtotal		**1043**	**523**	
1	**North Central**	Benue	500	500	Chicken
2		Abuja	40	37	Chicken, geese
3		Nasarawa	2298	509	Chicken, duck, turkey
4		Plateau	50	6	Chicken, duck
5		Taraba	103	89	Chicken, duck, turkey
	Regional subtotal		**2991**	**1141**	
1	**South West**	Lagos	60	High	Chicken
	Regional subtotal		**60**	**High**	
1	**South South**	Edo	500	500	Duck, turkey
	Regional subtotal		**500**	**500**	
		Total	**14512**	**11546**	

Table 4.3 HPAI in Nigerian states: commercial poultry distribution and mortality rates, 2006-2007.

Chicken type	**No. of Farms**	**Flock size**	**No. dead**	**Culled/stamped out (%)**	**Mortality rate (%)**	**Carcasses examined**
Layer	127	939620	104351	835269	11.11	248
Pullet	9	16421	4408	12013	26.84	25
Cockerel	8	3109	1415	1694	45.51	14
Broiler	9	6433	4755	1678	73.92	29
Ostrich	3*	20	10	10	50	-
Total	**156**	**965603**	**114939**	**850664**		**316**

*ostrich included only for computation of losses, not necropsied

Table 4.4. HPAI in Nigeria states: Backyard Poultry distribution and mortality rates, 2006-2007.

Backyard Poultry	No. farms	Flock size	No. dead (%)	Stamped out (%)	Mortality rate (%)	Carcasses examined
Chicken	37	12975	10657	2318	82.13	60
Duck	15	356	310	46	87.1	29
Turkey	14	505	407	98	80.59	37
Guinea fowl	8	579	119	460	20.55	14
Geese	6	97	53	44	54.64	12
Total	**80**	**14512**	**11546**	**2966**		**152**

The coordinates of the two hundred and thirty-three (233) HPAI-H5N1 infected farms and sixty-six (66) live bird markets in which HPAI-H5N1 was detected and were collected using Personal Digital Assistant (PDA) running Global Positioning System (GPS) and the non-spatial data collected through case reports were added to the Arc view GIS software for mapping on a state by state basis (Fig 4.1). This showed two clusters of outbreak points, one was in the northwestern-central region and the other was in the southwestern region of the country. Apart from these two clusters, isolated outbreak points were observed in the north-eastern and southern regions of the country. A geoecological map that shows the 24 wetlands (Saidu *et al*., 2008) in Nigeria was included to demonstrate the proximity and association of outbreaks points to wetlands (Fig 4.2). One of the outbreak cluster houses the Hadeija-Nguru wetland in Jigawa State which is significant for its wild bird wintering activities. Some isolated outbreak points especially in Yobe, Gombe and Kebbi also have presence of wetlands. The southwestern cluster, though houses some wetland, does not have significant wild bird activity. These two clusters also had the highest bird loss figures; high poultry density and majority of the farms affected are in these regions. The following maps below summarize the above findings.

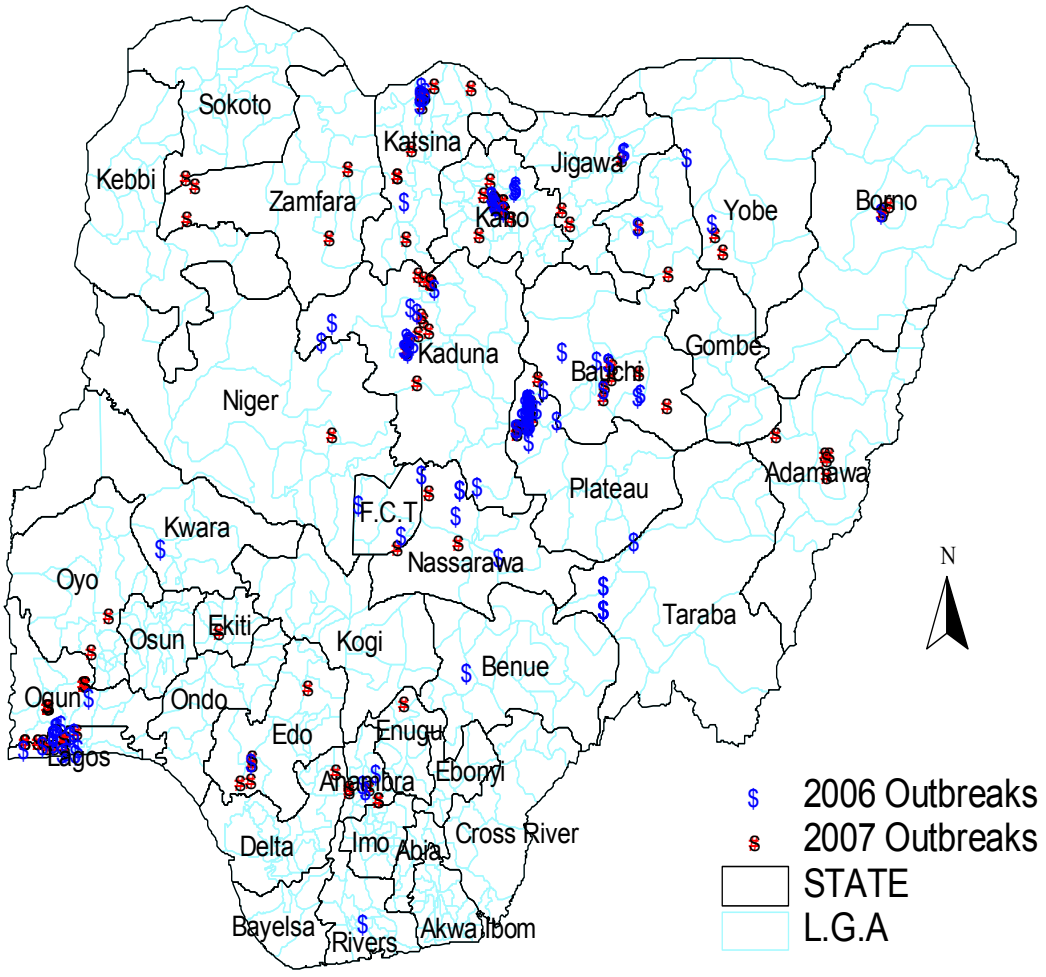

Figure 4.1. Spatial distribution of HPAI outbreaks in poultry in Nigeria from January 2006 to October 2007 and location of the single human case in south-western Nigeria

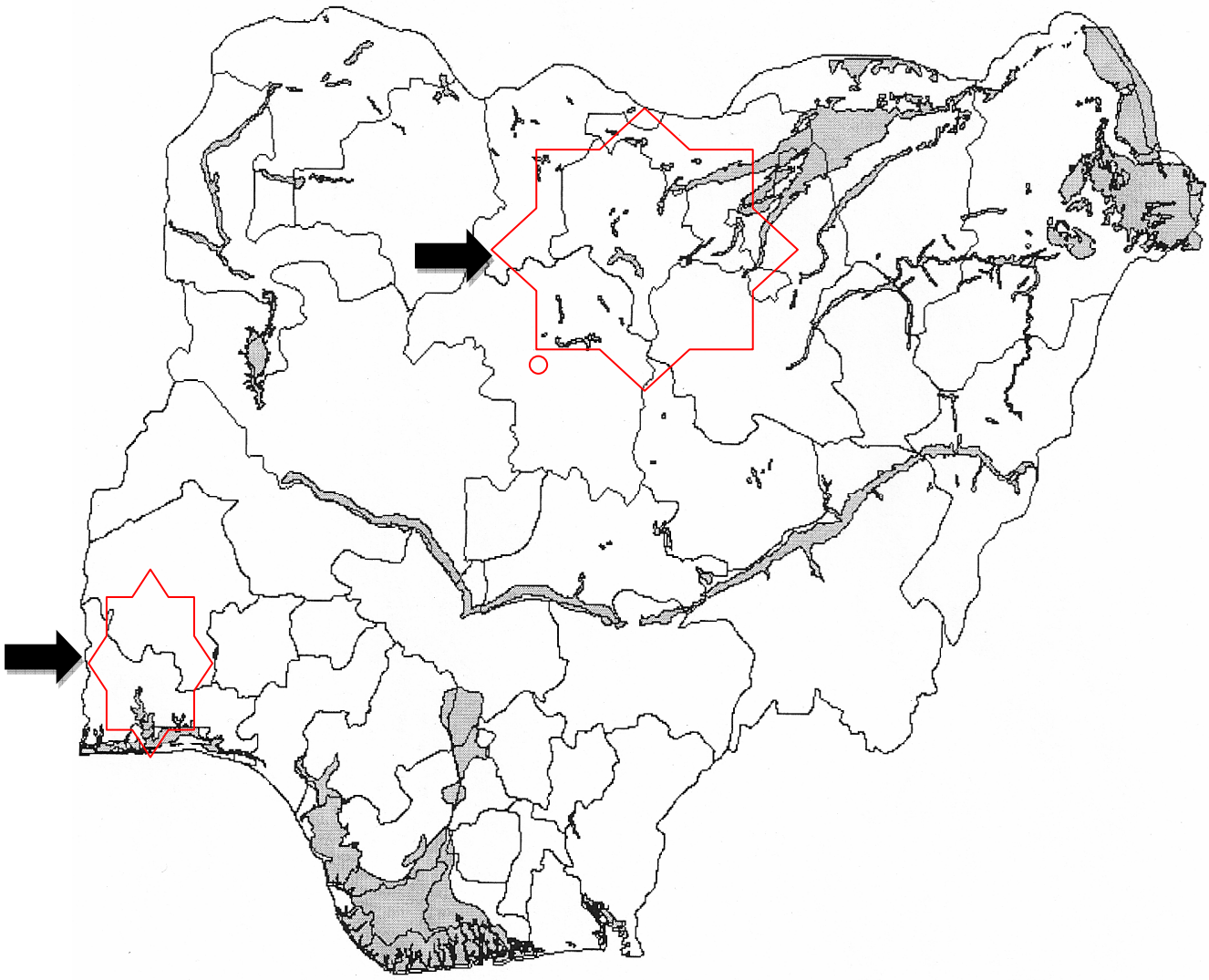

Figure 4.2. Geo-ecological map showing the 24 wetlands in Nigeria and HPAI outbreak clusters

4.1.2 Gross and Histopathology

Gross lesions observed included cyanosis and oedema of comb and wattle, facial and subcutaneous edema, haemorrhagic shanks, depression, paralysis, ataxia and torticolis. There were mucoid to catarrhal nasal discharges, dyspnea, coughing, sneezing and diarrhea. Main pathologic findings were observed in the nervous, circulatory, respiratory, integumentary, musculoskeletal, hemopoietic, gastro-intestinal and reproductive systems, occasionally lesions are multisystemic. Airsacculitis and pneumonia were also present in the commercial birds that died. There was petechiation and ecchymoses of the proventricular and intestinal mucosae. Integumentary system lesions are mainly cyanosis, subcutaneous edema and ecchymotic hemorrhages while there were inflammatory, degenerative and necrotic lesions in the musculoskeletal system. In adult commercial birds, mainly layers, reproductive signs and lesions were observed and they were mainly ovarian follicular ecchymotic hemorrhages and structure abnormalities such as shell-less and soft-shelled eggs. In younger birds (≤ 3 months old) and free-range chickens HPAI viruses caused acute mortality with minimal premonitory sign of disease while manifestation of clinical disease tended to be more overt with older flocks. The pathological features observed in HPAIV infected commercial chickens in Nigeria are consistent, lesions are mainly congested, hemorrhagic dermatitis with necrosis of the comb and wattle (Plate 4.1), subcutaneous edema (Plate 4.2), hemorrhagic tracheitis and proventricular hemorrhages (Plate 4. 3). The abdominal fat (Plate 4.4) had multifocal petechial hemorrhages.There was duodenal petechial and ecchymotic hemorrhage (Plate 4.5). Ovarian follicles were hemorrhagic and necrotic (Plate4.6). The shank and feet show petechiation and ecchymosis (Plate 4.7).

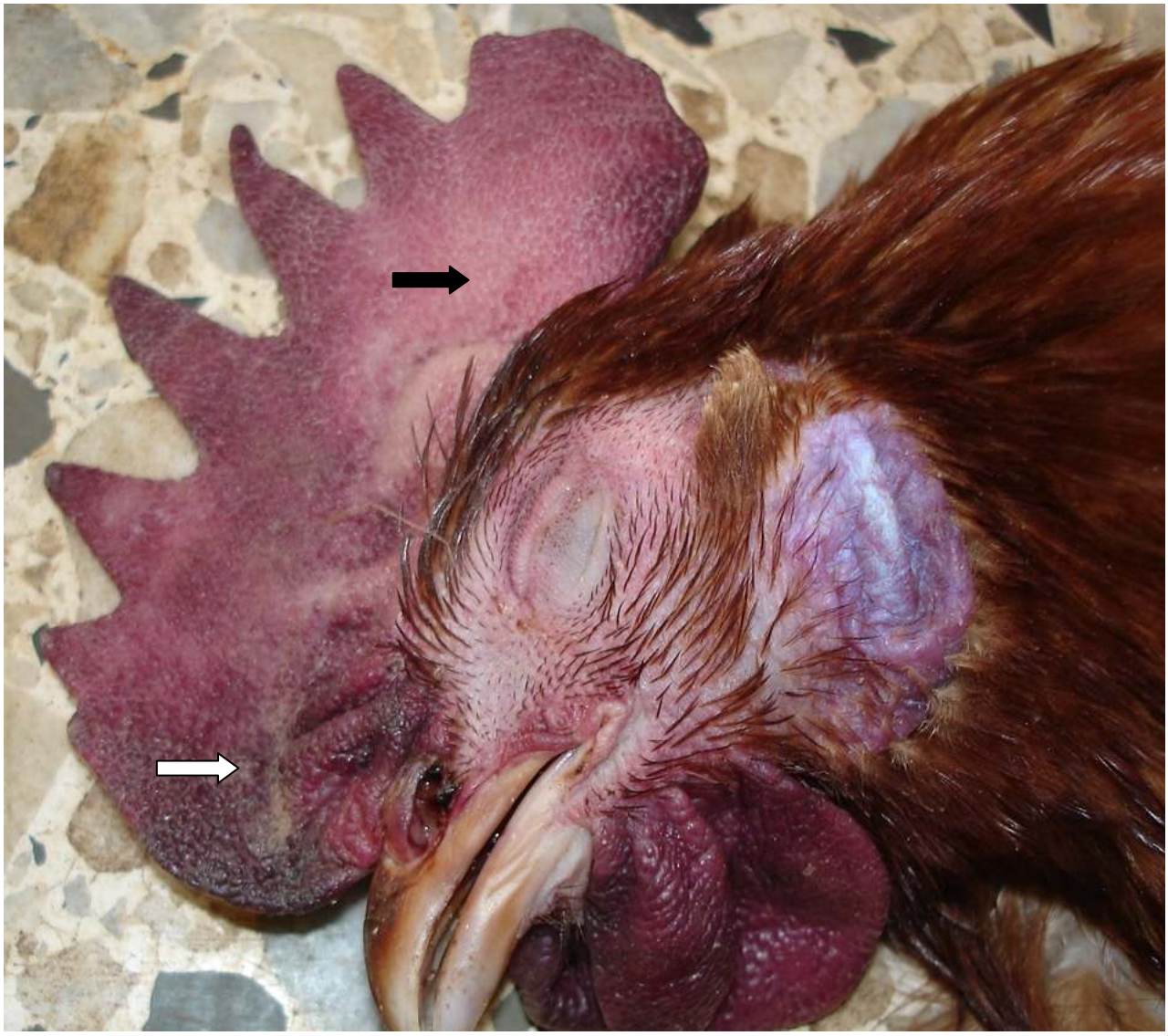

Plate 4.1. Comb and wattle, natural infection, clade 2.2 HPAI Cyanosis (black arrow) and hemorrhagic dermatitis (white arrow).

Plate 4.2. Wattle and neck, Chicken, natural infection, clade 2.2 HPAI. Congested trachea (white asterisk), wattle and neck subcutaneous edema (black asterisk).

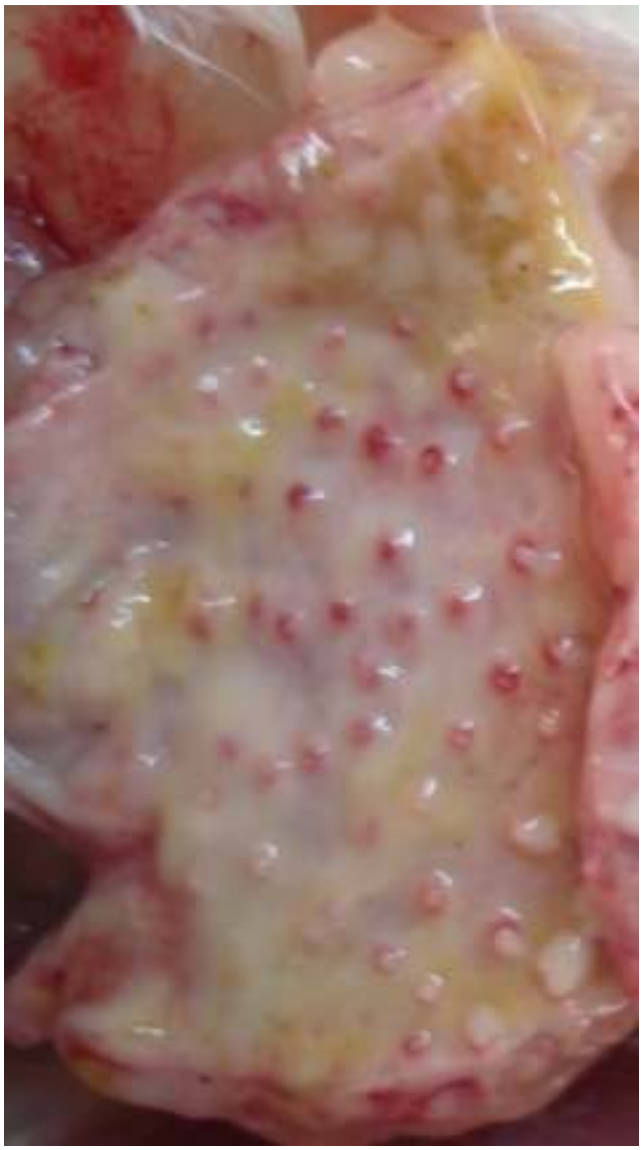

Plate 4.3. Proventriculus, Chicken, natural infection, clade 2.2 HPAI, mucoid glandular hypertrophy and hemorrhage of tips of proventricular glands.

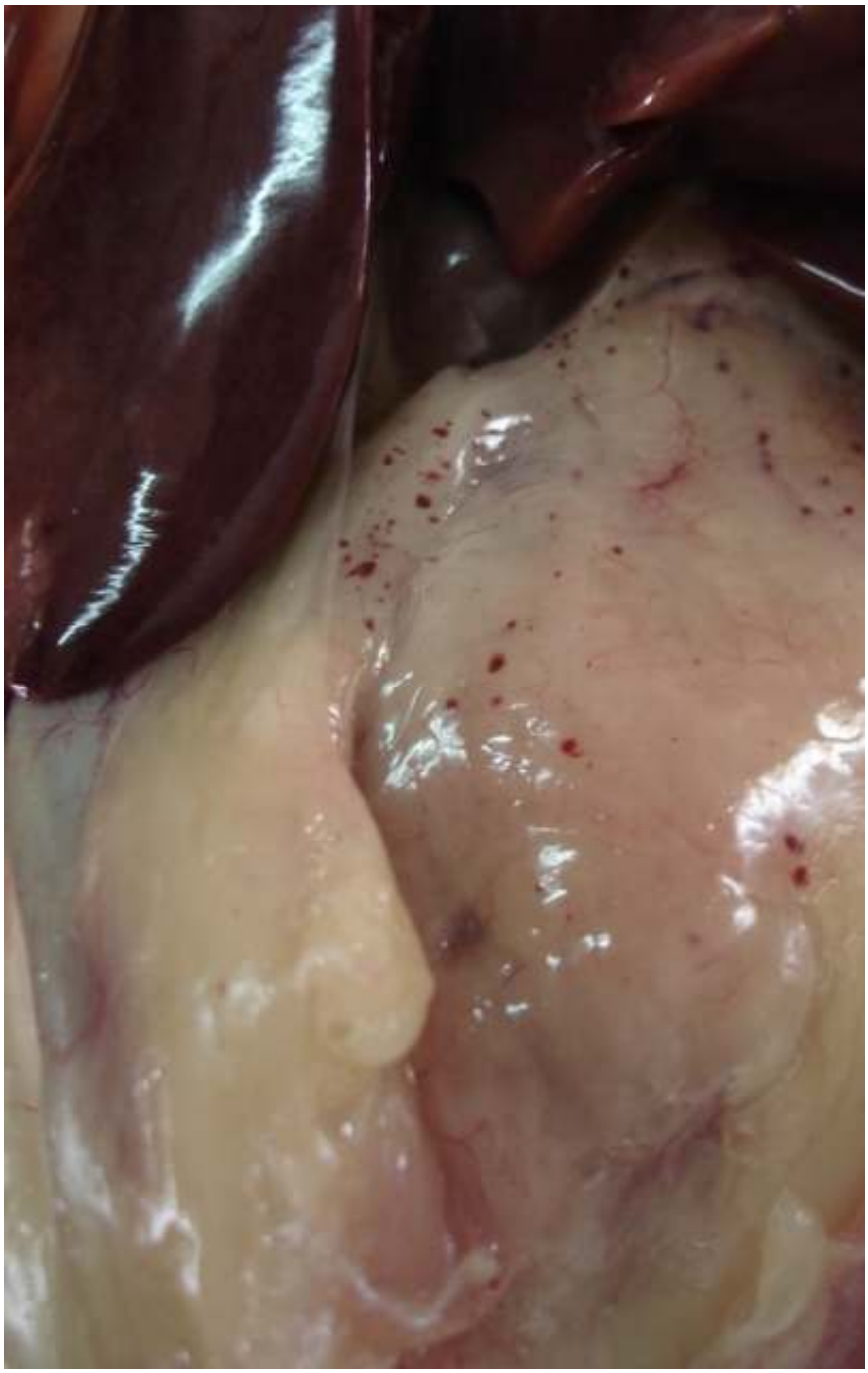

Plate 4.4. Liver and abdominal fat, Chicken, natural infection, clade 2.2 HPAI. Petechial and ecchymotic hemorrhages on the abdominal fat.

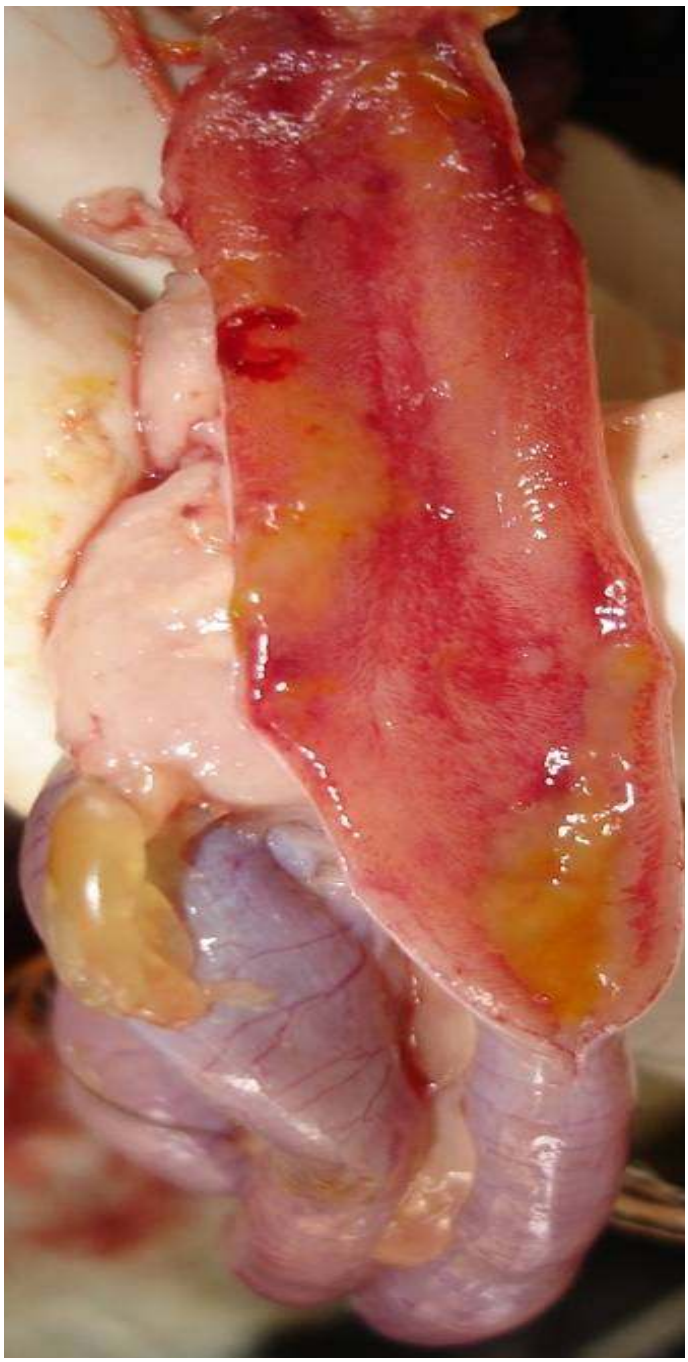

Plate 4.5. Duodenum: mucosal hyperemia and hemorrhage, Chicken, natural infection, clade 2.2 HPAI.

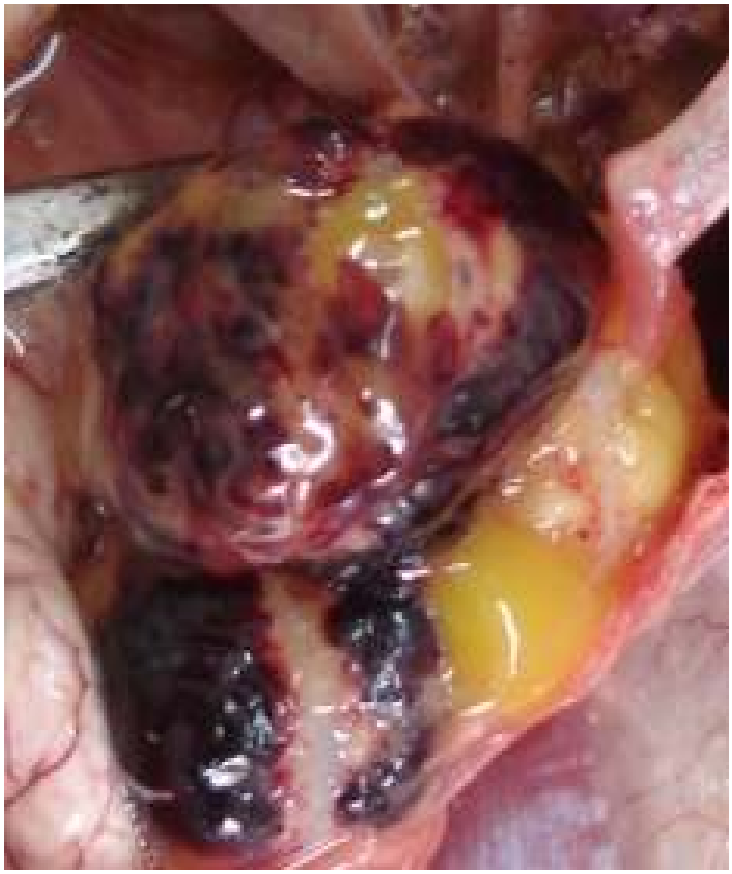

Plate 4.6. Ovarian follicles. Chicken, natural infection, clade 2.2 HPAI. Severe, diffuse hemorrhagic ovarian follicles.

Plate 4.7. Shank and Feet: Chicken, natural infection, clade 2.2 HPAI. Diffuse shank and feet hyperemia.

4.1.3.1 Layers

Most of the layers (67%) exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only 20% showed nervous signs and brain lesions of neuronal necrosis of cerebrum and Purkinje cell necrosis of cerebellum. Respiratory signs and lesions of nasal exudation, fibrinous airsacculitis and pneumonia was evident in 43.5% with more than half having pneumonia. Of the 60 clinical reports of diarrhea only 42 (70%) had enteritis and 18 (42.8%) had petechial to ecchymotic hemorrhages. Enteric petechiation and ecchymoses was observed in 45 (75%) of the carcasses. In all 42.3% of the 248 commercial layer carcasses examined showed GIT lesions including proventricular petechial to ecchymotic hemorrhages, pancreatic necrosis and pancreatitis. A few carcasses (6.4%) showed Integumentary system lesions, these are mainly cyanosis, edema and ecchymotic hemorrhages. Only 13.7% had muscular ecchymotic hemorrhages with necrosis, and or myositis. Only this class of chickens showed reproductive signs and lesions and these were observed in 15.3% only. The reproductive system lesions included lack of evidence of active laying in some adult layers, atrophy of follicles, underdeveloped and mishappened ovarian follicles, ovarian follicular congestion and haemorrhages. This also included egg structural abnormalities such as shelless egg, white eggs and soft shelled eggs. Forty-one (41) percent of the layers which died suddenly or from flocks with high mortality or that were anorexic or off feed weak and recumbence were classified as multisystemic signs/lesions. Lymphoid lesion included necrotic and hemorrhagic cecal tonsils.

4.1.3.2 Pullet

Of the twenty-five (25) pullet carcasses examined 60%, exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only 4% showed nervous signs of incordination and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory signs included respiratory rales, nasal exudation and dyspnea while lesions included fibrinous airsacculitis and pneumonia which were evident in 32%. Catarrhal enteritis was observed in 3 (12%) of the 25 carcasses examined. Twenty-eight (28) percent had muscular and shank hemorrhages with necrosis, and or myositis. Twenty-four (24) percent of the pullets which died suddenly or from flocks with high mortality or that were anorexic or off feed, weak and recumbence were classified as multisystemic signs/lesions. Lymphoid lesion included necrotic

and hemorrhagic cecal tonsils and occasionally, Bursa of Fabricius were edematous, enlarged and haemorrhagic. No lesion was observed in the integumentary and reproductive systems respectively in all carcasses examined.

4.1.3.3 Broiler

Of the twenty-nine (29) broiler carcasses examined 82.7%, exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only 4 (13.7%) showed nervous signs and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory signs included sneezing, bloody nasal discharge, coughing, and difficult breathing while lesions of nasal exudation, congested trachea with serous exudation, airsacculitis and pneumonia were evident in fourteen (48.2%) carcasses. Enteric lesion was observed in 9 (31%) of the 29 carcasses examined. Five (17.2%) carcasses had muscular and hemorrhages with necrosis, and or myositis. Ten (34.4%) of the broilers which died suddenly or from flocks with high mortality or that were anorexic or off feed, weak and recumbence were classified as multisystemic signs/lesions. Lymphoid lesion included necrotic and hemorrhagic cecal tonsils and occasionally, Bursa of Fabricius were edematous, enlarged and haemorrhagic. No lesion was observed in the integumentary and reproductive systems respectively in all carcasses examined.

4.1.3.4 Cockerel

Of the fourteen (14) cockerel carcasses examined 64%, exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only 5 (35.7%) showed nervous signs and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory signs and lesions of nasal exudation, airsacculitis and pneumonia were evident in five (35.7%) carcasses. Enteric lesion was observed in 6 (42.8%) of the 14 carcasses examined. Eight (57.1%) carcasses had muscular and shank hemorrhages with necrosis, and or myositis. Five (35.7%) of the cockerel which died suddenly or from flocks with high mortality or that were anorexic or off feed, weak and recumbence were classified as multisystemic signs/lesions. No lesion was observed in the integumentary and reproductive systems respectively in all carcasses examined.

4.1.3.5 Backyard Chickens

Twenty-four of the local chicken (40%) exhibited cyanosis of comb and wattle with occasional facial edema. Only 5 (8.3%) showed nervous signs of Torticollis; and neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory distress and lesions of nasal congestion and exudation, airsacculitis, congested trachea, pulmonary congestion, edema and pneumonia was evident in 19 (31.6%). Ecchymotic hemorrhages was seen on the mucosal surface of the proventricular, Intestinal petechiation and ecchymoses was observed in 21 (35%) of the carcasses. Lymphoid lesion included hemorrhagic and necrotic cecal tonsils, enlarged spleen with necrotic foci and enlarged and hemorrhagic bursa of fabricius

Only seventeen (28.3%) had muscular hemorrhages. Twenty-one (35%) of the local chickens which died suddenly or from flocks with high mortality or that were anorexic or off feed weak and recumbence were classified as multisystemic signs/lesions.

4.1.3.6 Duck

Sixteen of the ducks (55.1%) exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only four (13.7%) showed nervous signs and brain lesions which includes neuronal cell necrosis of cerebrum and and Purkinje cell necrosis of cerebellum. Respiratory signs and lesions of nasal exudation, airsacculitis, congested and hemorrhagic trachea and pneumonia was evident in sixteen (55.1%) with more than half having pneumonia. Enteric petechiation and ecchymoses was observed in 10 (34.4%) of the carcasses, occasional peritonitis with accumulation of caseous material was also recorded. Only one (3.4%) had muscular hemorrhages with necrosis, and or myositis. Seventeen ducks (58.6%) which died suddenly or from flocks with high mortality or that were anorexic or off feed weak and recumbence were classified as multisystemic signs/lesions. There was occasional congestion and hemorrhages in the spleen

4.1.3.7 Turkey

Thirty of the turkeys (81%) exhibited cyanosis of comb and wattle with occasional facial edema. Only one (2.7%) showed nervous signs and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. There was sero-mucous nasal exudation and rhinitis, congested trachea, cloudy airsac and airsacculitis, pulmonary consolidation and edema was evident in seventeen (45.9%).

Ecchymotic hemorrhages on the proventricular glands were commonly seen in majority of the carcasses. The liver was swollen and congested. Enteric petechiation and ecchymoses was observed in 13 (35.1%) of the carcasses and majority of them have yellowish-white fecal vent pasting. Only three (8.1%) had muscular hemorrhages with necrosis, and or myositis. Tweenty-seven turkeys (72.9%) which died suddenly or from flocks with high mortality or that were anorexic or off feed, weak and recumbence were classified as multisystemic signs/lesions.

4.1.3.8 Guinea fowl

Only three guinea fowls (21.4%) exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Only one (7.1%) showed nervous signs and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory signs and lesions of nasal exudation, airsacculitis and pneumonia was evident in four (28.5%) with more than half having pneumonia. Enteric petechiation and ecchymoses was observed in 6 (42.8%) of the carcasses. Only four (28.5%) had muscular hemorrhages with necrosis, and or myositis. Eight guinea fowls (57.1%) which died suddenly or from flocks with high mortality or that were anorexic or off feed weak and recumbence were classified as multisystemic signs/lesions.

4.1.3.9 Geese

Two geese (16.6%) exhibited one or more signs and lesions of the circulatory system, mainly cyanosis of comb and wattle with occasional facial edema. Seven (58.3%) showed nervous signs and brain lesions of neuronal and Purkinje cell necrosis of cerebrum and cerebellum respectively. Respiratory signs and lesions of nasal exudation, airsacculitis and pneumonia was evident in ten (83.3%) with more than half having pneumonia. Enteric petechiation and ecchymoses was observed in 4 (33.3%) of the carcasses. One goose (8.3%) which died suddenly after been recumbent was classified as multisystemic signs/lesions. Table 4.5 below summarizes and compares the clinical signs and systemic lesions seen in the poultry types.

Table 4.5**.** Prevalence of systemic lesions in different HPAI infected poultry groups in Nigeria

Bird type	Percentage of birds affected							
	Circulatory	Nervous	Respiratory	GIT diarrhea	Integu-mentary	Musculo-skeletal	Repro-ductive	multisystemic
Layer	67%	20%	43.5%	70%	6.4%	13.7%	15.3%	41%
Pullet	60%	4%	32%	12%		28%		24%
Broiler	82.7%	13.7%	48.2%	31%		17.2%		34.4%
Cockerel	64%	35.7%	35.7%	42.8%		57.1%		35.7%
Backyard chicken	40%	8.3%	31.6%	35%		28.3%		35%
Duck	55.1%	13.7%	55.1%	34.4%		3.4%		58.6%
Turkey	81%	2.7%	45.9%	35.1%		8.1%		72.9%
Guinea fowl	21.4%	7.1%	28.5%	42.8%		28.5%		57.1%
Geese	16.6%	58.3%	83.3%	33.3%				8.3%

Histological lesions were observed in multiple organs and were characterized by exudation, haemorrhage, necrosis, inflammation, or a combination of these. In particular, the lung, heart, brain, spleen, pancreas, kidneys, combs and wattles were the most consistently affected. Other histological findings in the outbreaks were moderate to severe congestion and edema of comb (Plate 4.8) and wattles with necrosis of vascular endothelia. In the cerebrum (Plate 4.9) and cerebellum, multifocal neuronal necrosis and necrosis of Purkinje cells were present respectively. In the lungs, hyperemia, necrosis of endothelia cells and atria lining cells, alveolar edema and moderate heterophilic infiltration into the interstitium were seen (Plate 4.10). Diffuse cardiomyocyte necrosis was seen in several histologic sections. Random foci of coagulation and hepatocellular necrosis with heterophilic infiltration were observed commonly in affected livers. Foci of superficial heterophilic infiltration and epithelial necrosis were seen in the proventriculus and at the esophageal-proventricular junction. GALT of small intestine showed lymphoid depletion. There was necrosis of enterocytes lining the duodenal villi with submucosal heterophilic infiltration. The pancreas had multifocal necrosis of the acinar cells and hemorrhages. Multifocally, proximal convoluted tubular epithelium showed evidence of necrosis with mild to moderate infiltrates of heterophils.

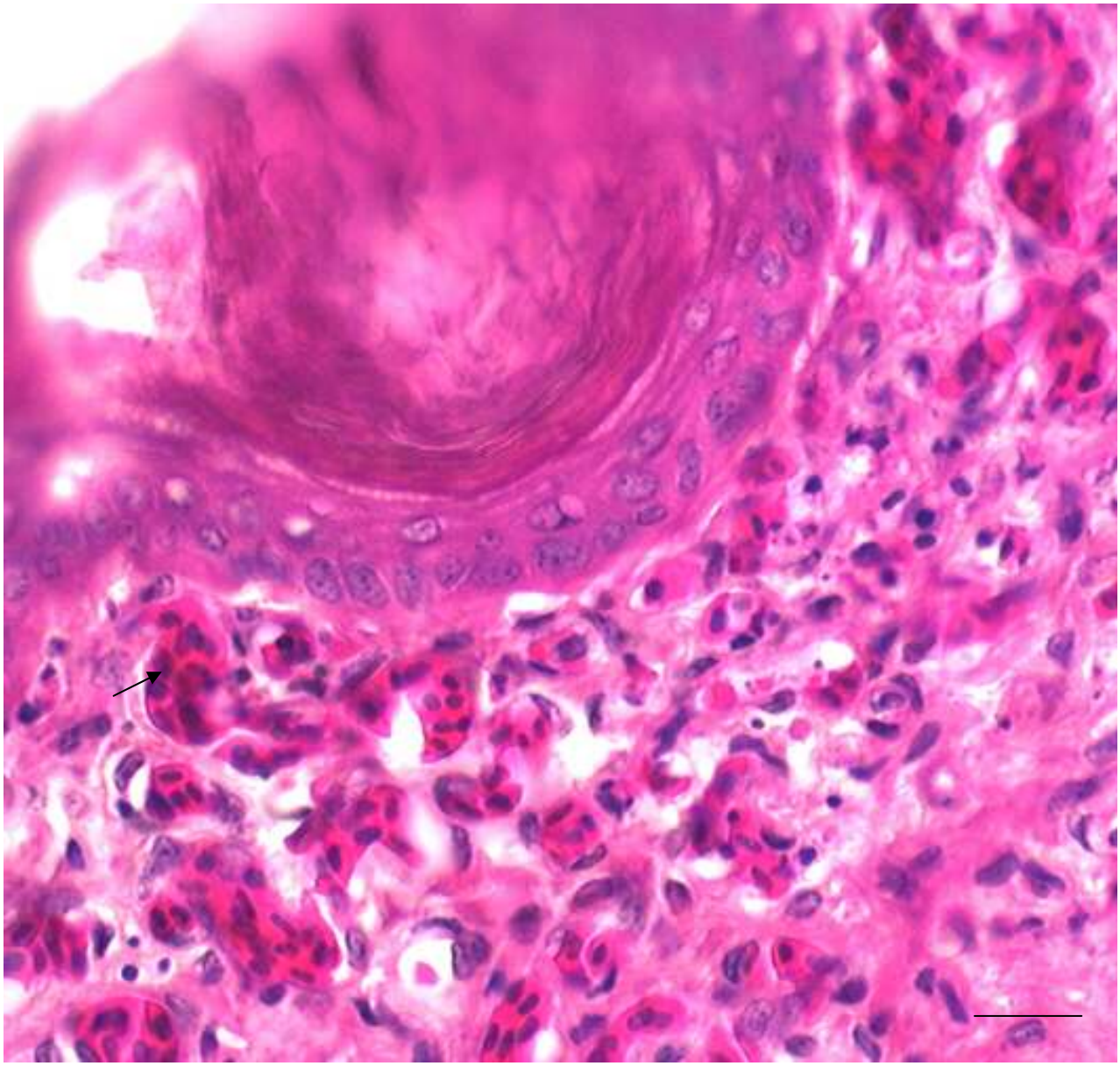

Plate 8. Comb; Chicken clade 2.2 HPAI. Natural infection, vascular congestion (black arrow), endothelial cell necrosis and heterophilic infitration in superficial dermis. HE Bar=100µm

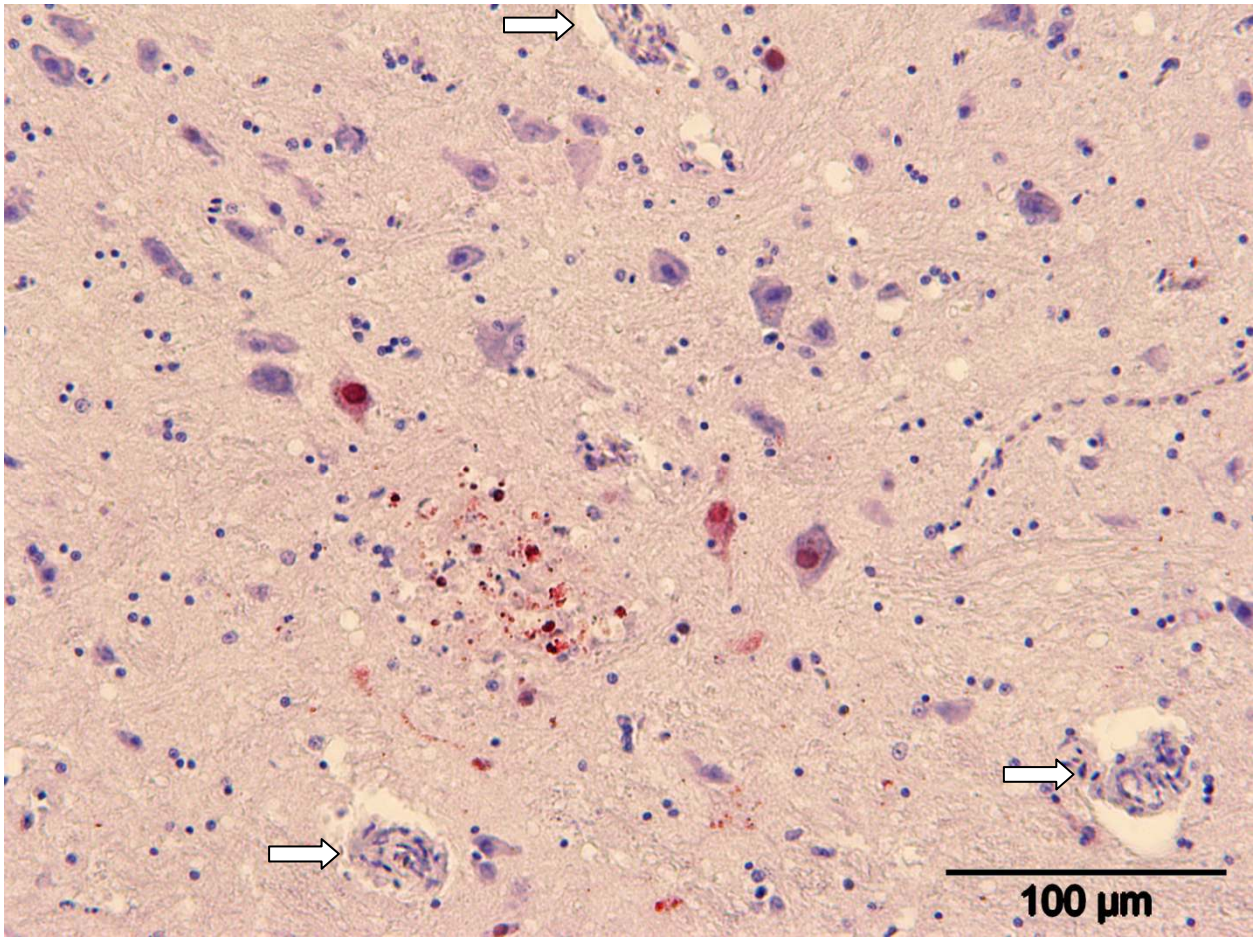

Plate 9. Cerebrum; Chicken clade 2.2 HPAI, natural infection. Encephalitis, severe diffuse with multifocal perivascular cuff formation (white arrow).

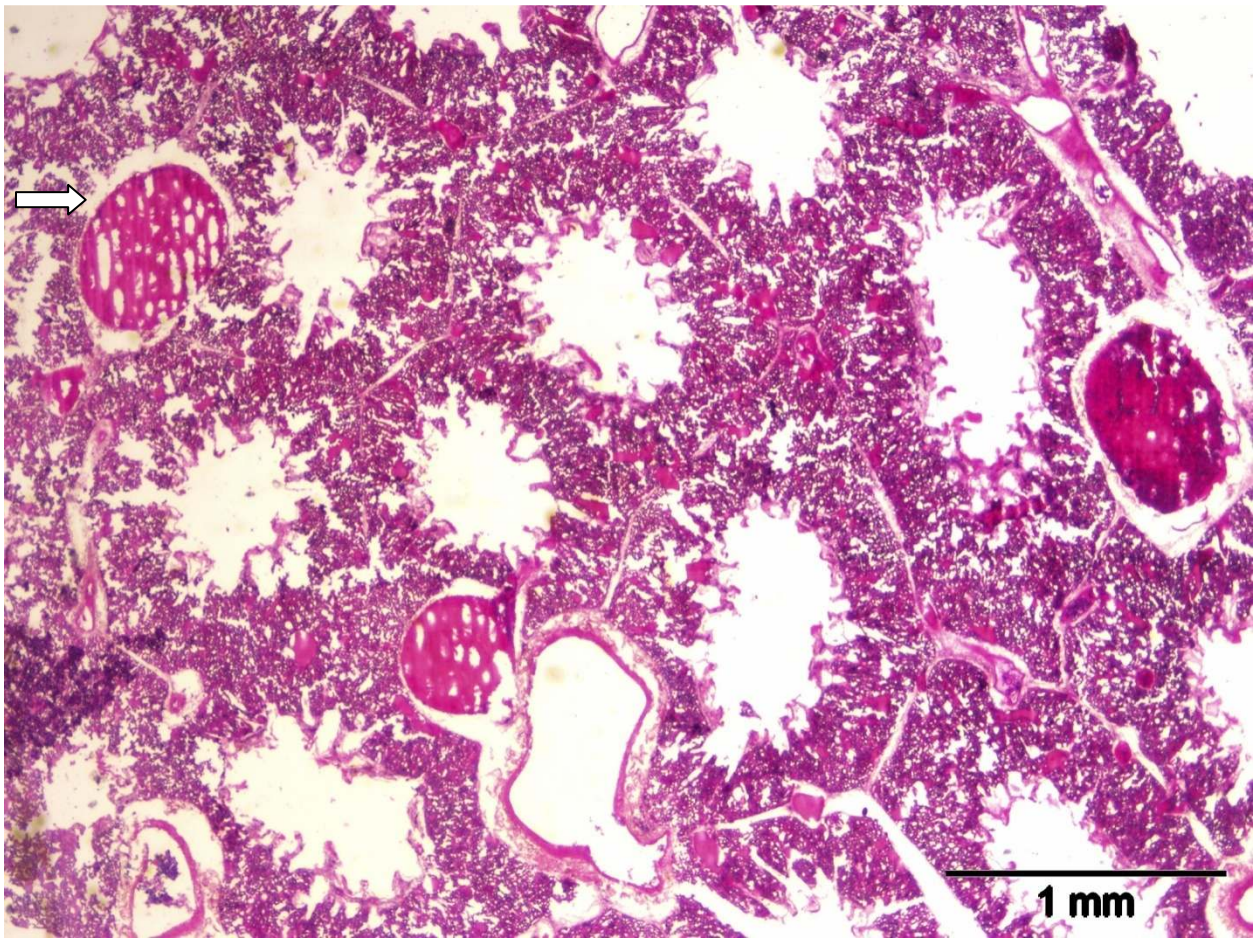

Plate 10. Lung; Chicken clade 2.2 HPAI, natural Infection. Severe peribronchial vascular congestion (white arrow).

4.2 STUDY 2: THE INFLUENCE OF MIXED SPECIES POULTRY FARMING ON THE MORTALITIES OF HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) H5N1 OUTBREAKS IN BACKYARD POULTRY BETWEEN 2006 AND 2008 IN NIGERIA.

4.2.1 Data

Analysis of the fifty-six (56) backyard poultry flocks included in this study on the basis of: a) backyard poultry flocks, b) HPAI infected and laboratory confirmed, c) fulfillment of being single species backyard flocks and/or mixed species backyard flocks, reveals the following:

Chicken only flocks HPAI outbreaks were reported the most from the North-central states followed by the north-eastern states and only three reports from the north-west. Duck only flocks HPAI outbreaks were reported most from the North-central states followed by the north-western states and only two reports from the north-eastern states and a single case from south-south state of Edo. Turkey only flocks HPAI outbreaks were reported the most from the North-western states; while one case each from the north east, north central and south-south. Only one case of Guinea fowl only flocks was reported. Six cases from Chicken and duck only flocks, three each from north central and north eastern states. Also, three cases were reported from Chicken, duck and turkey only flocks while three cases were reported from Chicken, duck and guinea fowl only flocks. The following tables show the distribution and mortality rates in single and mixed species flocks naturally infected with HPAI in Nigeria. Tables 4.6 and 4.7 below summarize the data on single species backyard flocks and mixed species backyard flocks.

Table 4.6. Distribution of single species backyard poultry flocks naturally infected with HPAI in Nigeria.

Species	Zone	State	Location	Flock size	Number dead	Mortality rate
Chicken	North Central					
		Jigawa	Hadeija	17	6	35.3
		Nasarawa	Akwanga west	58	58	100
		Nasarawa	Akwanga west	20	20	100
		Plateau	Jos	30	4	13.3
		Taraba	Ibi	18	17	94
		Taraba	Ibi	5	5	100
		Taraba	Ibi	5	4	80
		Taraba	Wukari	13	10	76.9
		Taraba	Wukari	11	7	63.6
		Abuja	Kuje	40	35	87.5
		Nasarawa	Andaha	1	1	100
	North east	Yobe	Potiskum & Jakusko	1	1	100
		Yobe	Jakusko	2	2	100
		Yobe	Jakusko	2	2	100
		Katsina	Kankara	20	20	100
		Adamawa	Girei	20	19	95
		Bauchi	Misan	20	3	15
		Bauchi	Bauchi	6	2	33.3
		Bauchi	Bauchi	21	21	100
		Bornu	maiduguri	50	29	58
	North west	Jigawa	Dutse	1	1	100
		Zamfara	Bungudu LGA	1	1	100
		Katsina	Katsina	36	36	100

Table 4.6. Contd: Distribution of single species backyard poultry flocks naturally infected with HPAI in Nigeria.

Duck	Zone	State	Location	Flock size	Number dead	mortality rate
	North Central	Nasarawa	Akwanga west	15	15	100
		Nasarawa	Akwanga west	4	4	100
		Plateau	Jos	20	2	10
		Nasarawa	Akwanga west	12	12	100
		Taraba	Wukari	11	7	63.6
	North east	Yobe	Jakusko	1	1	100
		Bauchi	Azare	2	1	50
	North west	Jigawa	Dutse	8	2	25
		Kaduna	Zaria	2	2	100
		Katsina	Kankara	20	20	100
	South south	Edo	Oredo,Benin	200	200	100
Turkey						
	North west	Katsina	Dutsen safe	2	2	100
		Katsina	Katsina	5	5	100
		Jigawa	Dutse	28	17	60.7
		Jigawa	Dutse	19	11	57.9
		Kaduna	Zongo Madubi rd	20	14	70
		Kaduna	Zongo Madubi rd	50	28	56
	North east	Adamawa	Lamorde	5	5	100
	North central	Nasarawa	Akwanga west	14	14	100
	South south	Edo	Oredo,Benin	300	300	100
Guinea fowl						
	North east	Adamawa	Jimeta Yola	125	13	10.4

Table 4.7. Distribution of mixed species backyard poultry flocks naturally infected with HPAI in Nigeria.

Type	Zone	State	Flock size				Number dead				Percent Dead			
Chicken & Duck			Total	Chicken	Duck		Total	Chicken	Duck		Total	Chicken	Duck	
	North central	Bauchi	57	43	14									
		Bauchi	41	34	7		17	12	5		41.46	35.29	71.42	
		Plateau	50	30	20		6	4	2		12	13.33	10	
	North east	Taraba	53	13	40		49	10	39		92.45	77	97.5	
		Taraba	22	11	11		14	7	7		63.6	63.6	63.6	
		Yobe	6	5	1		6	5	1		100	100	100	
Chicken Duck & Turkey			Total	Chicken	Duck	Turkey	Total	Chicken	Duck	Turkey	Total	Chicken	Duck	Turkey
	North central	Nasarawa	91	58	19	14	91	58	19	14	100	100	100	100
		Nasarawa	66	50	12	4	65	49	12	4	98.48	98	100	100
	North	Jigawa	37	1	8	28	20	1	2	17	54	100	25	60.71
Chicken Duck & G. fowl			Total	Chicken	Duck	G. fowl	Total	Chicken	Duck	G. fowl	Total	Chicken	Duck	G. fowl
		Jigawa	259	1	8	250	8	1	2	5	3	100	25	2
		Katsina	44	20	20	4	44	20	20	4	100	100	100	100
		Bauchi	342	300	2	40	273	235	1	37	79.82	78.33	50	92.5

4.2.2 Statistical Analysis

The result of the Chi-square test for the comparison of two proportions (from independent samples) expressed as percentage performed for all other poultry species against the chicken showed that the difference in percent proportion of the number of dead from duck only flocks was 13.78% higher than in chicken only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence interval, meaning that there is a significant difference between the two proportion. When the difference in percent proportion of the number of dead from the mixed species flock group in this case, Chicken and duck only flocks were compared with the single species flock group in this case, chicken only flocks it was found that the percent proportion in Chicken and duck only flocks was 22.89% higher than in chicken only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence interval, meaning that there is a significant difference between the two proportion. While the percent proportion in Chicken, duck and turkey only flocks was 14.34% higher than in chicken only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence interval, meaning that there is a significant difference between the two proportion. Also, the percent proportion in Chicken, duck and guinea fowl only flocks was 25.99% higher than in chicken only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence interval, meaning that there is a significant difference between the two proportion. The duck only flocks were compared with all other poultry species in the backyard poultry flock. This comparison of percent proportion reveals that duck only flocks was 0.78% higher than in turkey only flocks with P-value higher than 0.05 ($P=0.8279$) at 95% confidence interval, meaning that there is no significant difference between the two proportion. While the duck only flocks was 79.77% higher than in guinea fowl only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence interval, meaning that there is a significant difference between the two proportion. When the difference in percent proportion of the number of dead from another mixed species flock group in this case, Chicken, duck and turkey only flocks were compared with the single species flock group in this case, duck only flocks, it was found that the percent proportion in Chicken, duck and turkey only flocks was slightly higher (0.55%) than in duck only flocks with P-value more than 0.05 ($P=0.9634$) at 95% confidence interval, meaning that there is no significant difference between the two proportions. When the comparison of percent proportion of the number dead between chicken, duck and turkey only flocks was compared against chicken, duck and guinea fowl only flocks, it was revealed that the percent proportion of chicken, duck and turkey only flocks was 40.33% higher than in chicken, duck and guinea fowl only flocks with P-value less than 0.05 ($P<0.0001$) at 95% confidence

interval, meaning that there is a significant difference between the two proportions. The odd ratio analysis reveals that the odds of occurrence of death in a duck only flocks is 2.8 higher than in chicken only flocks, while it is 2.6 higher in turkey only flocks than in chicken and 0.03 less in guinea fowl only flocks than in chicken. While the odds of occurrence of death in a chicken and duck only flocks is 0.35 lesser than in chicken only flocks. The odds was 3.02 higher in chicken, duck and turkey only flocks than in chicken only flocks and the odds was 0.31 lesser in chicken, duck and guinea fowl only flocks than in chicken only flocks, representing the highest odd ratio in this study. This shows that there is a higher incidence of mortality due to HPAI in multiple, mixed species backyard poultry than in single species backyard poultry production. When the mortality rates in single and mixed species Backyard poultry flocks 3 days post-onset of disease from Natural HPAI infection in Nigeria were compared on a bar chart, it was found out that duck only flock had the highest mortality rate (90.16%) among single species and chicken, duck and turkey had the highest mortality rate (90.72%) among mixed species flock (Fig 4. 3).

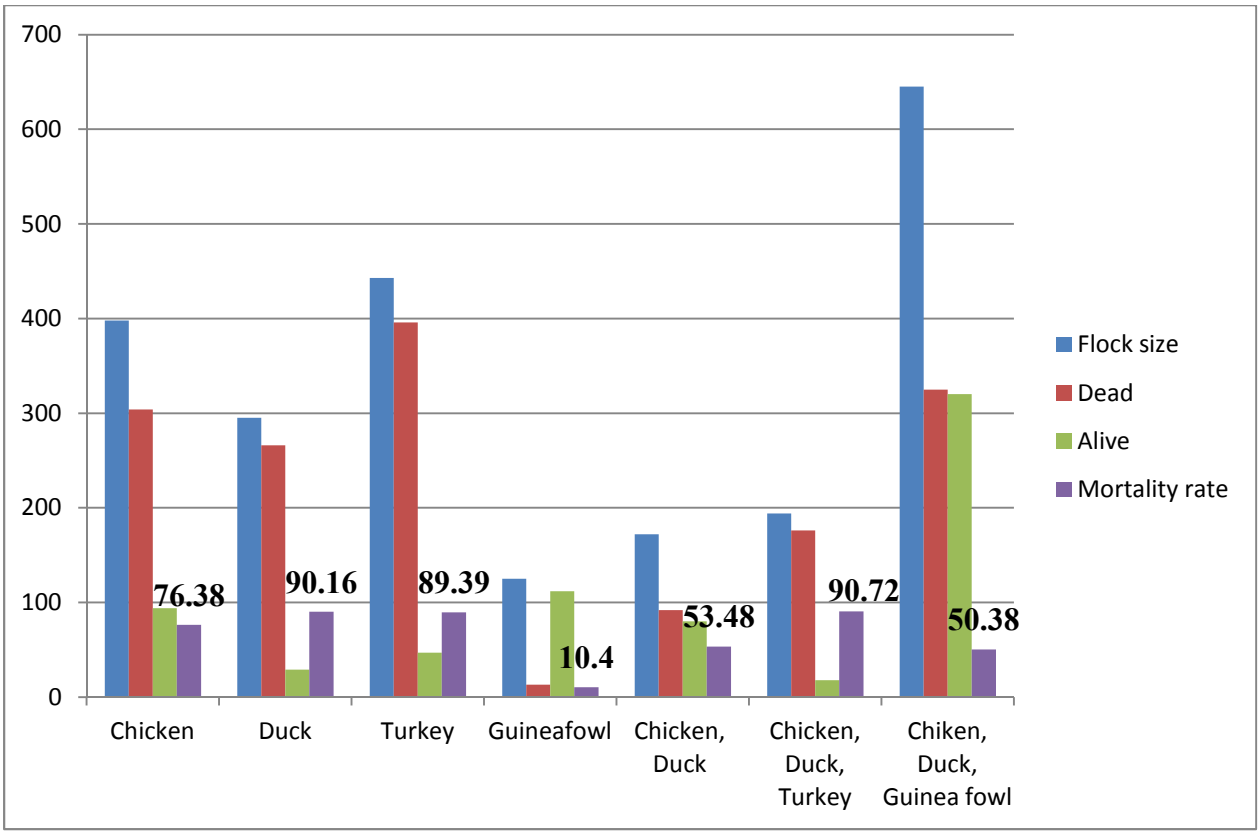

Figure 4.3. Mortality rates in single and mixed species backyard poultry flocks 3 days post-onset of disease from natural HPAI infection in Nigeria.

4.3 STUDY 3: DETERMINATION OF THE USEFULNESS OF ARCHIVAL FORMALIN-FIXED PARAFFIN-EMBEDDED (FFPE) TISSUES OF CHICKENS NATURALLY INFECTED WITH HPAI H5N1 VIRUS FOR RETROSPECTIVE DIAGNOSIS OF AI IN NIGERIA

4.3.1 Immunohistochemistry on FFPE sections

Immunohistochemical demonstration of influenza H5 virus antigen was possible in tissues obtained from all outbreaks. Although immunopositivity varied significantly between the different outbreaks and in different tissues used in this experiment. Some tissues in the majority of the cases were consistently immunohistochemically positive. It was possible to show that comb and wattle which are not included in the six traditional tissue samples (Heart, Trachea, Lung, Liver, Spleen and Intestine) taken for AI diagnosis, were consistently positive in 7 of the 10 cases investigated. Heart tissue was next in positivity with 6 of 10. The table below shows the immunohistochemical detection in FFPE tissue samples from 10 outbreaks (A-J) of HPAI-H5N1 in commercial chickens in Nigeria, in 2006 and 2007 (Table 4.8).

Table 4.8. Results of Immunohistochemistry of HPAI FFPE tissues of selected HPAI H5N1 outbreaks in 2006 and 2007 in Nigeria.

Outbreaks Cases	ID. Of Animal	Immunohistochenical detection in tissues										
		Comb & Wattle	Brain	Heart	Trachea	Lung	Liver	Spleen	Kidney	Proventriculus	Intestine	Pancreas
A/07	1	+++				+		+	++			
B/07	2-3		++			++			+		++	+
C/06	4-6		++	++					++			
D/06	7-10	++										
E/06	11-15	+		++				++				
F/06	16-19	+++		++	++	++					++	++
G/07	20			+						+		
H/06	21-22	+		+						+		
I/07	23-24	+		+		++	++				+	
J/06	25-26	+				+						

Immunohistochemistry: Mildly Positive +; moderately Positive ++; strongly positive +++.

Immunopositivity of vascular endothelium of comb (Plate 4.11) and wattle (Plate 4.12) was strong and cardiomyocytes were also immunoreactive. Neurons of cerebrum (Plate 4.13) cerebellum, pulmonary atria epithelia cells (Plate 4.14), pancreas cortical and medullar cell are focally and strongly intranuclearlly immunoreactive to H5 nuclear protein (Plate 4.15). In addition, the surface epithelium of the ventriculus, vascular endothelium in the lamina propria of proventriculus; and intramural ganglion of intestine (Plate 4.16), occasionally within epithelial cells stained positive for AIV antigen. Immunoreativity was evident in splenic vessels, and myocardial vessels. Renal tubular epithelium and endothelium (Plate 4.17) were also strongly immunopositive.

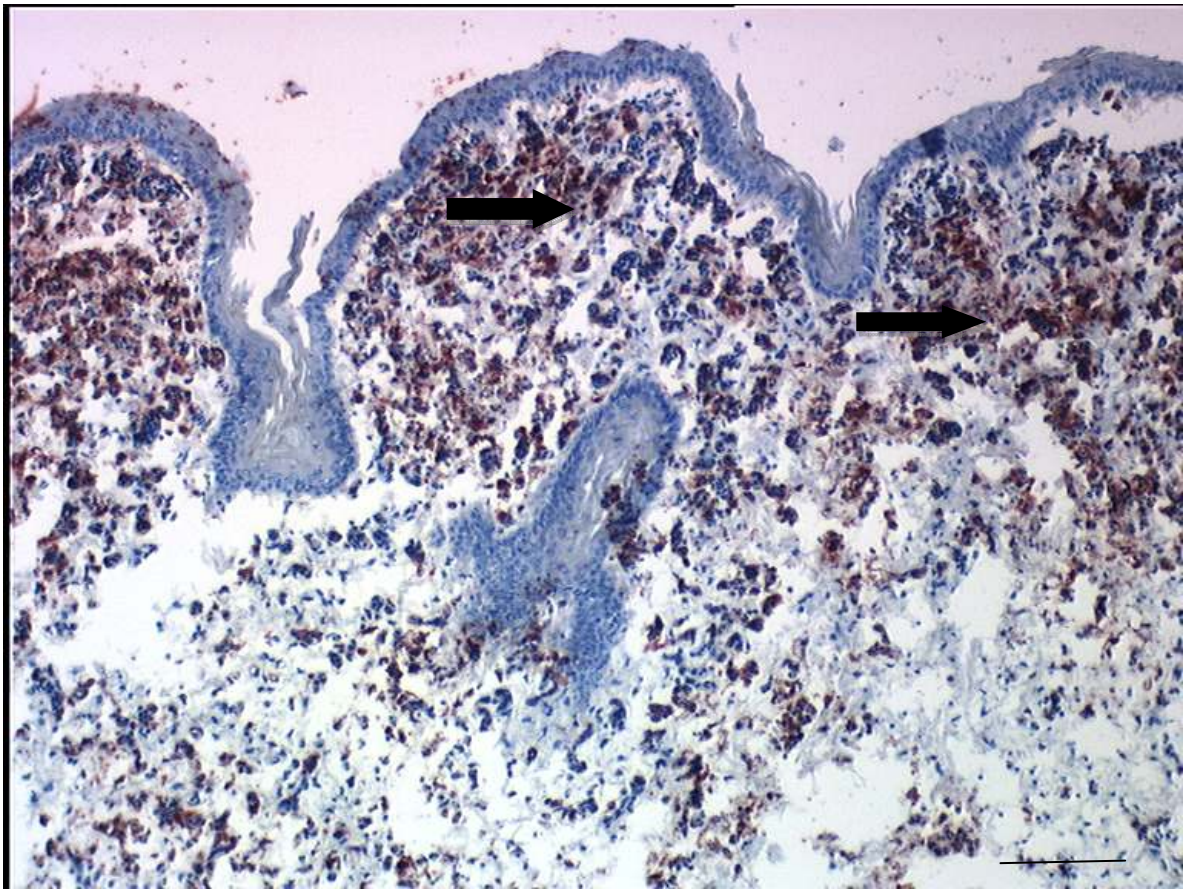

Plate 4.11. Comb; Chicken clade 2.2 HPAI, Natural Infection. Vascular endothelia cells are reddish brown i.e diffusely and strongly immunoreactive (black arrow) to H5 nuclear protein. Bar=100µm.

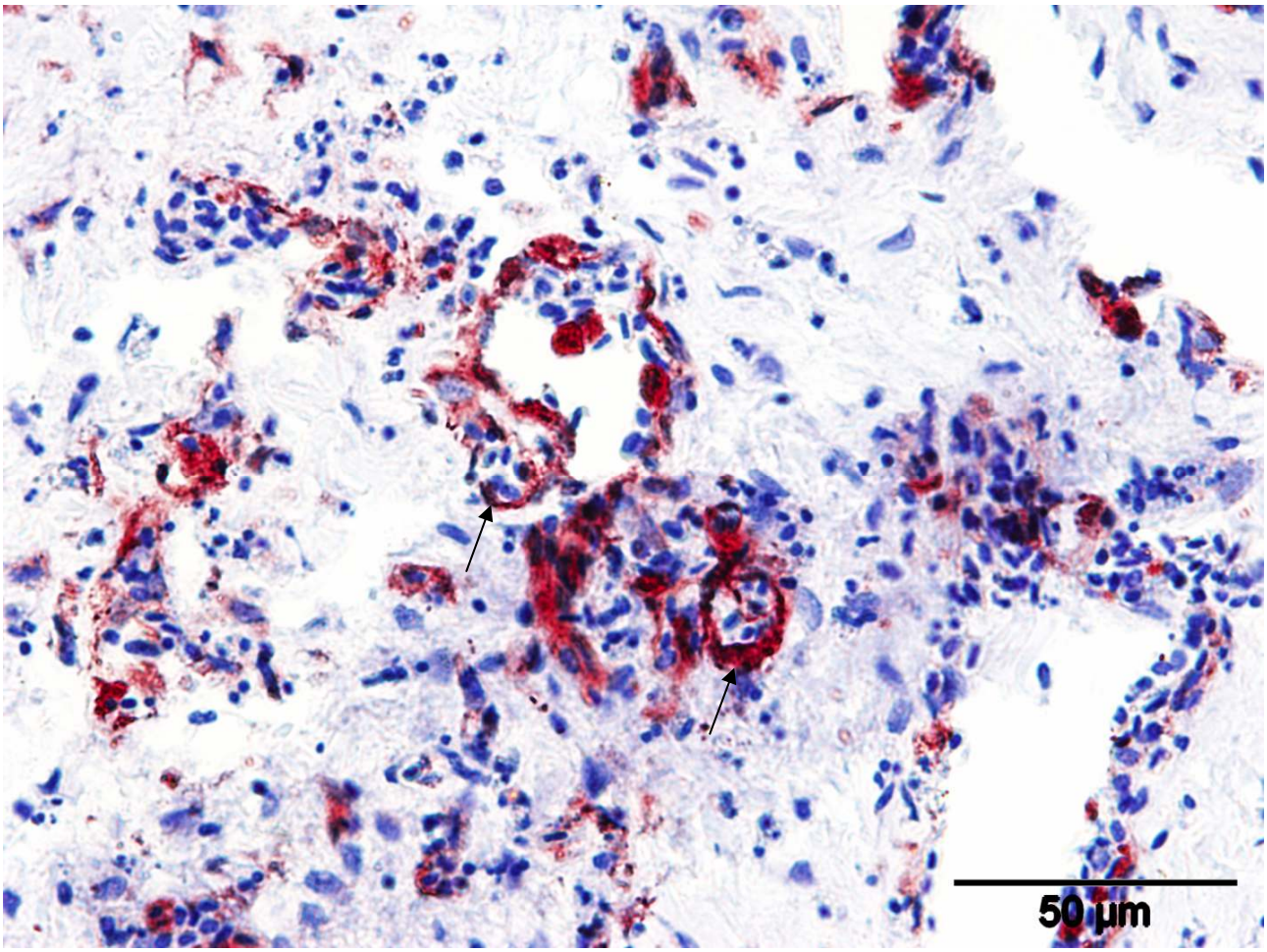

Plate 4.12. Wattle; Chicken (A/07) clade 2.2 HPAI, natural infection. Vascular endothelia cells are reddish brown i.e diffusely and strongly immunoreactive (black arrows) to H5 nuclear protein.

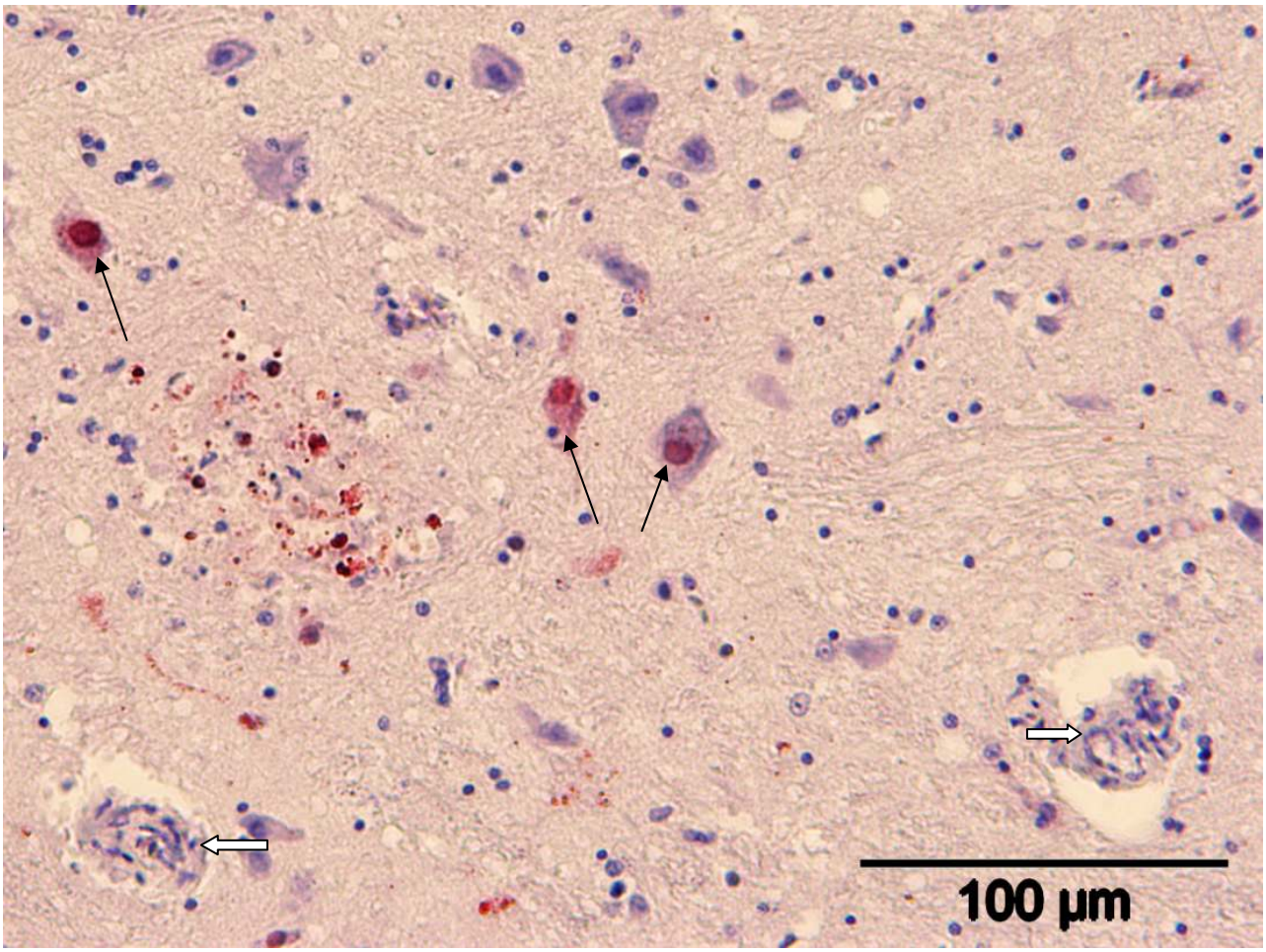

Plate 4.13. Cerebrum; Chicken clade 2.2 HPAI, natural infection. Encephalitis, severe diffuse with multifocal perivascular cuff (white arrow) formation. Neurons are diffusely and strongly immunoreactive (black arrow) to H5 nuclear protein.

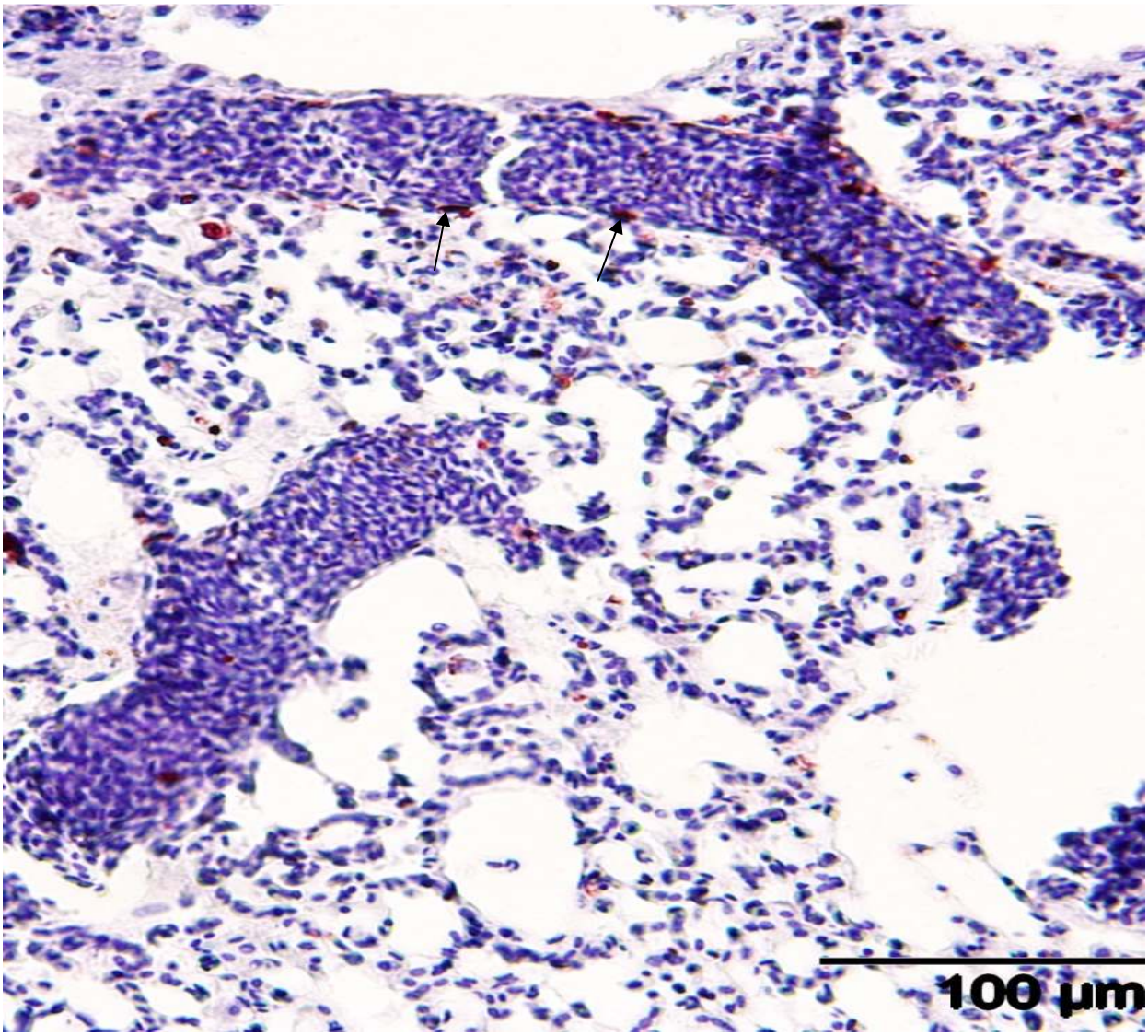

Plate 4.14. Lung; Chicken clade 2.2 HPAI, natural Infection. Vascular congestion. vascular endothelia cells and atria cells are multifocally and strongly immunoreactive (black arrow)to H5 nuclear protein.

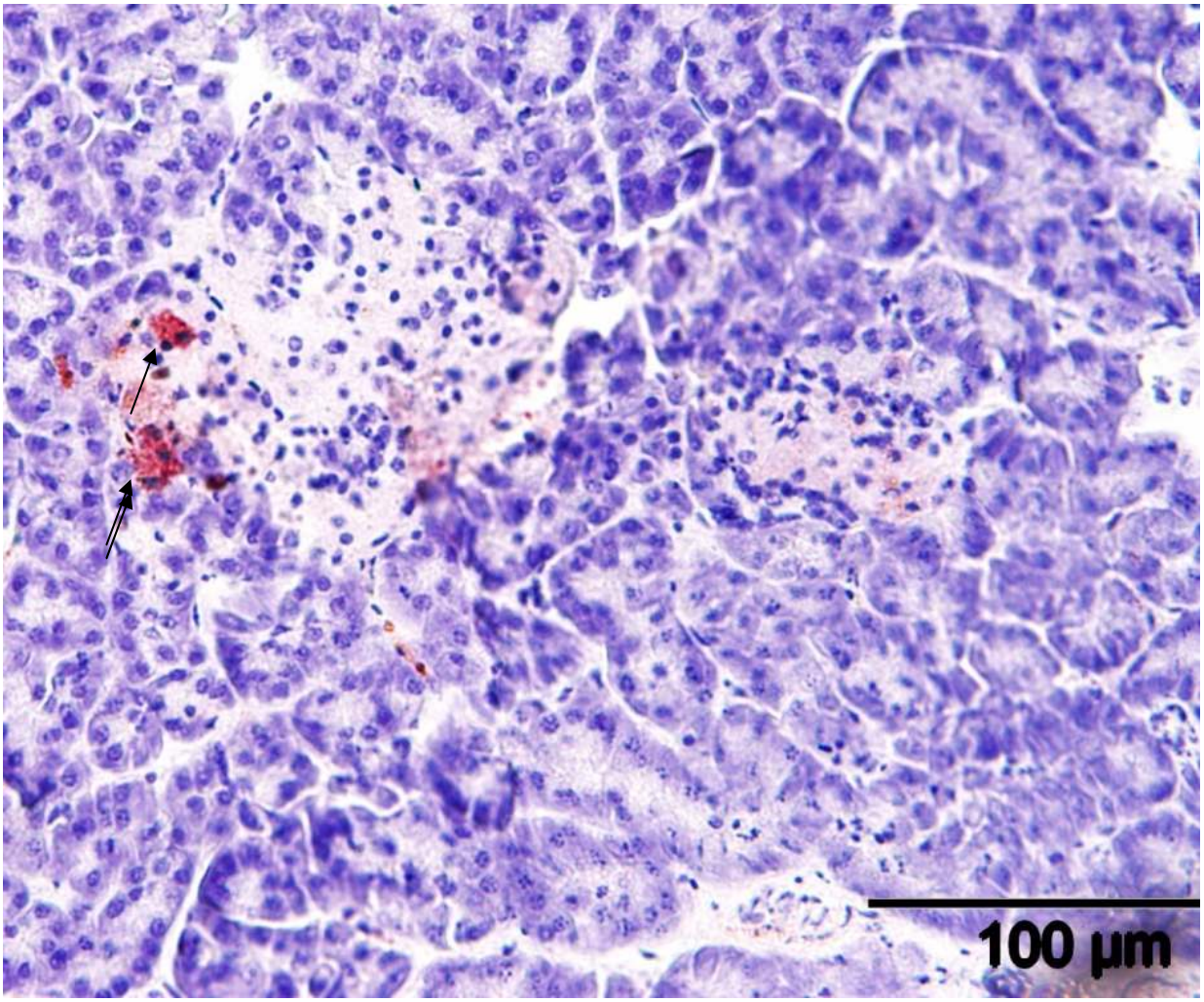

Plate 4.15. Pancreas; Chicken clade 2.2 HPAI, natural infection. Acinar cell necrosis, with cells focally and strongly immunoreactive (arrow) to H5 nuclear protein.

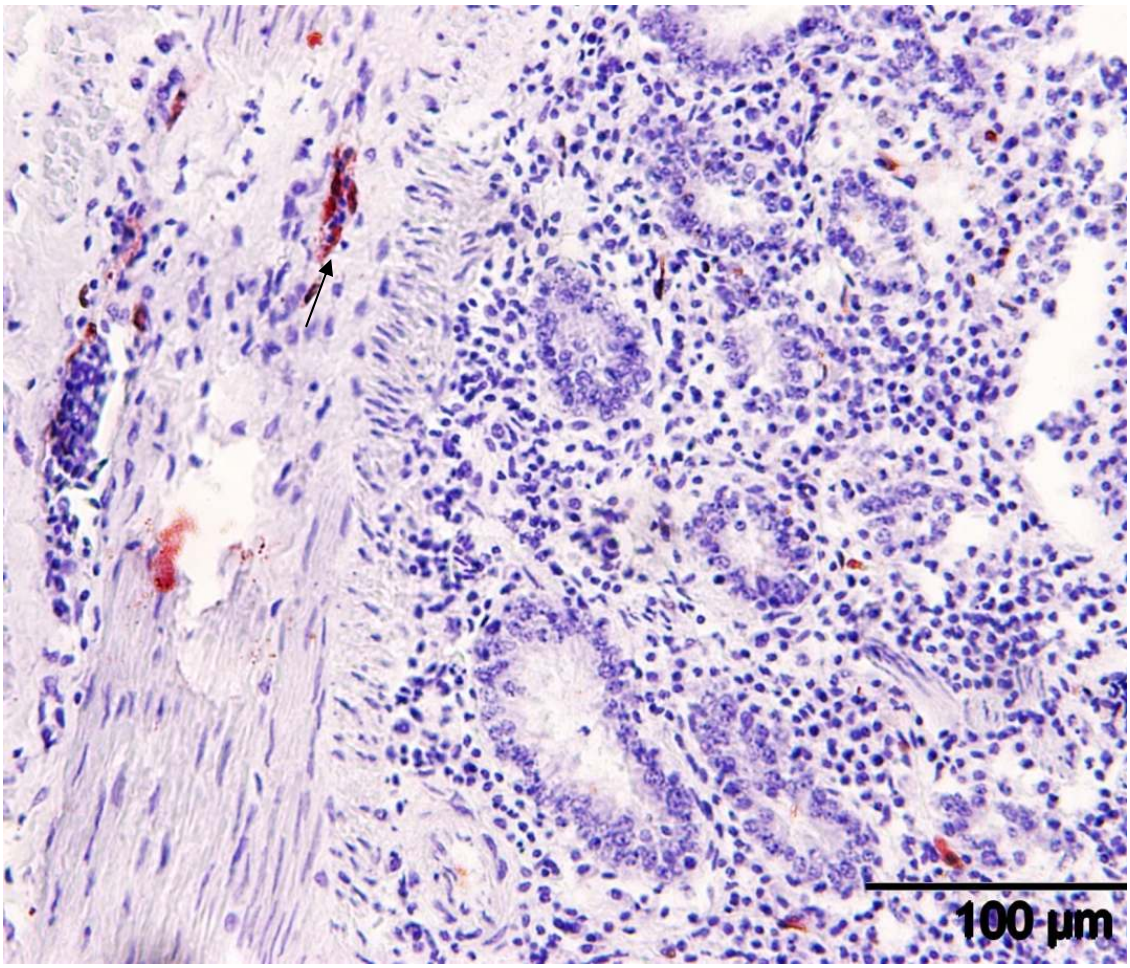

Plate 4.16. Intestine-Ileum; Chicken clade 2.2 HPAI, natural infection. Intramural ganglia and endothelia cells are focally and strongly immunoreactive (arrow) to H5 nuclear protein.

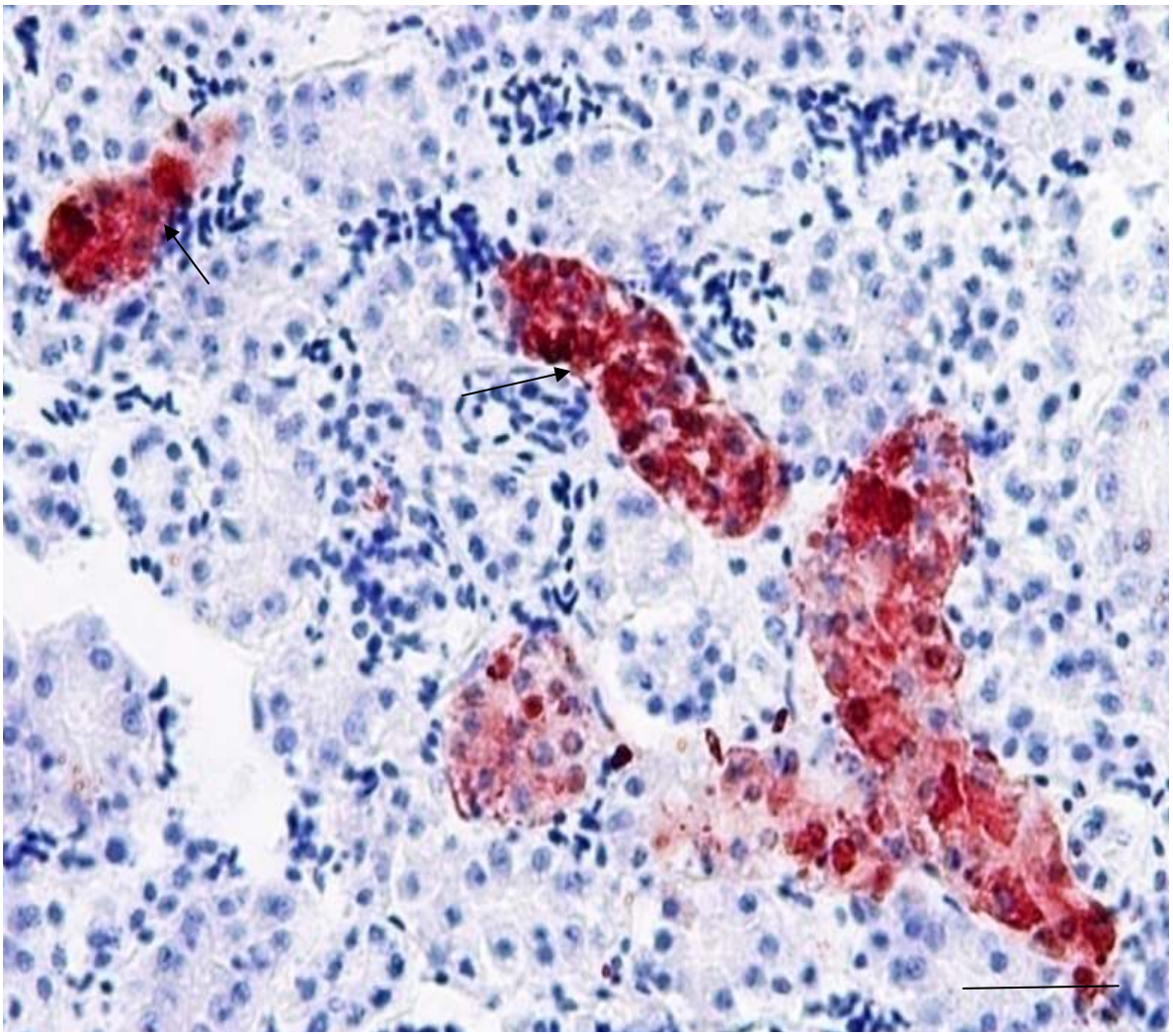

Plate 4.17. Kidney; Chicken clade 2.2 HPAI, natural infection. Glomerular and Tubular epithelia cells are multifocally and strongly immunoreactive (black arrow) to H5 nuclear protein. Bar=100µm.

4.3.2 RT-PCR, sequencing and molecular analysis of FFPE

All experiments to amplify parts of or the whole H5 gene by conventional RT-PCR assays were not successful. The real-time PCR assay with the probe specific for the HA cleavage site of clade 2.2 viruses revealed weakly positive signals for two samples (A/07 and B/07) which were however, too weak for direct sequencing. One of them, originating from outbreak A was sequenced after cloning into the bacterial vector pGEM-T Easy (Promega). The deduced HA cleavage site amino acid sequence is consistent with those of clade 2.2. Viruses: PQGERRRKKR*GLF. In contrast, matrix gene rRT-PCR assays of pooled tissues from each of the selected outbreaks revealed threshold cycle (ct) values between 24.5 and 38.4, whereas three samples remains negative (ct-value 40.3 or no ct). CT values of the H5 HA2 rRT-PCR were lower (28 to 39) but allowed purification and sequencing of the PCR products of four samples (A/07, B/07, D06 and I/06). Sequencing data covering 154 nucleotides (nt) (1531-1684) generated by this rRT-PCR could be obtained. The homology between sequences from outbreaks A/07, D/06 and I/06 were 100%, whereas the fourth sample from outbreak B/07 differed at 4 nucleotides (97, 4% homology*)*. Three of the differences were silent mutations whereas the fourth mutation led to an amino acid exchange (544 Ala → Thr). AIV sequences with highest homology to the two different Nigerian viruses determined by BLAST search are shown in Table 4.9. The three identical viruses- A/07, D/06, I/06 showed highest homology to European viruses in 2005/06. In contrast, the fourth virus- B/07 clustered mainly with sequences from Vietnam 2005.

Table 4.9. Sequences with highest homology to Nigerian H5N1 viruses (154nt of the HA2-part) identified by BLAST search.

Animal ID	**Accession numbers**	**HPAI-H5N1 isolates**	**Percentage homology (%)**
*B/07	EF535027	A/chicken/Vietnam/14/2005	96.1
	EU015404	A/Indonesia/TLL002/2006	96.1
	EU118135	A/chicken/Vietnam/TY25/2005	96.1
*A/07,D/06, I/07	FJ785122	A/chicken/Calarasi/RO-AI-091/2005	97.4
	EU889073	A/tufted/duck/Sweden/V998/2006	97.4
	DQ840533	A/swan/Astrakhan/Russia/Nov-2/2005	97.4

*see appendix for full sequence of Nigerian isolates

4.4 STUDY 4: SUSCEPTIBILITY, TRANSMISSION, PATHOGENESIS AND PATHOLOGY OF INFECTION OF CLADE 1 H5N1 VIRUS- INFLUENZA A/DUCK/VIETNAM/TG24-01/05 AND CLADE 2.2 H5N1 VIRUS- INFLUENZA A/CYGNUS CYGNUS/GERMANY/R65/2006 IN MUSCOVY, PEKIN AND MALLARD DUCKS AND THEIR IN-CONTACT CHICKENS AND DUCKS.

4.4.1 *Influenza A/duck/Vietnam/TG24-01/05* of clade 1 subtype H5N1 virus infection in ducks

4.4.1.1Clinical signs

Severe neurological signs (Plate 4.18), which included unsteady gait, ataxia, tremor and opisthotonus (Plate 18) were exhibited amongst eight (80%) of the 3- weeks old Muscovy ducks infected with HPAI A/duck/Vietnam/TG24-01/05 of clade 1 subtype H5N1 virus while the remaining 20% died suddenly at 3 days post infection (dpi). Though Mallard and Pekin ducks showed no observable signs at 3 dpi, they were however weak at 4 dpi and one mallard duck showed corneal opacity and another one exhibited lateral deviation of the head and neck (torticollis) at 7 dpi. Within 5 and 8 dpi, 30% of Pekins and 25% of Mallard died naturally while the remaining of both species were sacrificed either as planned or for animal welfare reasons after exhibiting unsteady gait, ataxia, tremor and opisthotonus.

4.4.1.2 Gross Pathology

Grossly, only one Muscovy had multifocal pancreatic necrosis with duodenal vascular congestion. All other Muscovy that died or were sacrificed at 3dpi showed no visible lesion. On 4dpi Pekin ducks, there was severe diffuse myocardial and pancreatic necrosis and pancreatitis. As the days post-infection increases, more gross lesions were recorded in the Pekin and Mallard ducks examined 6 dpi and these includes myocardial necrosis and myocarditis, hydrothorax, pulmonary congestion and edema, hepatic and pancreatic necrosis and pancreatitis. In both Mallard which were examined 8 dpi, gross lesion was not evident. Grossly, in mallard ducks that were sacrificed 4dpi lesions were sparse in these carcasses except for one that showed severe diffuse pancreatic necrosis.

4.4.1.3 Histopathology and Immunohistochemistry

Histopathology lesion included multifocal perivascular lymphocyte cuff in the cerebrum. Occasionally within the eye, corneal and iridial multifocal ballooning degeneration, pigmented epithelia necrosis, and sclera edema and moderate heterophil infiltration while the harderian gland often had focal extending epithelia necrosis. There is also lymphoplasmacytic optic neuritis. The airsacs were edematous with mild heterophil infiltration and epithelia hypereosinophilia material (fibrin), and sometimes with subepithelia lymphocyte and plasma cell infiltrates. The Bursa of Fabricius showed multifocal follicular depletion with glandular cystic formation while the spleen and thymus had multifocal periateriolar necrosis with hemosiderin laden macrophages. In the ducks (Muscovy, Mallard and Pekin) there were lesions of cerebral and cerebellar neuronal degeneration and necrosis with multifocal perivascular lymphocytic cuffs, (Plate 4.19) and severe lymphoplasmacytic optic neuritis (Plates 4.20 and 4.21). There was respiratory epithelial necrosis with intraluminal cellular debri in the nasal septal and severe vascular congestion (Plate 4.22) and lymphoplasmacytic airsacculitis (Plate 4.23). There was severe myocarditis and pulmonary edema (Plate 4.24) and severe diffuse myocardial necrosis (Plate 4.25). The following cell types were mildly to strongly immunoreative to H5 intranuclear proteins using the ABC immunohistochemical methods. The feather follicle and keratinocytes within the dermis of the Comb, cerebral and cerebellar neurons and glia cells, in particular the meninges, and purkinje cells of the cerebellum, ependymal cells of the choroids plexus, ependyma cells, neurons and glia cells of the spinal cord, also immunoreativity was detected in the central canal. The smooth muscle cells, spindle cells in uvea and outer conjunctival epithelia, fibrocytes and nerve ganglia epineurium, corneal epithelium, sclera and pigmented cells in uvea, including the harderian gland and pectin glandular epithelium were all immunoreactive. Upper and lower cells of filtration angle, anterior chamber are also immunoreative. The glandular epithelium and smooth muscle cells of the eyelid. Mononuclear cells in thymus, Bursa and spleen, the cardiomyocytes were also immunoreactive. The nasal cavity and palatine epithelium, trachea columnar epithelium, parabronchial epithelium of the lung, the esophageal endothelia cells, myocytes in gizzard, jejunum, rectal endothelia cells and hepatocytes and bile duct epithelium, including renal tubular epithelia cells and skeletal muscle myocytes were all immunoreactive. Pekin ducks examined at 4 dpi, as compared to the only Muscovy duck showed histological lesion of multifocal lymphocytic myocarditis (Plate 4.26). There was multifocal

pancreatic necrosis with duodenal vascular congestion (Plate 4.27), only evident in Muscovy duck no. 4. Bursa of Fabricius (Plates 4.28 and 4.29) show lymphoid depletion and immunohistochemical staining. All tissues of Muscovy ducks examined at 3 dpi were immunopositive by immunohistochemistry, but no positivity was seen in the entire GIT tissues of Pekin. There were consistent histopathologic lesions in these Muscovy ducks at 3 dpi. The spleen and thymus had multifocal periateriolar necrosis with hemosiderin laden macrophages. Furthermore, histopathology lesions observed in the Pekin ducks included comb keratinocyte necrosis, upper respiratory focal epithelia necrosis and desquamation, oedematous and fibrinous lymphoplasmacytic airsacculitis, pulmonary congestion and edema, splenic hemosiderosis, lymphoid depletion in thymus, spleen and Bursa of Fabricius, adrenal and duodenal intramural lympho-plasma-histiocytic ganglioneuritis, lymphocytic myocytis, severe, diffuse pancreatitis and acinar necrosis. Histopathological lesions included severe diffuse meningo-encephalitis with multifocal lymphocytic perivascular cuffs. Bursa of Fabricius in Pekin duck shows follicular necrosis and infiltration by mononuclear inflammatory cells and the heart had diffuse necrotic myocarditis. Mallard duck show neuronal necrosis with diffuse infiltration of mononuclear cells, mainly lymphocytes and plasma cells; and vascular congestion with optic neuritis. The hallmark histopathological lesions in the Pekin and Mallard at 4 dpi included meningoencephalitis with lymphocytic perivascular cuffs. Additionally, there was adrenal necrosis with lympho-plasma-histiocytic ganglioneuritis and the duodenal intramural lympho-plasma-histiocytic ganglioneuritis which was evident in the 4th and 5 dpi Pekin ducks. Although hallmark of histopathological lesions were fewer, the disease was accompanied by lymphoplasmacytic meningoencephalitis and polioencephalitis with lymphocytic perivascular cuffs, which was also seen in the eyelid and multifocal lymphocytic myocarditis. In the Pekin ducks, the following cell types were mildly to strongly immunoreative to H5 intranuclear proteins using the ABC immunohistochemical methods. The feather follicle and keratinocytes within the dermis of the Comb, cerebral and cerebellar neurons and glia cells, in particular the meninges, and purkinje cells of the cerebellum, ependymal cells of the choroids plexus, ependyma cells, neurons and glia cells of the spinal cord, also immunoreativity was detected in the central canal linning cells and, also the corneal epithelium. The glandular epithelium and smooth muscle cells of the eyelid. Mononuclear cells in thymus, spleen and adrenal, also the cardiomyocytes, The nasal cavity medial ophthalmic branch of trigeminal nerve and palatine epithelium, trachea columnar epithelium, parabronchial epithelium of the lung, including renal tubular and testicular seminiferous interstium and skeletal

muscle myocytes. In the Mallard, the feather follicle and keratinocytes within the dermis of the comb, cerebral and cerebellar neurons and glia cells, in particular the meninges, and purkinje cells of the cerebellum, ependymal cells of the choroids plexus, ependyma cells, neurons and glia cells of the spinal cord, also immunoreativity was detected in the central canal linning cells and, also the corneal epithelium, and the glandular epithelium and smooth muscle cells of the eyelid. Mononuclear cells in thymus, spleen and adrenal, also the cardiomyocytes, The nasal cavity medial ophthalmic branch of trigeminal nerve and palatine epithelium, trachea columnar epithelium, parabronchial epithelium of the lung, ncluding renal tubular and testicular seminiferous interstium and skeletal muscle myocytes.

Generally Immunoreactivity was successful in all ducks but with variation in the GIT tissues. It was seen in the endothelia cells of some GIT tissues and in Bursa of Fabricius in Muscovy ducks examined at 3 dpi, but none was seen in the entire GIT tissues of Pekin. The Pekin only showed immunoreactivity in the nasal cavity medial ophthalmic branch of trigeminal nerve (Plate 4.30) and palatine epithelium. Although the serosal lining cells of the GIT tissues were IHC positive in Mallard at 5 dpi, they remain negative in the Pekin still, and in both at 6 dpi. Immunoreativity was restricted to the cerebral neurons and glia cells and around the vessels only in one Mallard and not detected in the second Mallard 8 dpi.

Plate 4.18. Muscovy duck 3days p.i, clade 1 HPAI. Torticollis and partial paralysis of right limb.

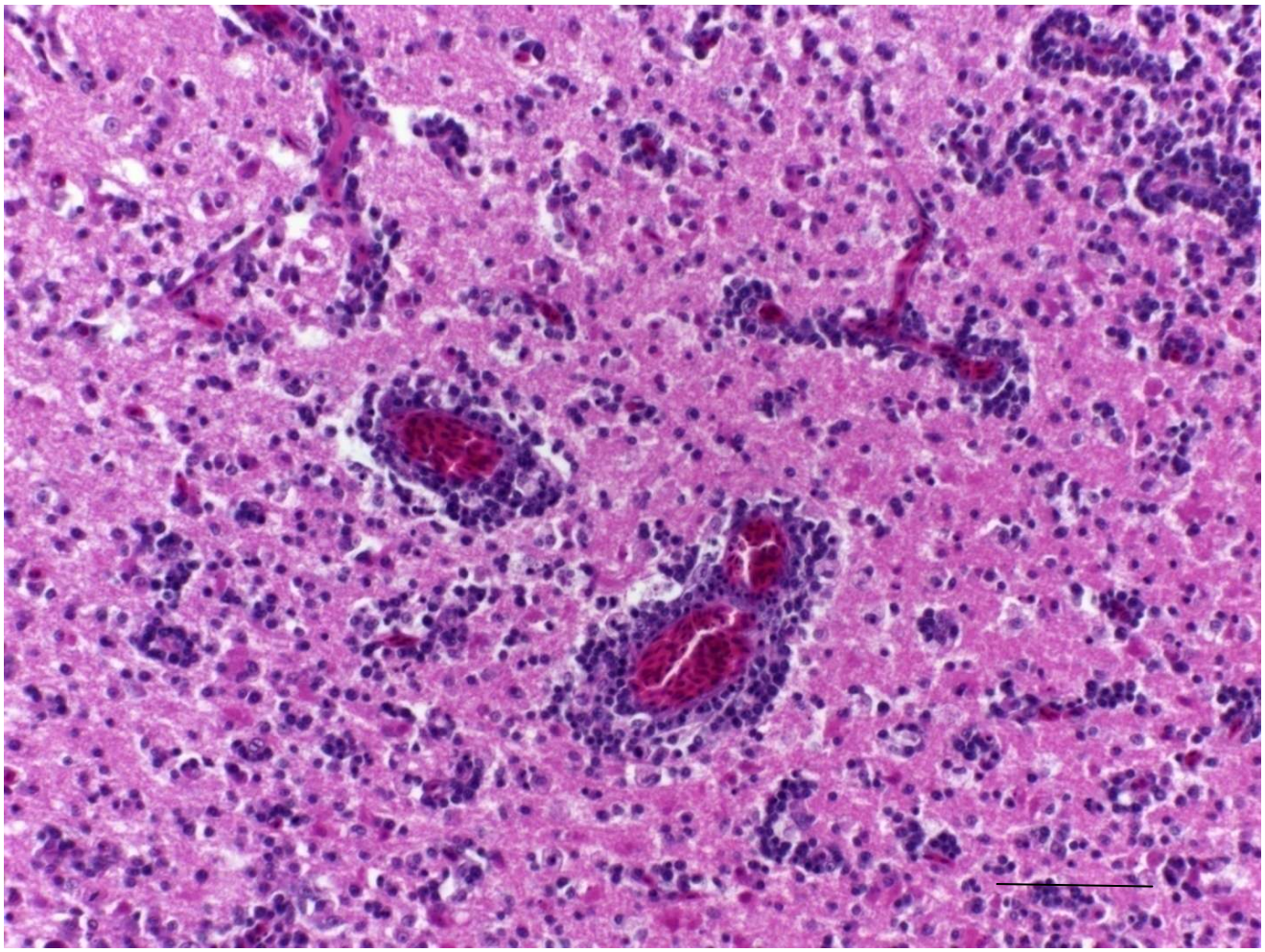

Plate 4.19. Cerebrum; Mallard duck 4 days p.i, clade 1 HPAI. Multifocal Perivascular lymphocytic cuffing, neuronal necrosis, with multifocal infiltration of mononuclear cells, mainly lymphocytes; vascular congestion. HE. Bar=100µm

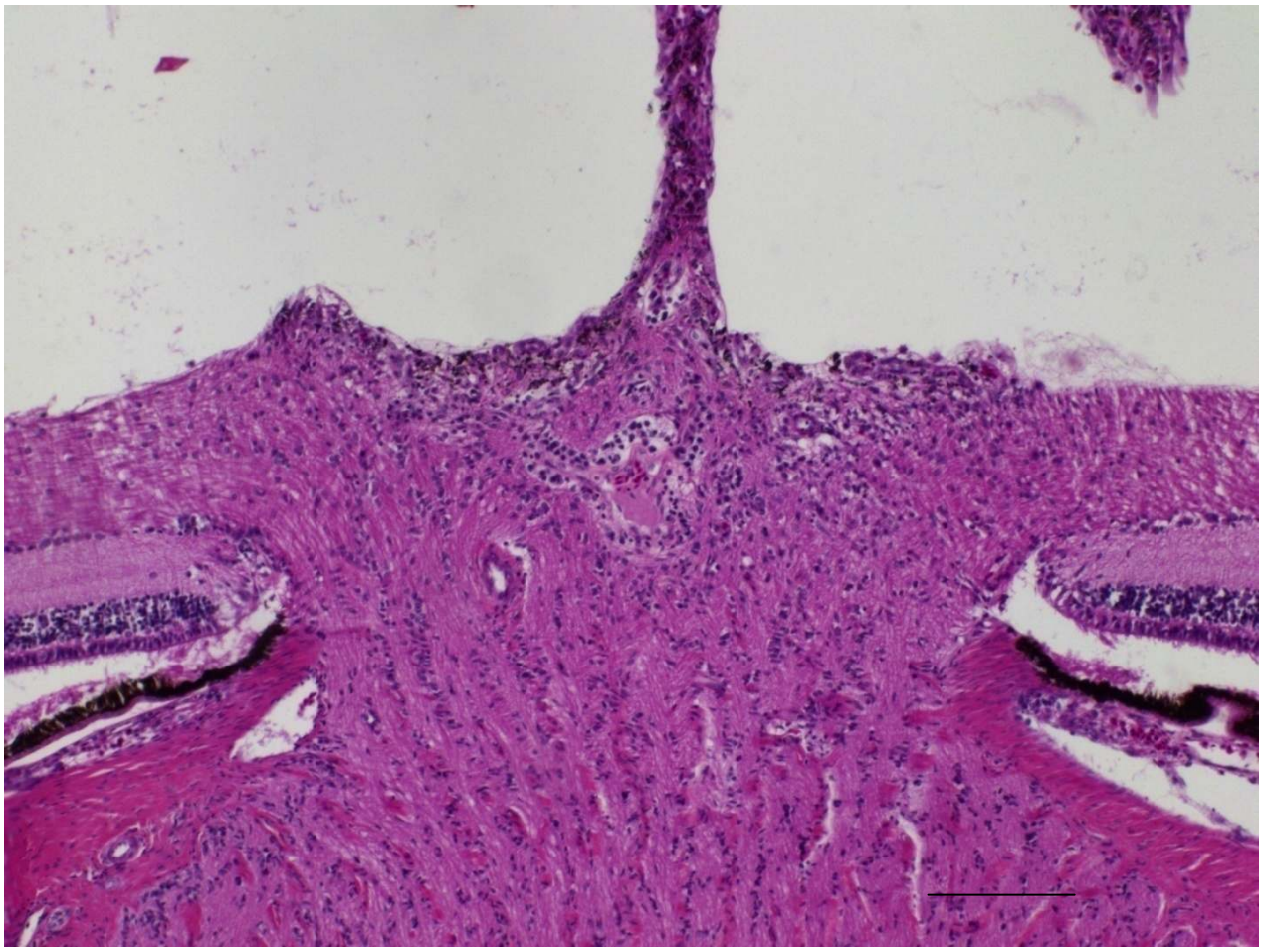

Plate 4.20. Optic nerve - Eye; Muscovy duck 3 days p.i, clade 1 HPAI Optic neuritis with diffuse infiltration of mononuclear cells, mainly lymphocytes and plasma cells; and vascular congestion. HE. Bar=100µm

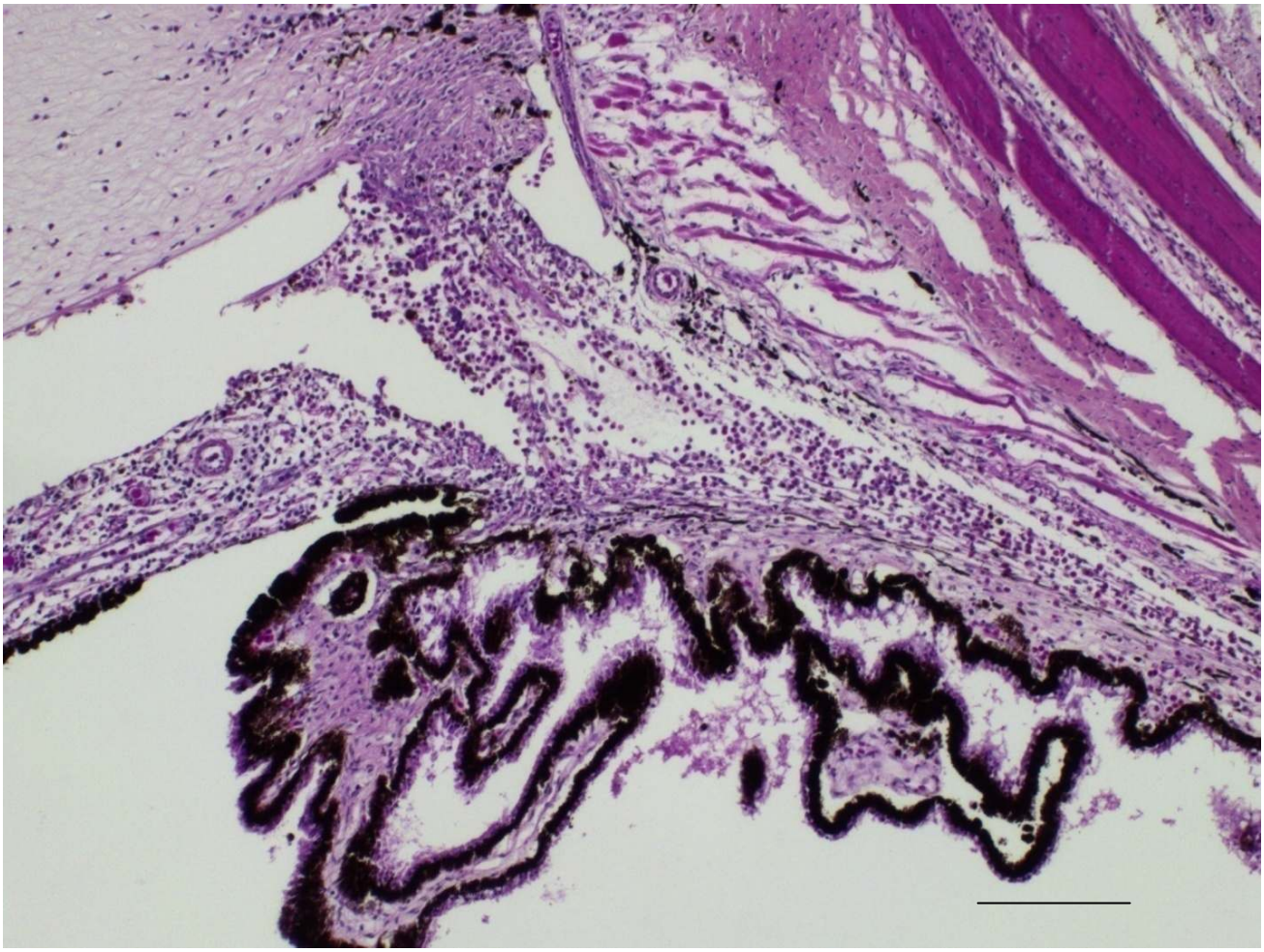

Plate 4.21. Optic nerve and pectin - Eye; Mallard duck 4 days p.i, clade 1 HPAI Neuronal necrosis with diffuse infiltration of mononuclear cells, mainly lymphocytes and plasma cells; and vascular congestion. HE. Bar=100µm

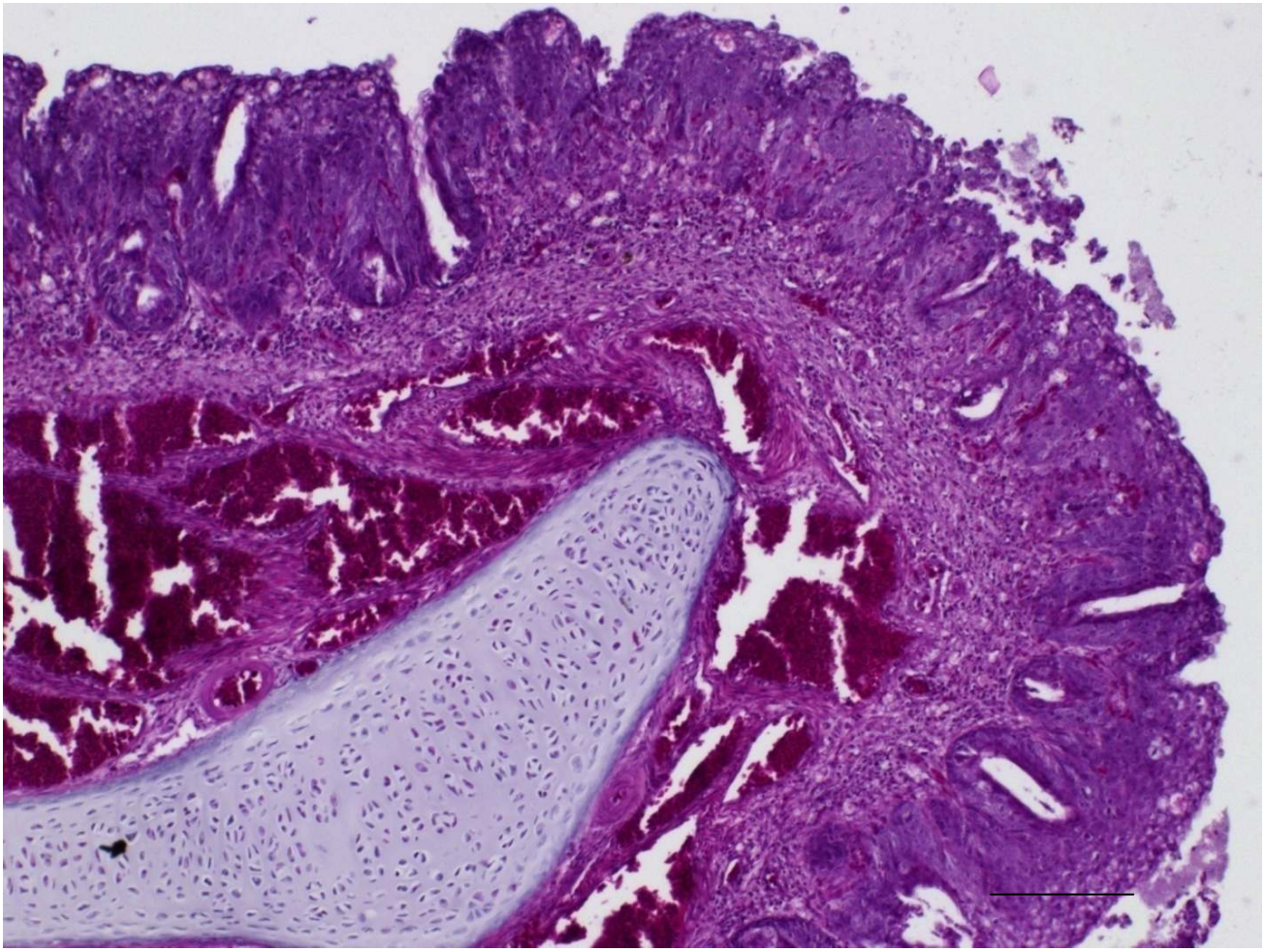

Plate 4.22. Muscovy duck 3 days p.i, clade 1 HPAI. Respiratory epithelium. Necrotizing and hemorrhagic rhinitis. Mucous epithelium is hypertrophied, matted and necrotic, infiltrated by mononuclear cells with multifocal desquamation. Vascular congestion and hemorrhage HE. Bar=100µm

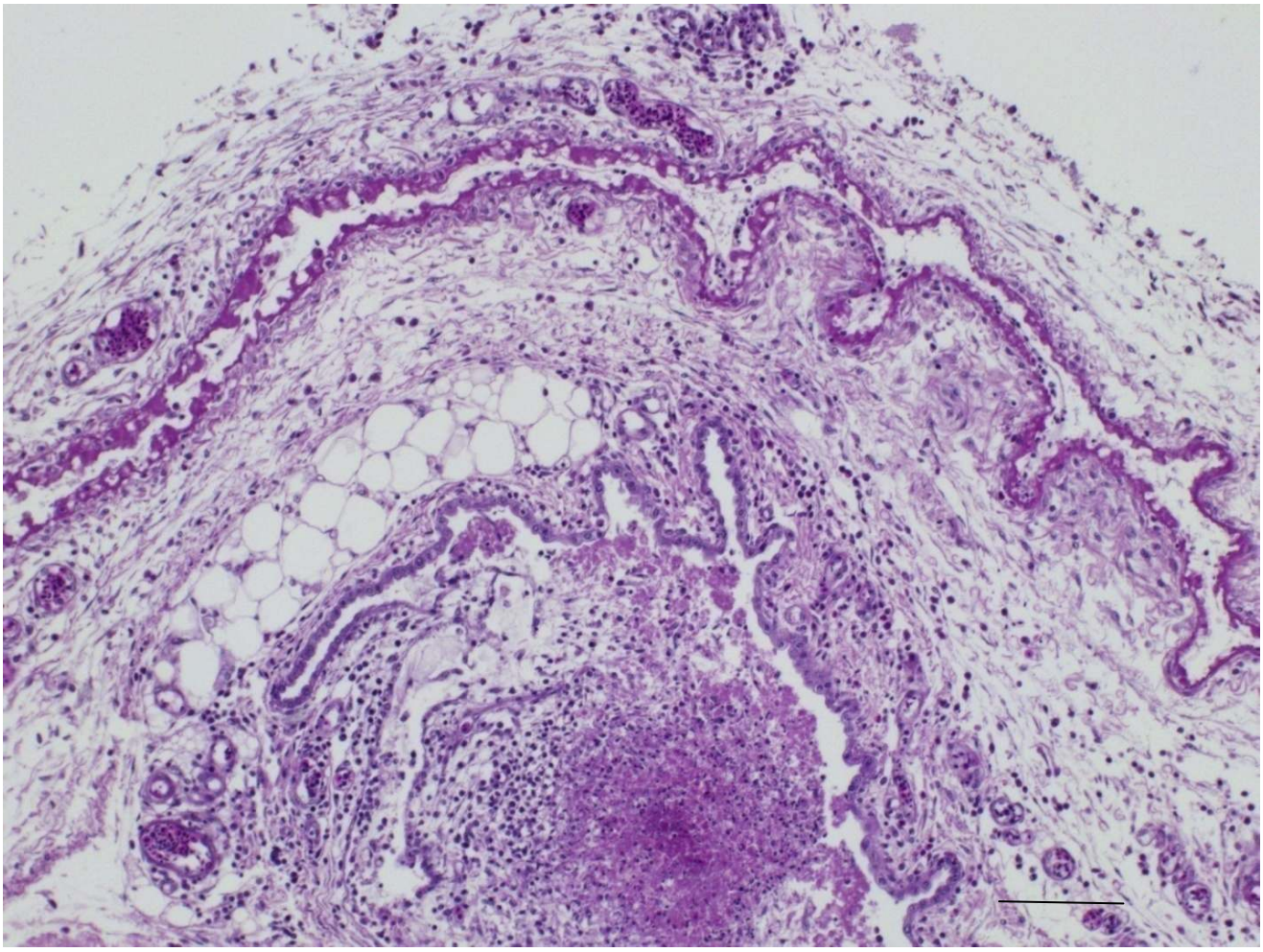

Plate 4.23. Airsac-Pulmonary; Pekin duck 4 days p.i, clade 1 HPAI. Airsacculitis : the mesothelium is diffusely infiltrated by mononuclear cells, mainly lymphocytes HE. Bar=100µm

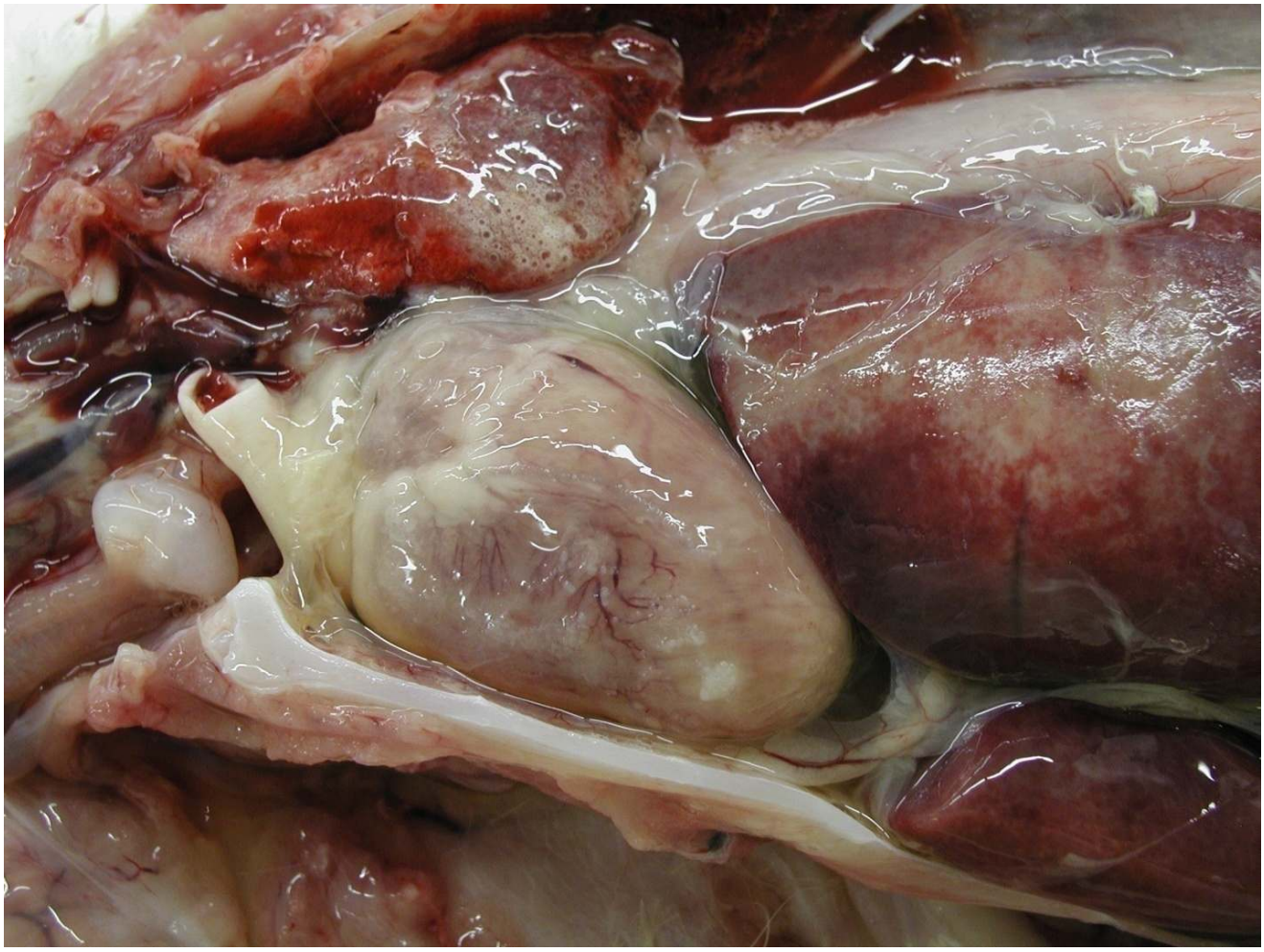

Plate 4.24. Heart: Pekin duck 6 days p.i, clade 1 HPAI. Fibrinous pericarditis and myocardial necrosis, diffuse. Lung: pulmonary congestion and edema.

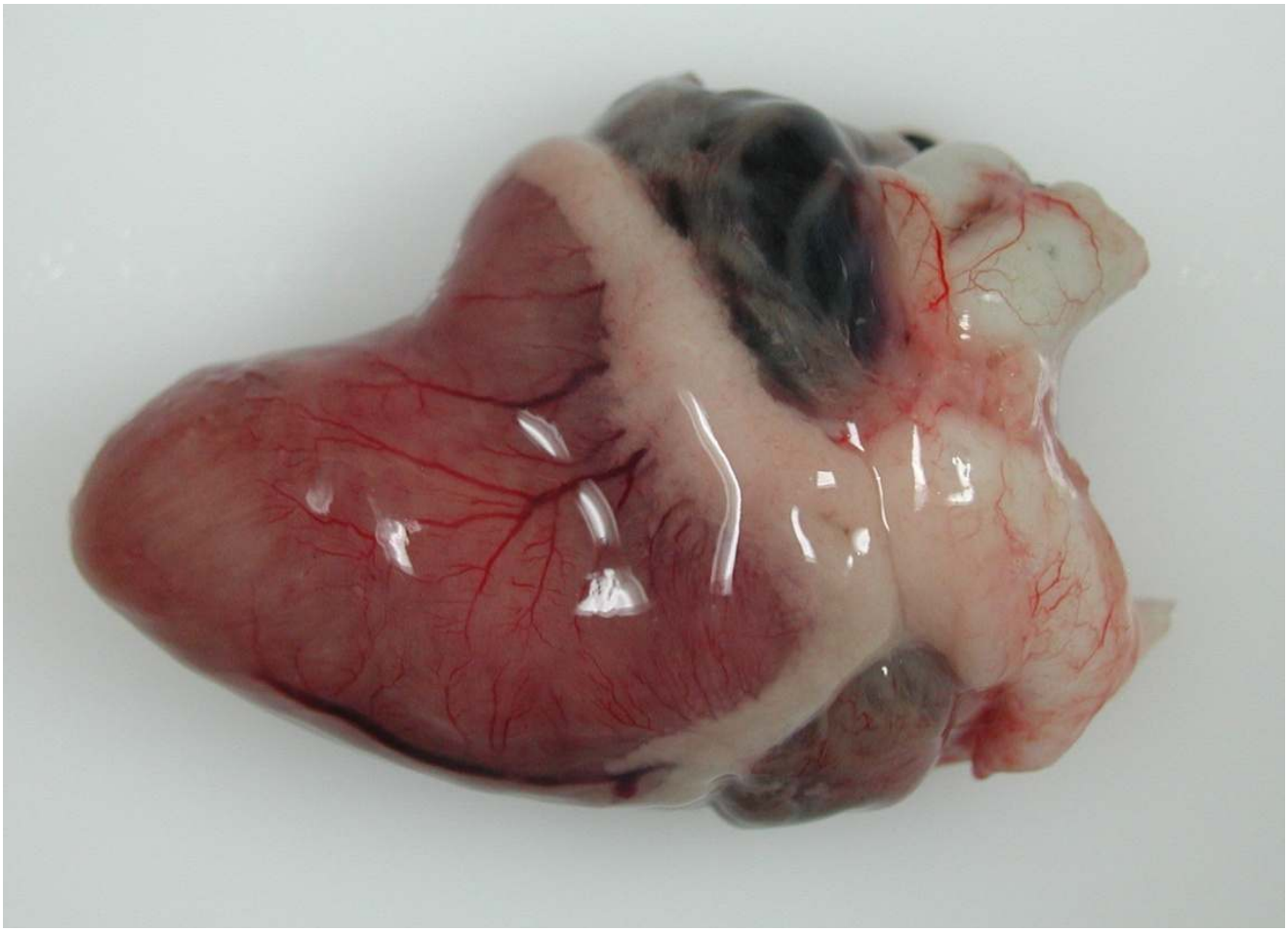

Plate 4.25. Heart: Pekin duck 5 days p.i, clade 1 HPAI. Myocarditis, diffuse and coronary vascular congestion.

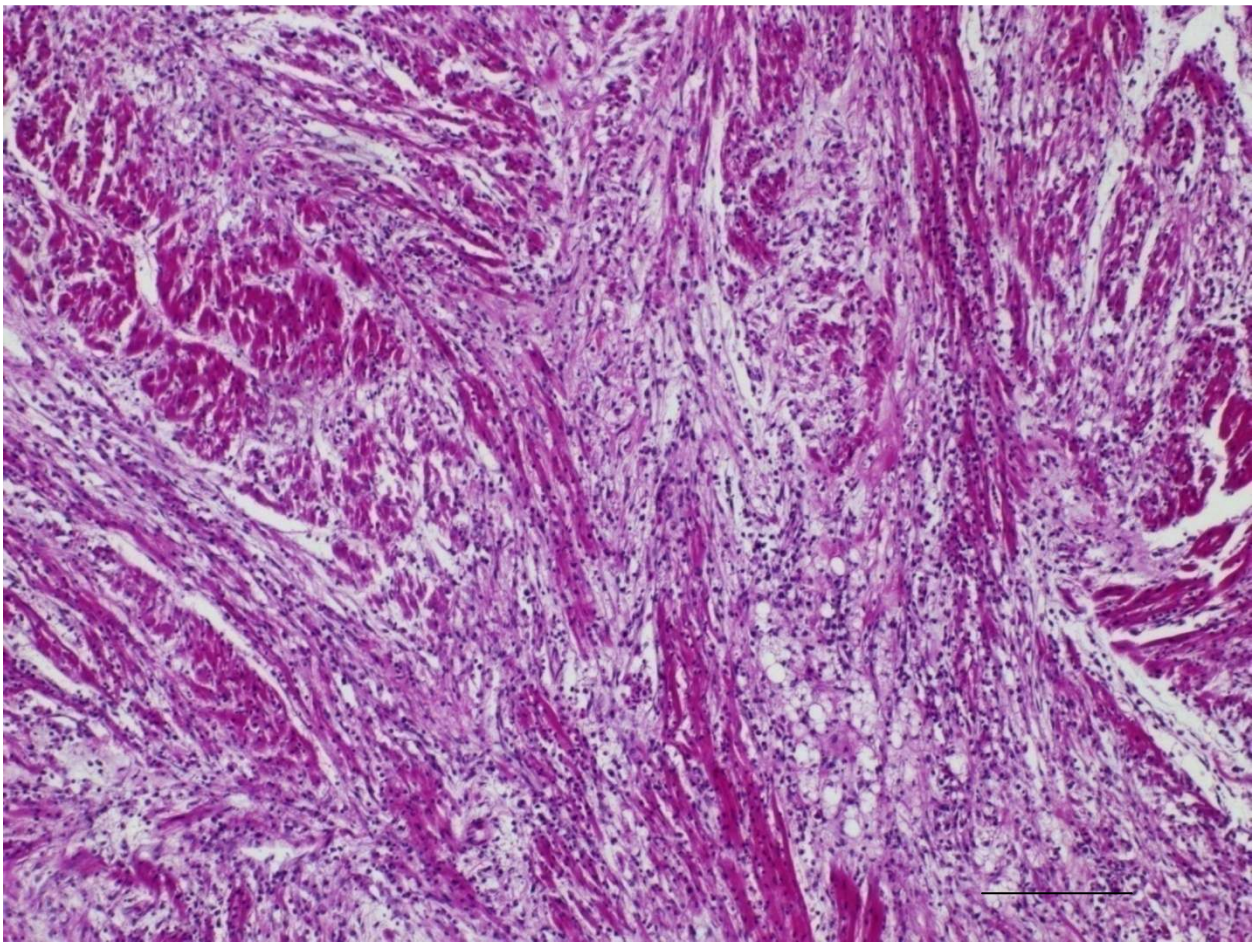

Plate 4.26. Heart; Pekin duck 6 days p.i, clade 1 HPAI. Myocarditis, severe diffuse: Cardiomyocyte necrosis and mononuclear cell infiltration, mainly lymphocyte and plasma cells . HE. Bar=100µm.

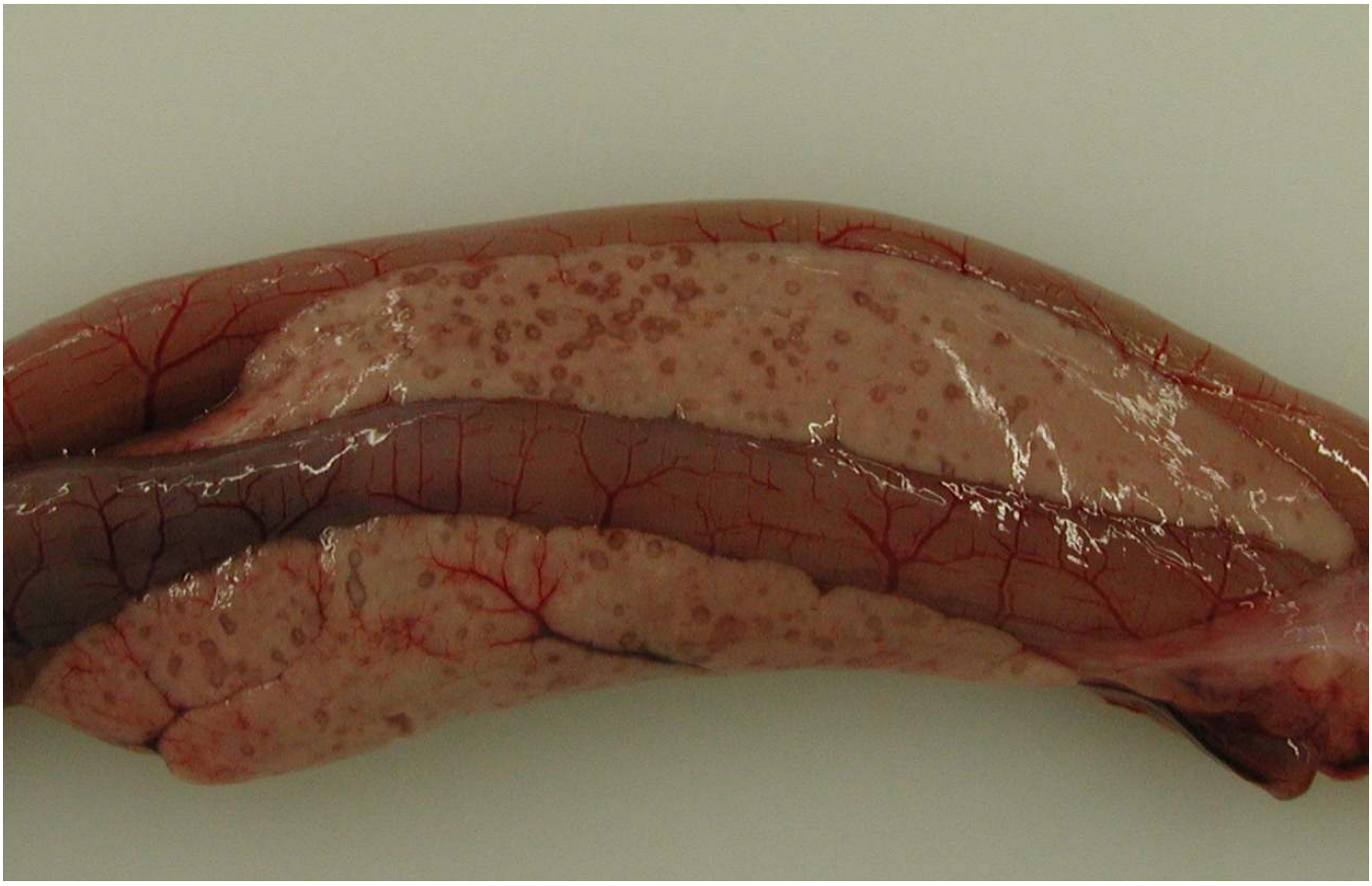

Plate 4.27. Pancreas: Muscovy duck 3 days p.i, clade 1 HPAI. Necrotic pancreatitis, multifocal with vascular congestion.

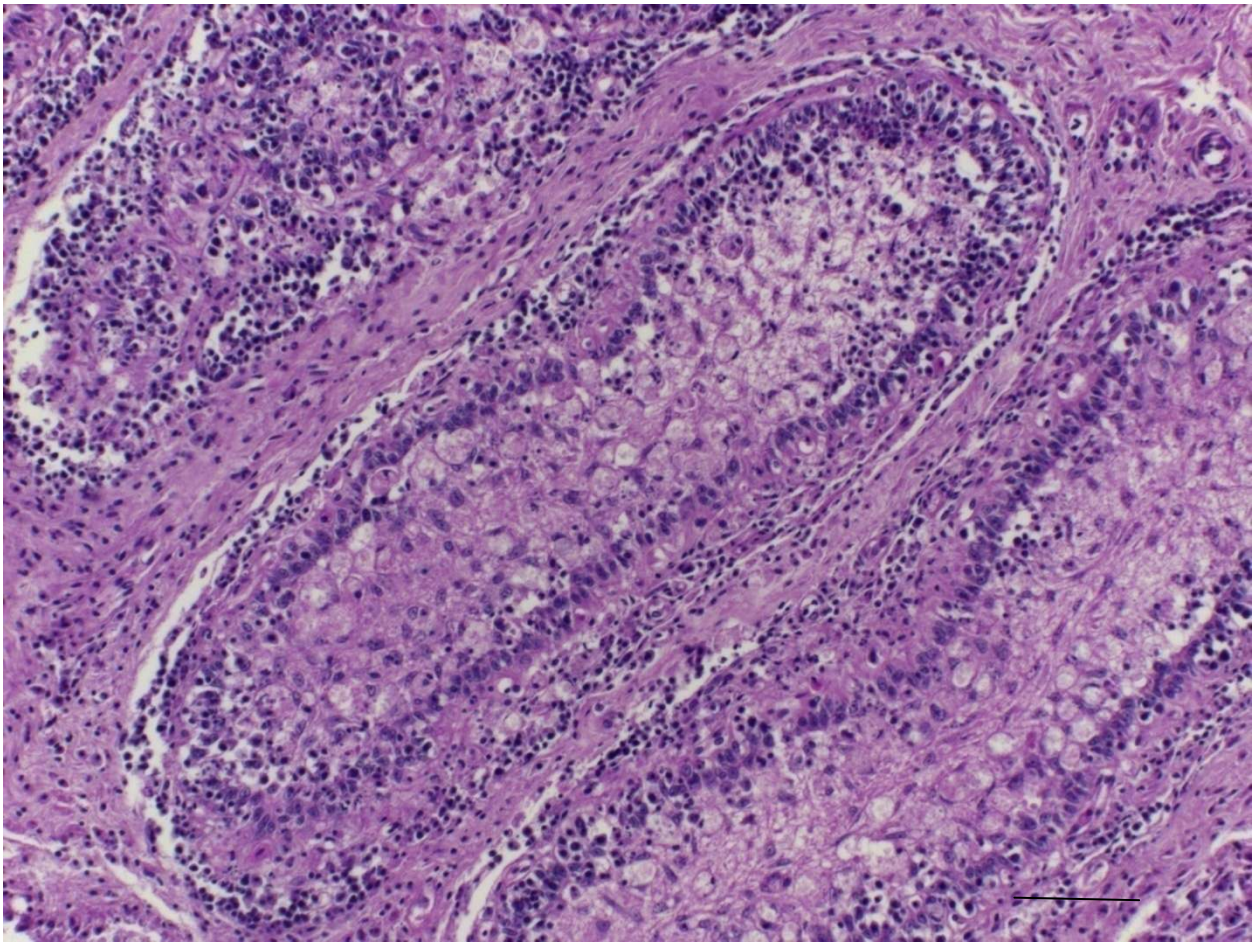

Plate 4.28. Bursa of Fabricius; Pekin duck 4 days p.i, clade 1 HPAI. Bursitis, follicular necrosis and lymphoid depletion and lymphocytic repopulation. HE. Bar=100µm

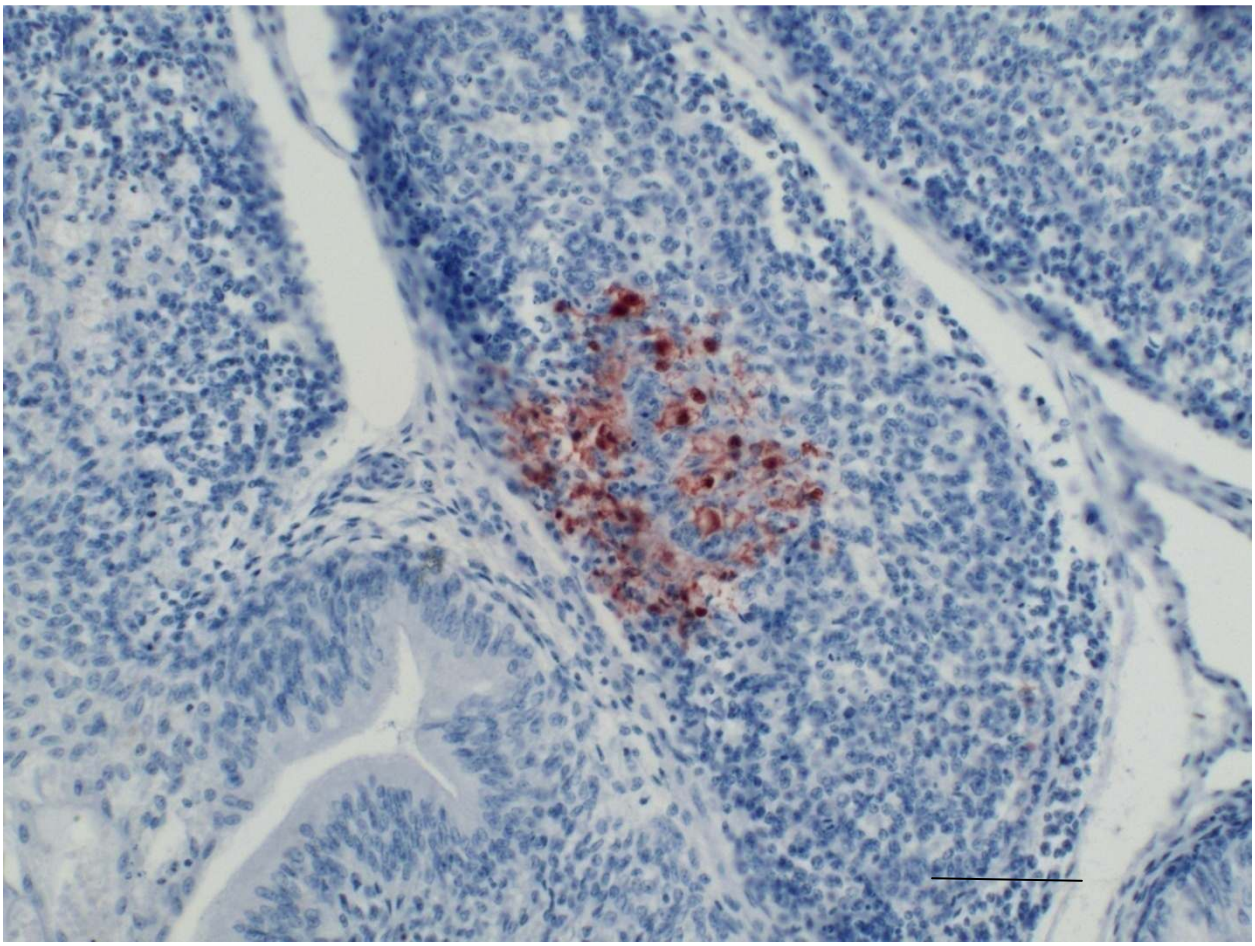

Plate 4.29. Bursa of Fabricius; Muscovy duck 3 days p.i, clade 1 HPAI. Follicular mononuclear cells are focally and strongly immunoreactive to H5 nuclear protein. Bar=100µm

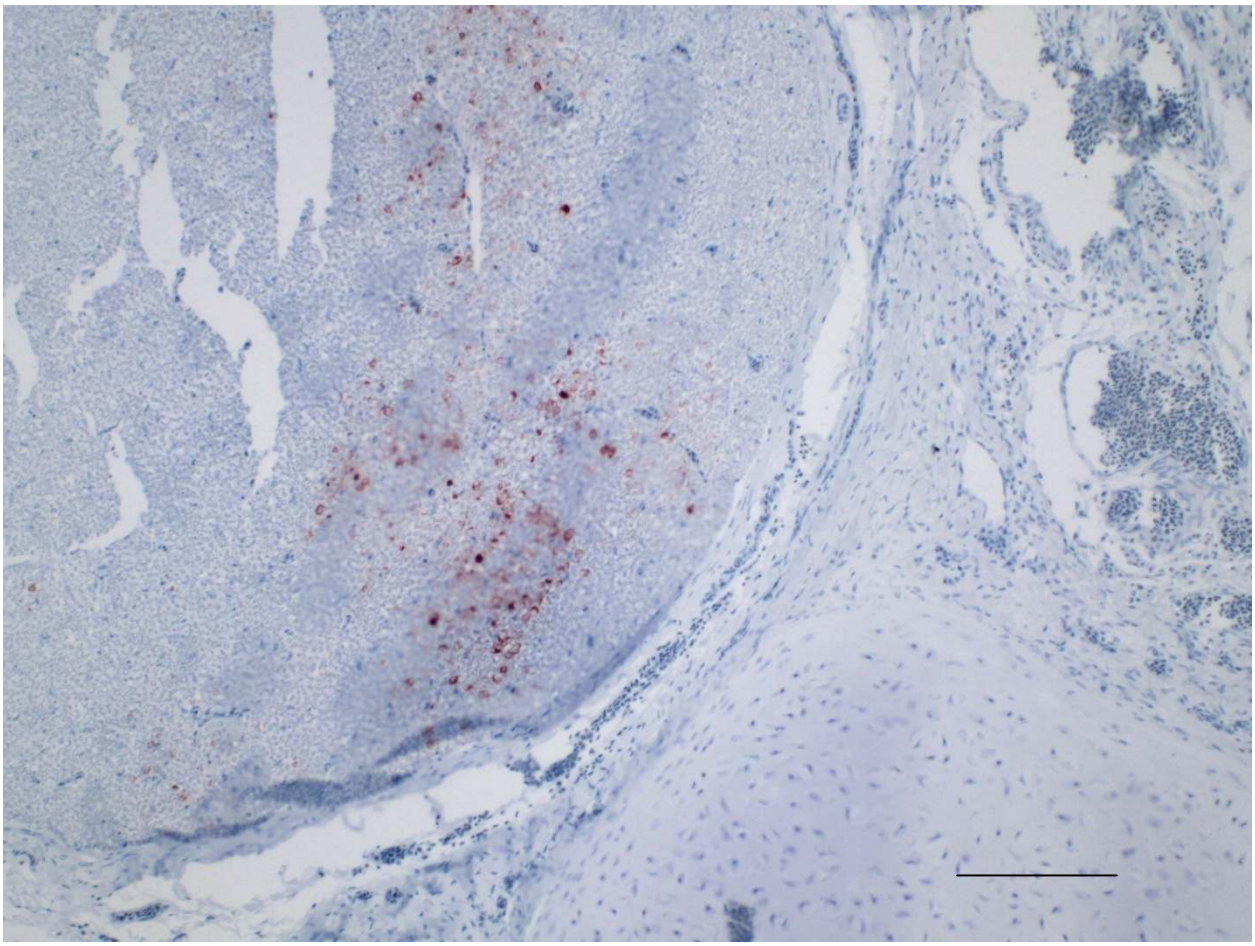

Plate 4.30. Trigerminal nerve-Nasal septum; Pekin duck 4 days p.i, clade 1 HPAI. Neuronal and axonal necrosis with focally diffuse infiltration of lymphocytes. The neurons are diffusely and strongly immunoreactive to H5 nuclear protein. Bar=100µm

4.4.1.4 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in swabs

All ducks tracheal and cloacal swabs were negative (ct-value ≥ 40) on the day of infection with clade 1 H5N1 virus by real time RT-PCR. 1st, 2nd and 3rd day post infection (dpi) tracheal swabs for all Muscovy were very positive (high viral genome load) and at 2 and 3dpi, most had a ct-value of less than 20 (Fig 4.4). While most cloacal swabs for 2nd and 3rd dpi were most positive, although viral genome load in few individuals were negative on 1st dpi, most were moderately positive (Fig 4.5). 3dpi tracheal swabs of Pekin ducks were most positive, having highest viral genome load (Fig 4.6) as compared to other days PI. While the cloaca swab ct-value varied widely with some Pekin duck having moderate positivity values at 3dpi (Fig 4.7).Two peaks were observed in the Pekin ducks mean ct-values of the tracheal swabs at 3 d.p.i. (μ=26.1), been lowest and at 6 d.p.i (μ=28.80), these were higher than the mean cloacal swab ct-value for the 3dpi (μ=31.59) and no surge was observed. The tracheal swab RRT-PCR ct-values of Mallard ducks at 3dpi were very positive (Fig 4.8), while cloaca swab ct-values varied widely with most ducks having moderate viral genome load (RRT-PCR ct-value≤ 36) on 3rd and 4th dpi (Fig 4.9).

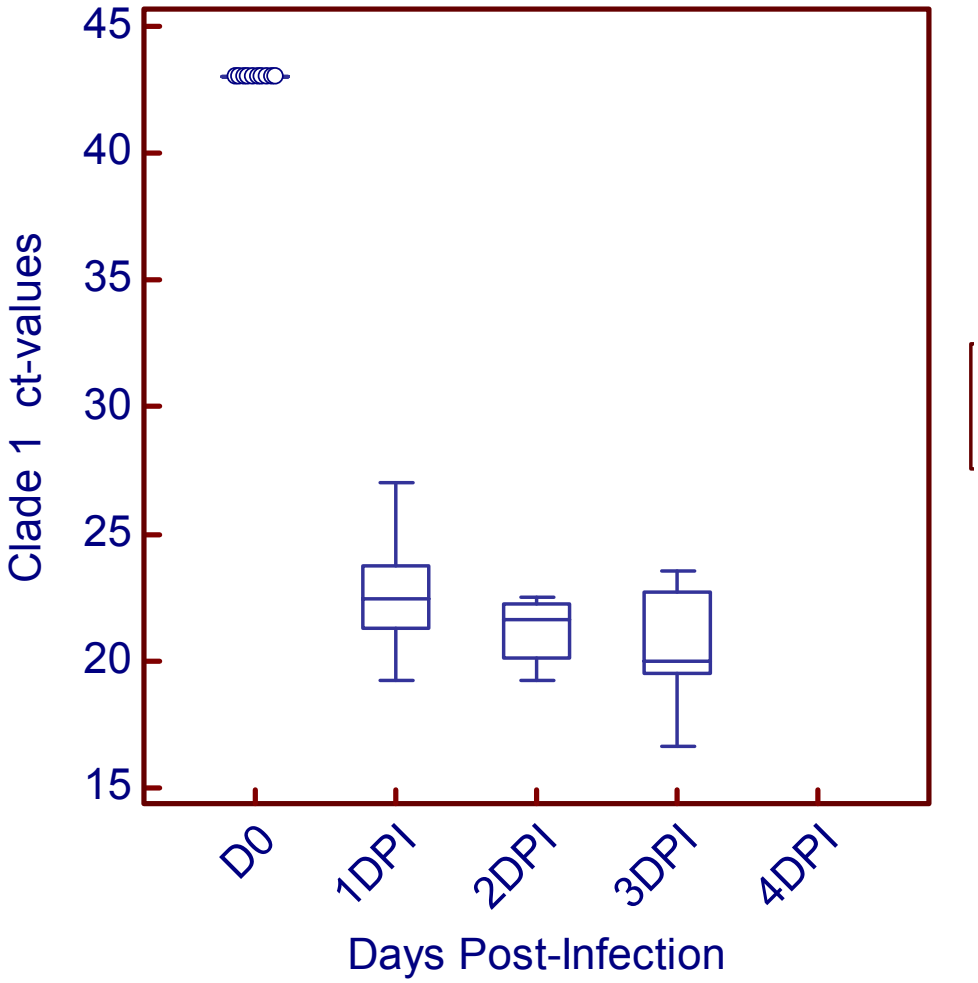

Figure 4.4. Clustered multiple variables graph: Muscovy Tracheal Swab ct-values of Clade 1

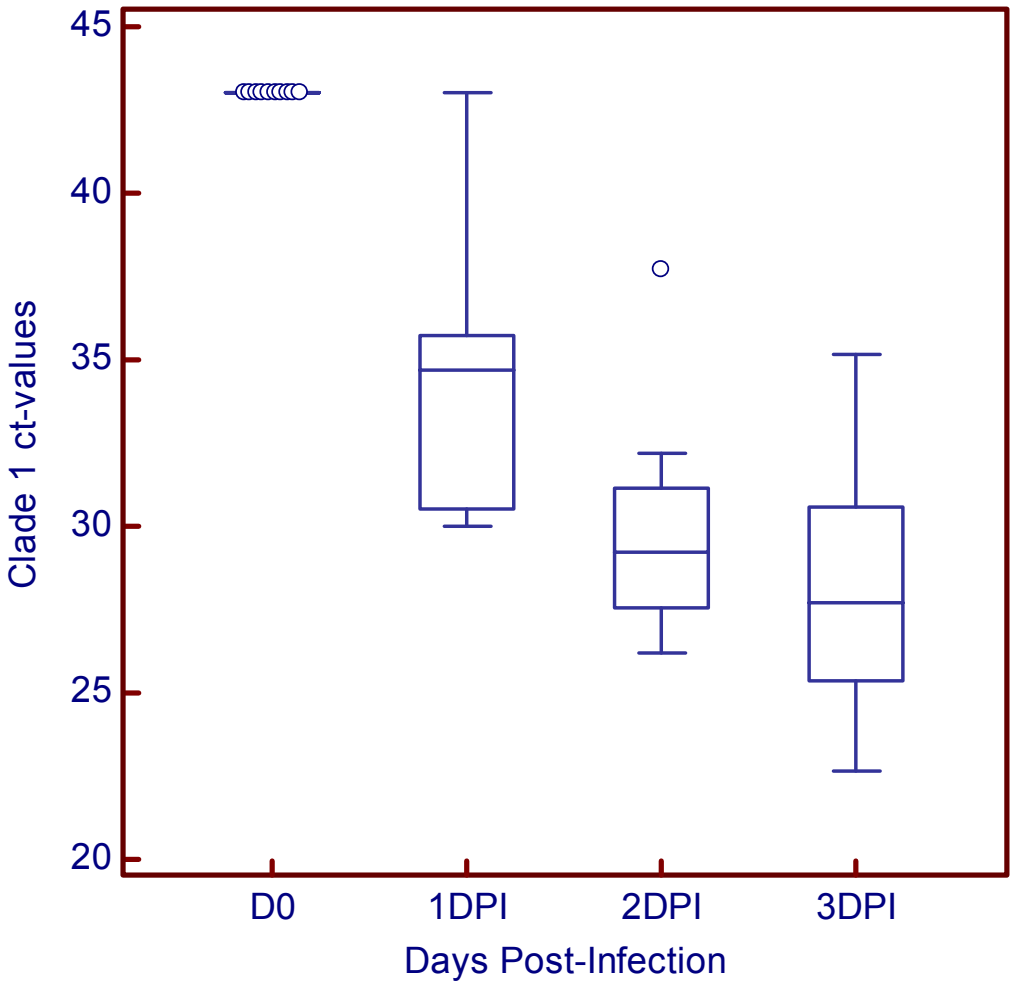

Figure 4.5. Clustered multiple variables graph: Muscovy Cloaca Swab ct-values of Clade 1

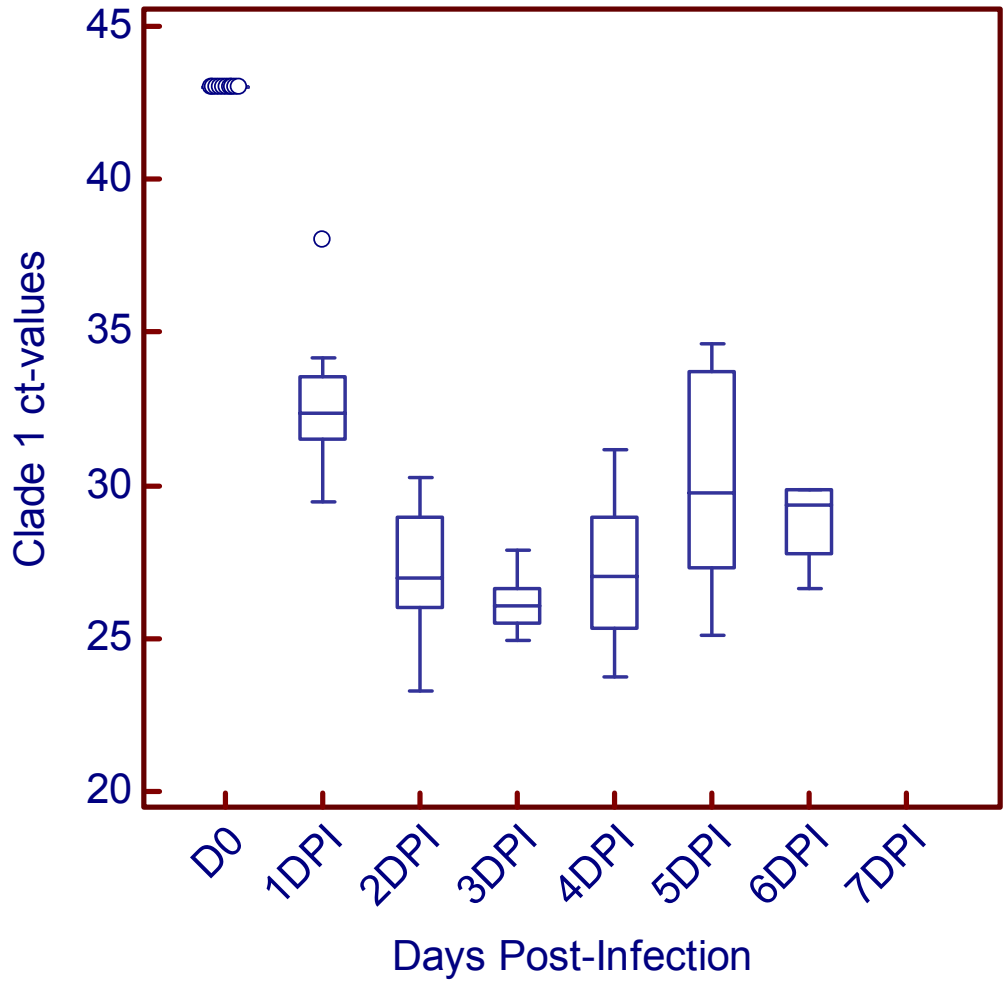

Figure 4.6. Clustered multiple variables graph: Pekin Tracheal Swab ct-values of Clade 1

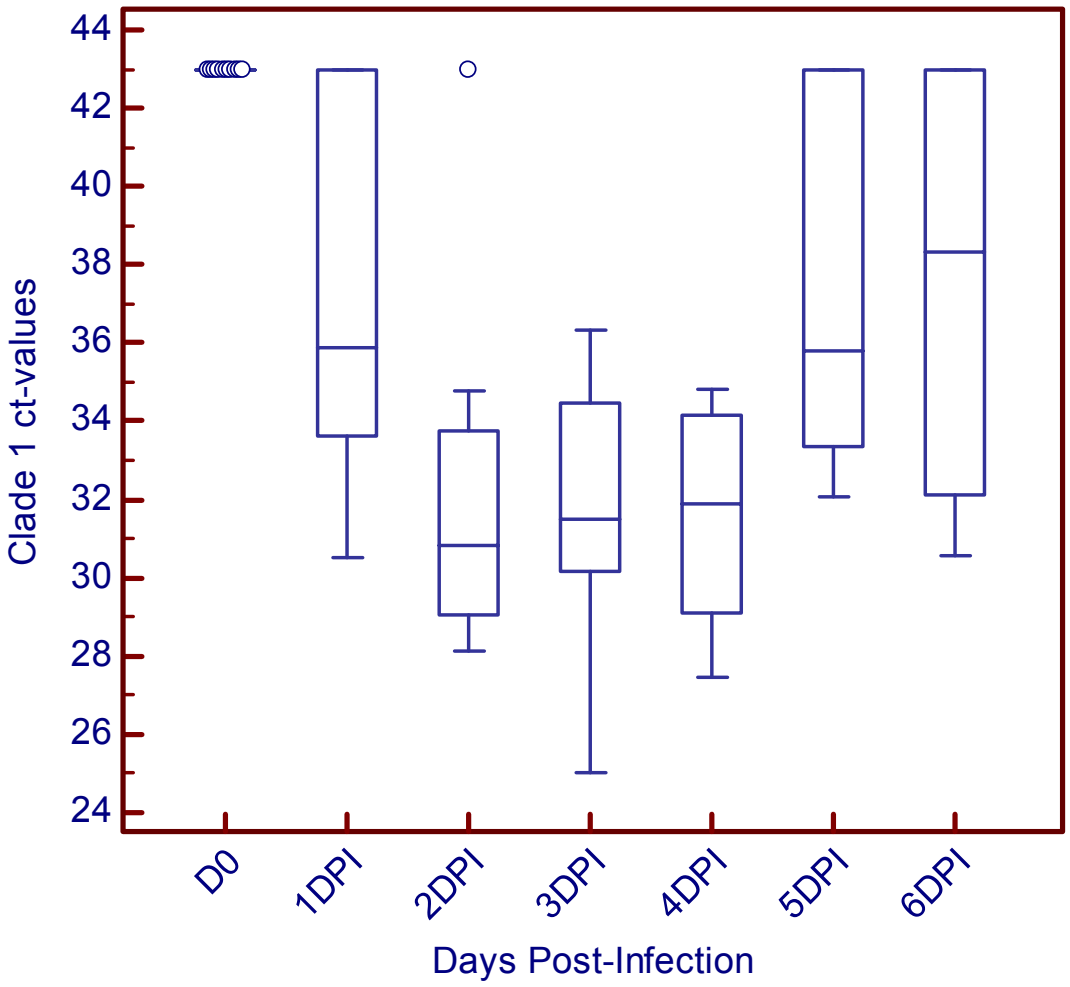

Figure 4.7. Clustered multiple variables graph: Pekin Cloaca Swab ct-values of Clade 1

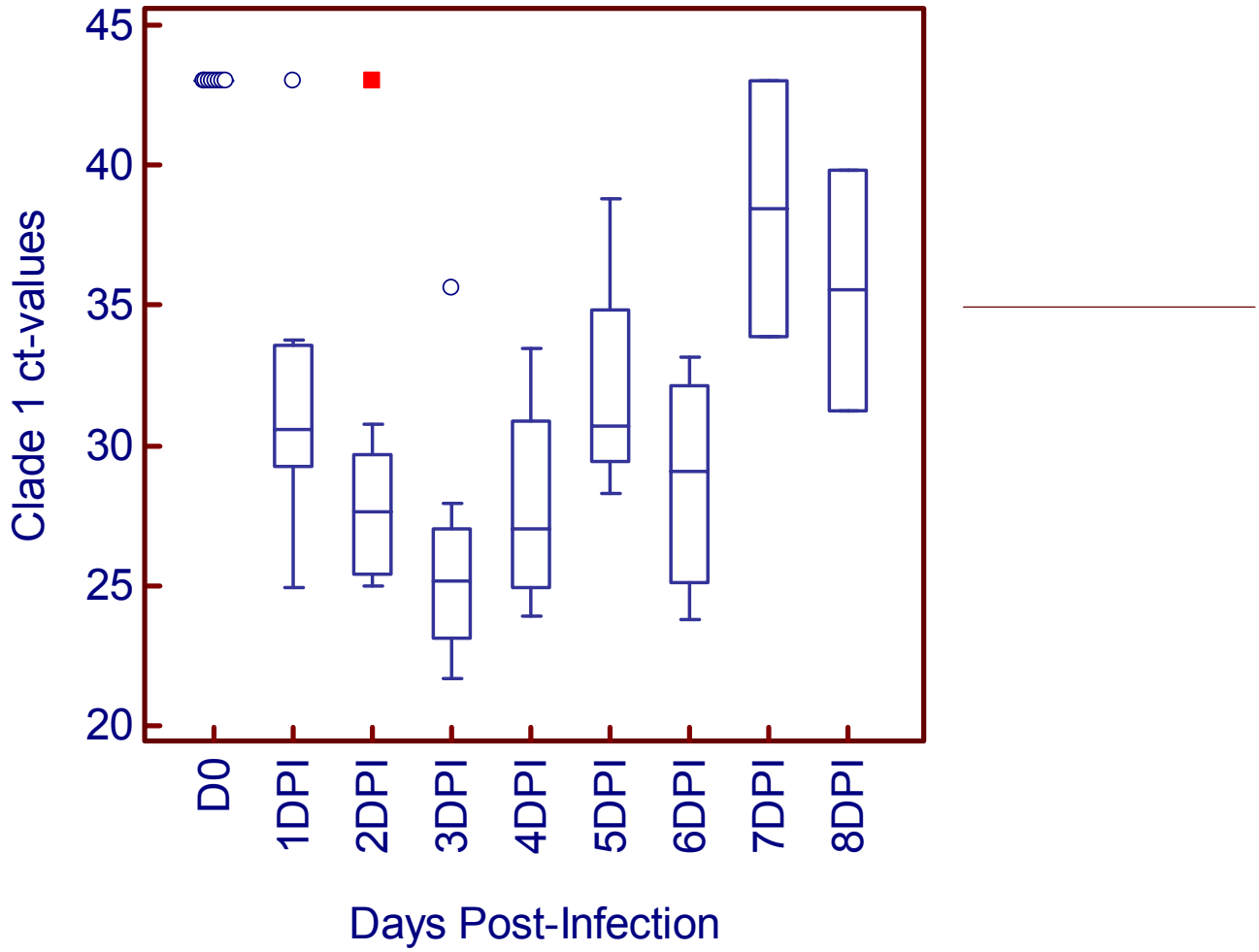

Figure 4.8. Clustered multiple variables graph: Mallard Tracheal Swab ct-values of Clade 1

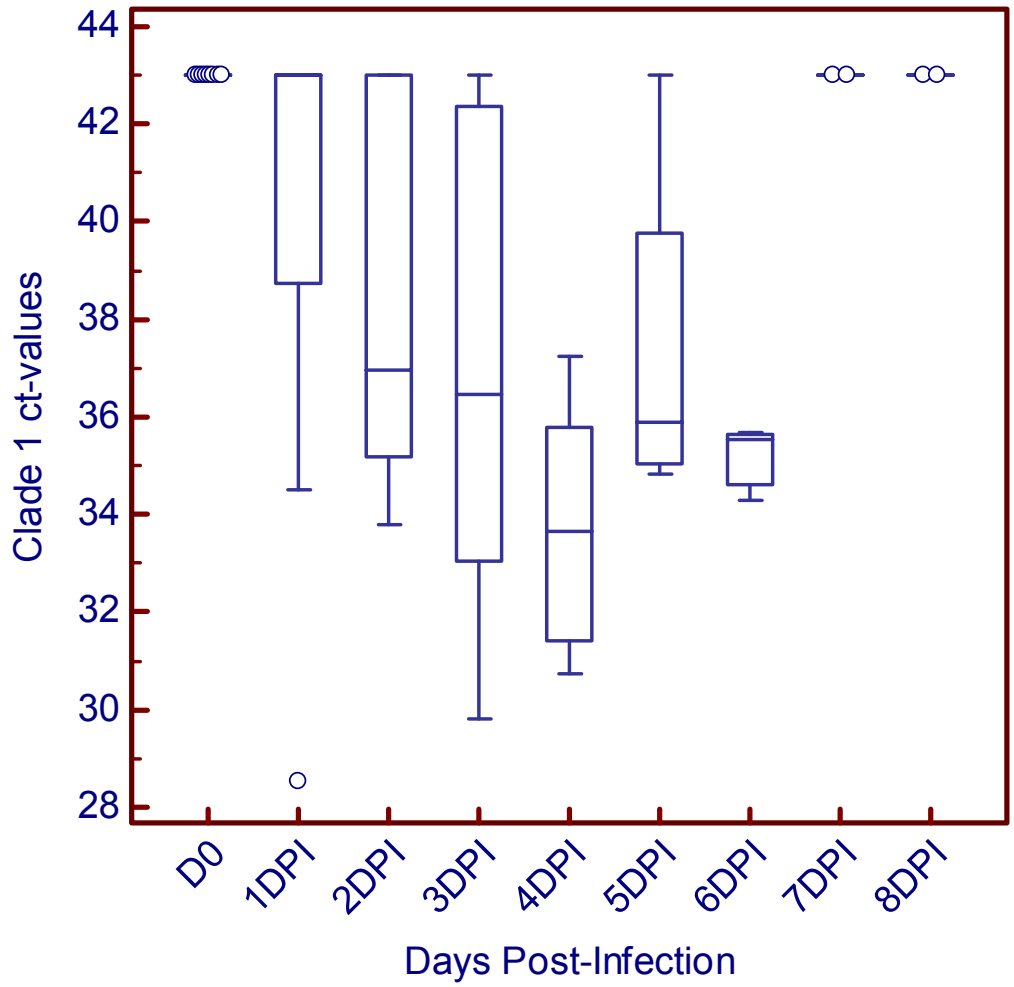

Figure 4.9. Clustered multiple variables graph: Mallard Cloaca Swab ct-values of Clade 1

4.4.1.5 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in tissues

Tissues of Muscovy duck examined at 3dpi were all positive for the virus; with cerebrum, nasal cavity, lung, heart, ovary, testis and spleen having very high ct-values has detected by RRT-PCR (Fig 4.10). All tissues of Mallard ducks were all positive at 4dpi, with ovary and testis been the most positive tissue samples, closely followed by nasal cavity. Others include cerebrum, heart, muscle, spleen and kidney (Fig 4.11 and 4.12). All tissues of Pekin ducks examined at 4dpi were positive except for the spleen in one. Nasal cavity was most positive; other very positive tissues included airsac, lung, heart, pancreas, ovary and testis. This also applies to the Pekin duck sacrificed at 5dpi. At 6dpi, all tissue of Pekin duck examined was positive. Cerebrum was most positive and other very positive tissues included ovary, testis, muscle, heart and gizzard (Fig 4.13).

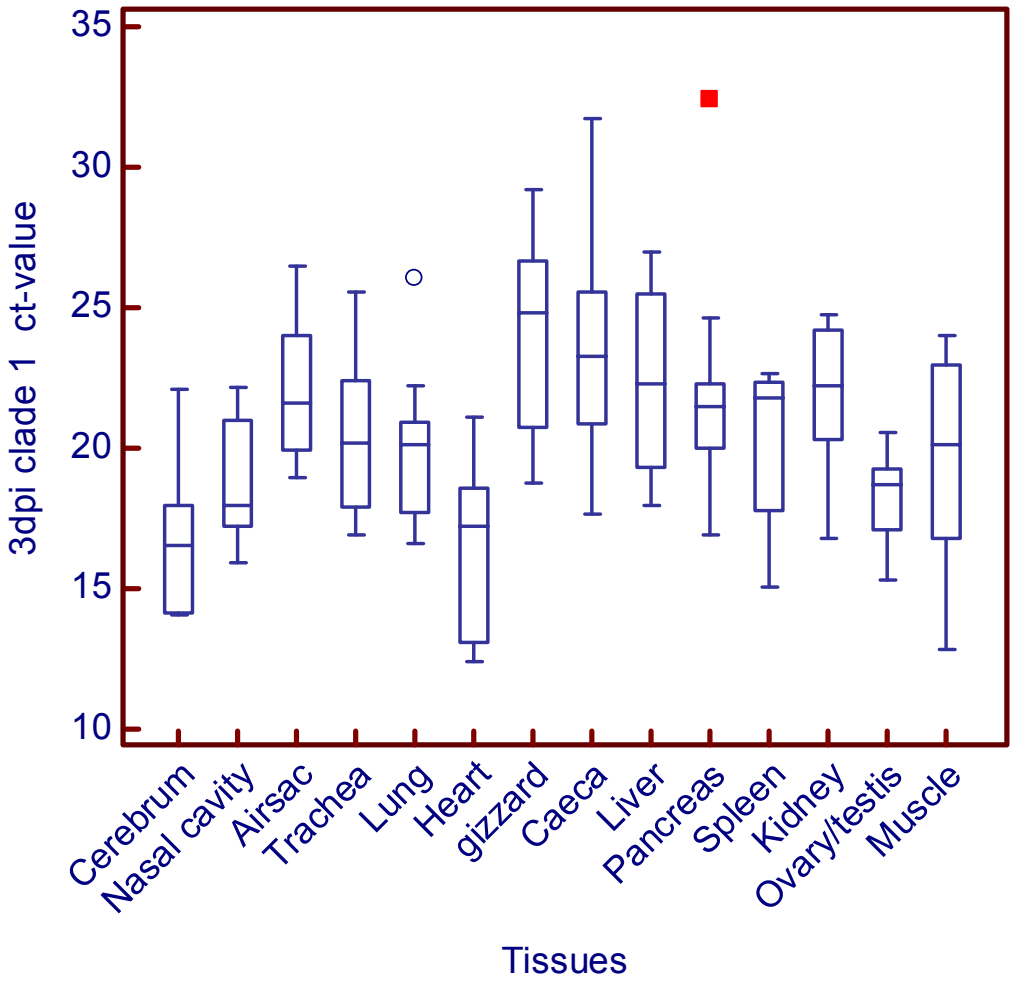

Figure 4.10. Clustered multiple variables graph: Muscovy Tissue ct-values of Clade 1 3DPI

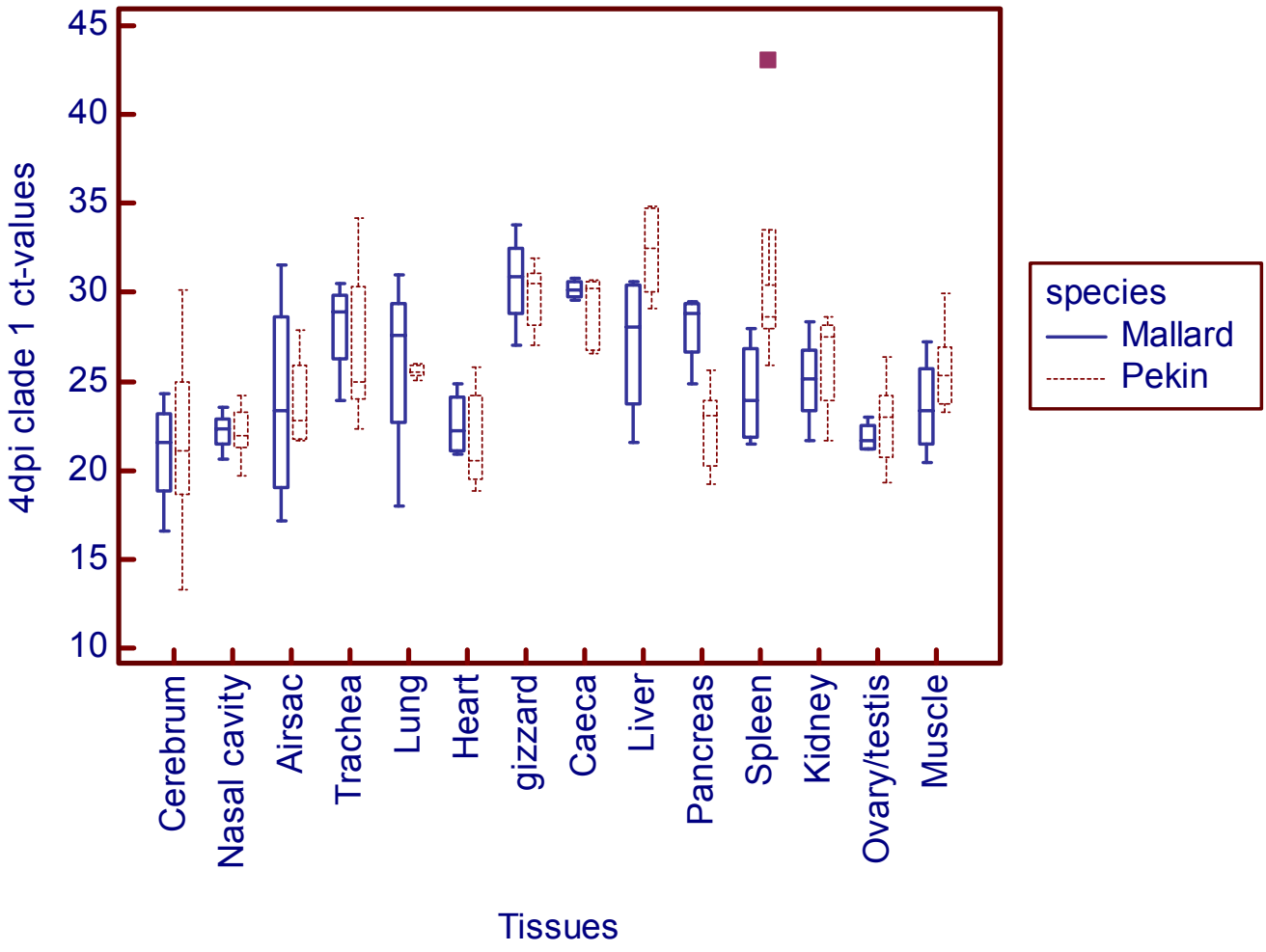

Figure 4.11.Clustered multiple variables graph: Mallard and Pekin duck Tissue ct-values of Clade 1 4DPI

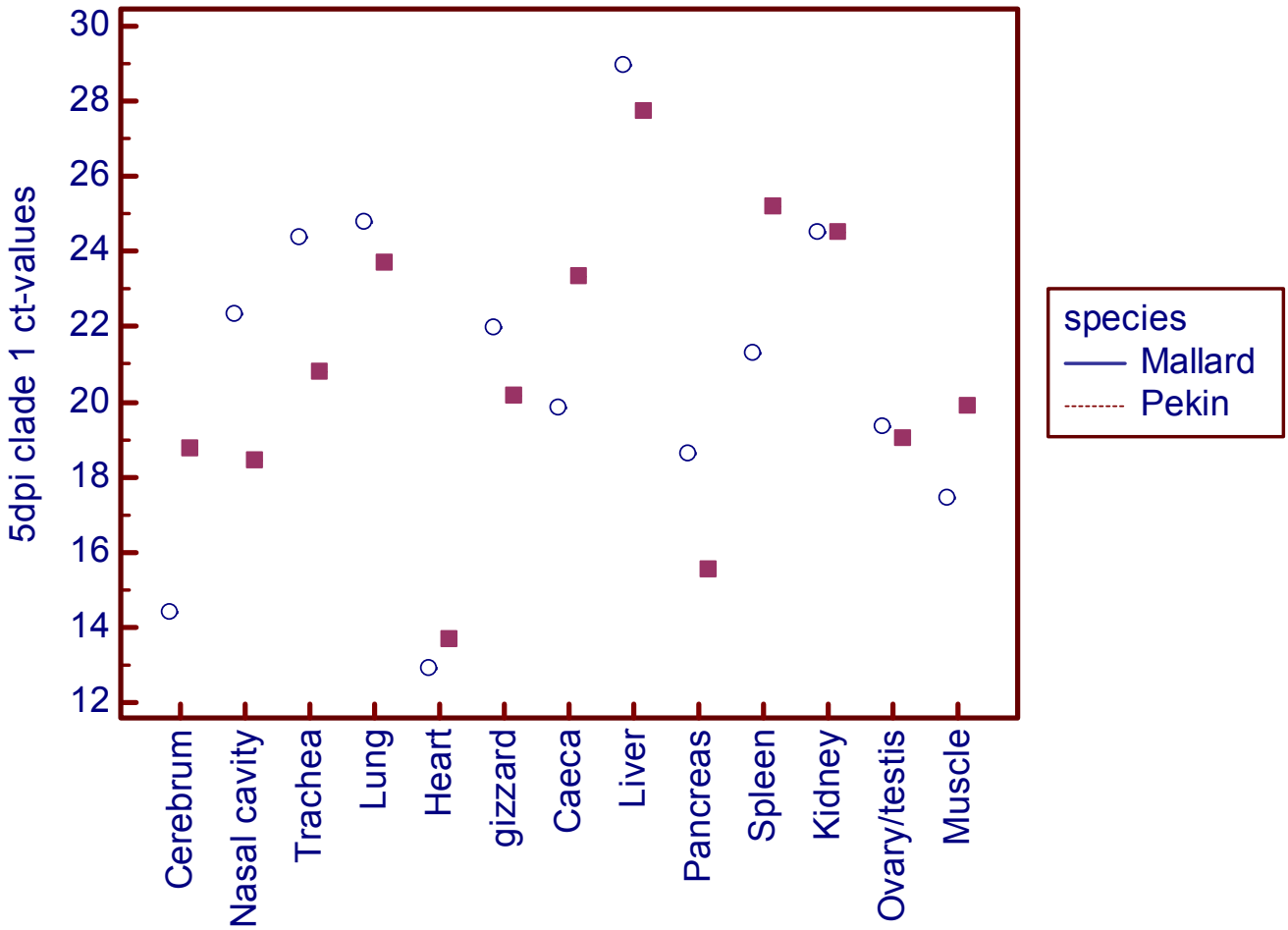

Figure 4.12.Clustered multiple variables graph: Mallard and Pekin Tissue ct-values of Clade 1 5DPI

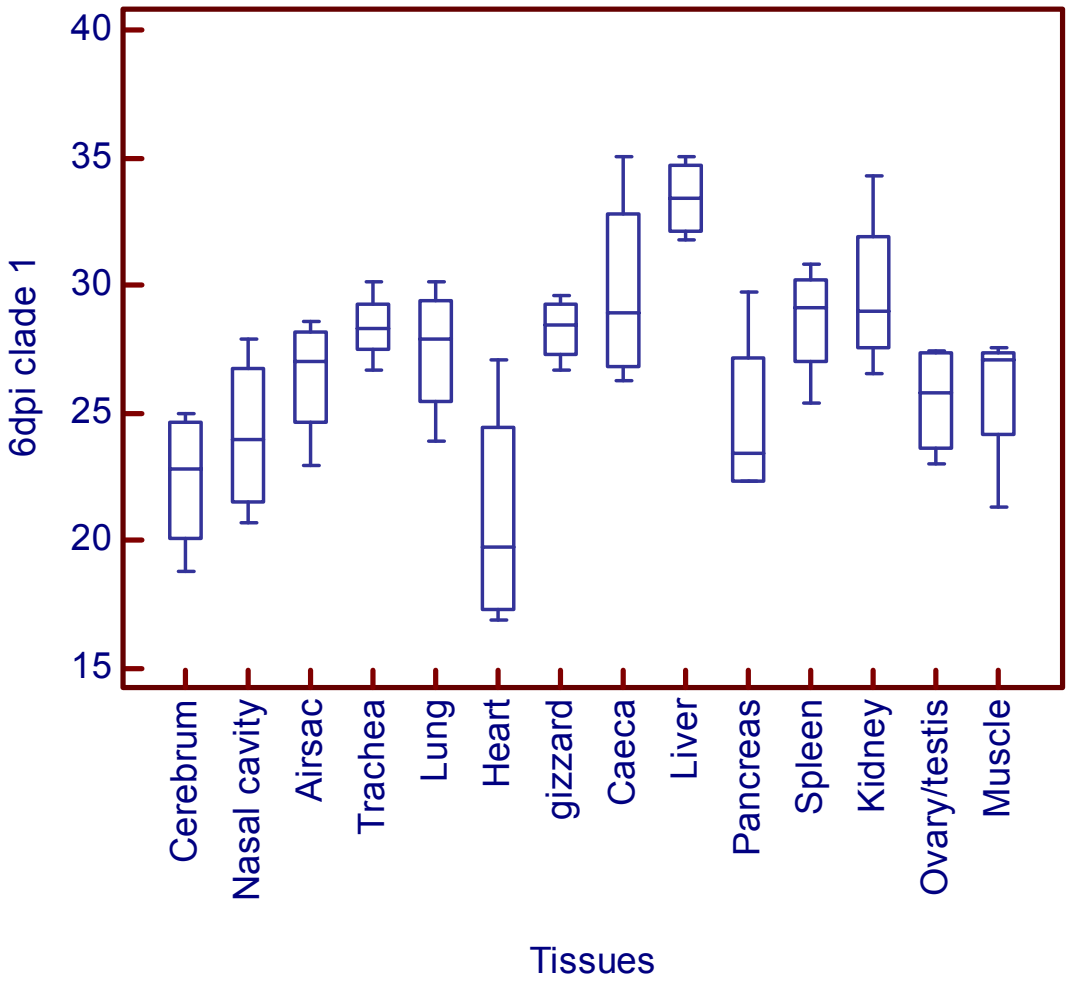

Figure 4.13.Clustered multiple variables graph: Pekin Tissue ct-values of Clade 1 6DPI

4.4.2 *Influenza A/Cygnus cygnus/Germany/R65/2006* of clade 2.2 subtype H5N1 virus infection in ducks

4.4.2.1 Clinical signs

As early as 2 days p.i all the Muscovy ducks infected with Highly Pathogenic Avian Influenza A/Cygnus cygnus/Germany/R65/2006 of clade 2.2 subtype H5N1 virus, were generally weak, while Pekin and Mallard ducks exhibited no sign of disease. The sign of clinical disease exhibited as weakness in the Muscovy ducks was more evidentful when two (20%) of them died suddenly and six (60%) exhibited severe neurological signs, which included unsteady gait, ataxia, tremor and opisthotonus at 3 d.p.i and were sacrificed for animal welfare reasons. No clinical sign was observed in the Pekin and Mallard ducks. General weakness was seen in six Pekin ducks 4d.p.i, while one Muscovy exhibited neurological signs, but still the Mallard show no observable signs. At the termination of the experiment 8 d.p.i, no Pekin or Mallard duck died as a result of the infection but were however sacrificed as planned.

4.4.2.2 Gross Pathology

Multifocal pancreatic necrosis with duodenal serosal vascular congestion was the gross lesion in Muscovy ducks at 3 dpi. The ducks examined at 3dpi showed no gross and histopathological lesion in the cardiovascular and GIT systems, except intestinal intramural ganglia degeneration and necrosis. Main gross lesions in Pekin ducks at 4dpi, are myocardial ecchymotic hemorrhages, fatty liver and multifocal pancreatic necrosis. At 8 d.p.i, the surviving Muscovy had no visible gross lesion. Gross and histopathological lesions are consistent in the surviving Pekin ducks at 8 d.p.i as in 4 d.p.i but with severe pneumonia. Mallard ducks had no visible gross lesion at 4 d.p.i. Mallard ducks show no visible gross lesion at 8 d.p.i.

4.4.2.3 Histopathology and Immunohistochemistry

Immunoreactivity was only in the intramural ganglia of the duodenum, jejunum, ileum, caeca and rectum and cardiomyocytes, hepatocytes and acinar cells of the pancreas also show staining. These intramural ganglia immuno-staining was also seen in the testis. Theca cells of the ovary also show immunoreactivity. Lymphoid depletion was present in the thymus and Bursa of Fabricius in these ducks. Histopathological lesions in Pekin ducks at 4dpi, was duodenal lymphoplasmacytic ganglioneuritis , airsacculitis, necrotizing with perivascular lymphocytic cuffing with lymphocytic infiltration of the mesothelium. The mesothelium of the airsac is diffusely immunoreactive to H5 nuclear protein. Mallard ducks at 4days p.i showed histopathological lesions of cerebral and cerebellar meningo-encephalitis with multifocal lymphocytic perivascular cuffs and neuronal necrosis together with jejunal intramural lymphocytic ganglioneuritis, which was seen in the Pekin also. Medullary lymphoid depletion in Bursa of Fabricius and caeca lymphoid tissue in Mallard 9 &16. Immunohistochemical detection was rare in Mallard ducks at 4 d.p.i. At 8 d.p.i, the surviving Muscovy had histopathologic lesions consistent with 3 days p.i, except for lymphoplasmacytic conjunctivitis, heterophilic duodenitis, jejunitis, ileitis, cecaitis and typhilitis and all the tissues were not immunoreactive by ABC method. Histopathological lesions are consistent in the surviving Pekin ducks at 8 d.p.i as in 4 d.p.i but with severe fibrinous pneumonia, mild lymphocytic adrenalitis, medullary depletion with numerous pyknotic lymphocytes in the Bursa of Fabricius, splenic hemosiderosis with severely enlarged PALS, severe multifocal myocarditis and myofibrillar necrosis and duodenal lymphoplasmacytic ganglioneuritis. Immunohistochemical detection was in central canal and neurons of spinal cord, and the medullar cells of the adrenal gland in addition to immunostaining in 4days p.i. Mallard ducks show the following histopathological lesion at 8 days p.i; cerebral glia proliferation, Epithelia proliferation in the nasal cavity, thymic lymphoid depletion and atrophy and splenic hemosiderosis and lymphocytic myocytis. Only one mallard duck show immunohistochemical detection of H5 antigen in the ganglion and inner nuclear layer of the retina. The following Figures 4.31-4.41 show the gross and histopathological lesions, including immunohistochemically detection.

Plate 4.31. Muscovy duck 4 days p.i clade 2.2 HPAI. Torticollis

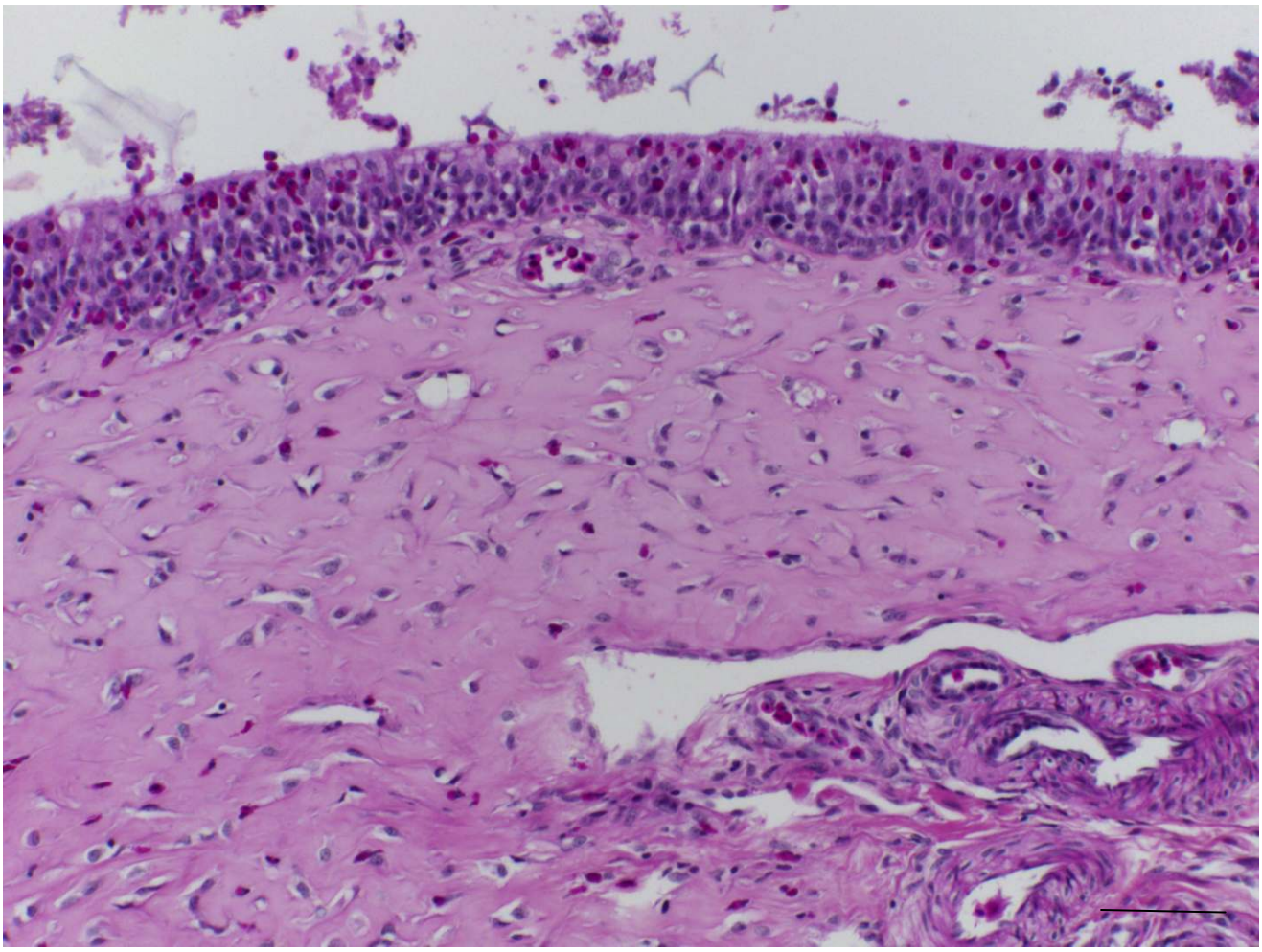

Plate 4.32. Conjuctiva- Eye; Muscovy duck 3 days p.i clade 2.2 HPAI. Conjuctivitis and Vasculitis. HE. Bar=100µm.

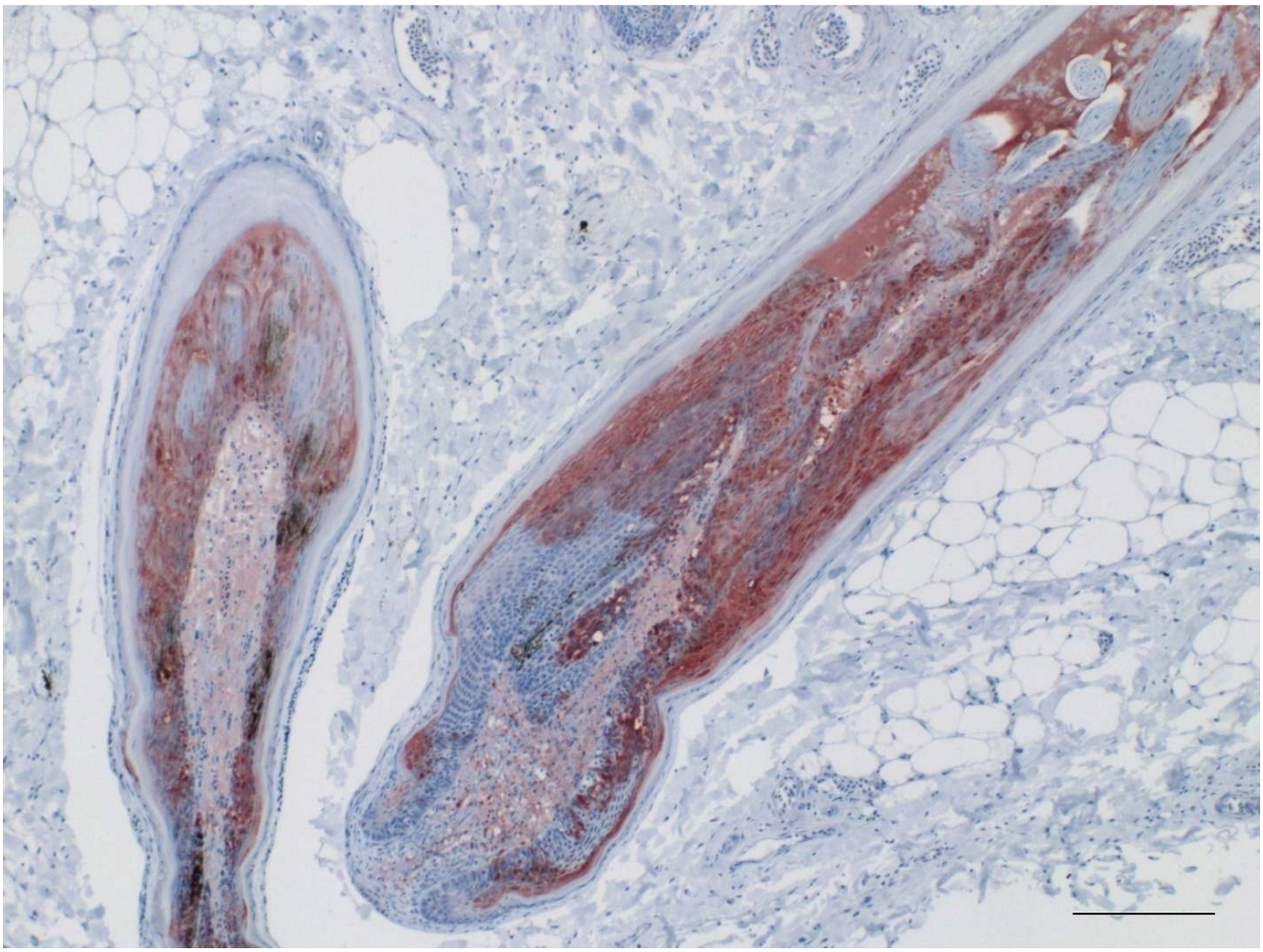

Plate 4.33. Feather follicle- Skin; Muscovy ducks 3 days p.i clade 2.2 HPAI. The keratinocytes are diffusely and strongly immunoreactive to H5 nuclear protein. Bar=100µm.

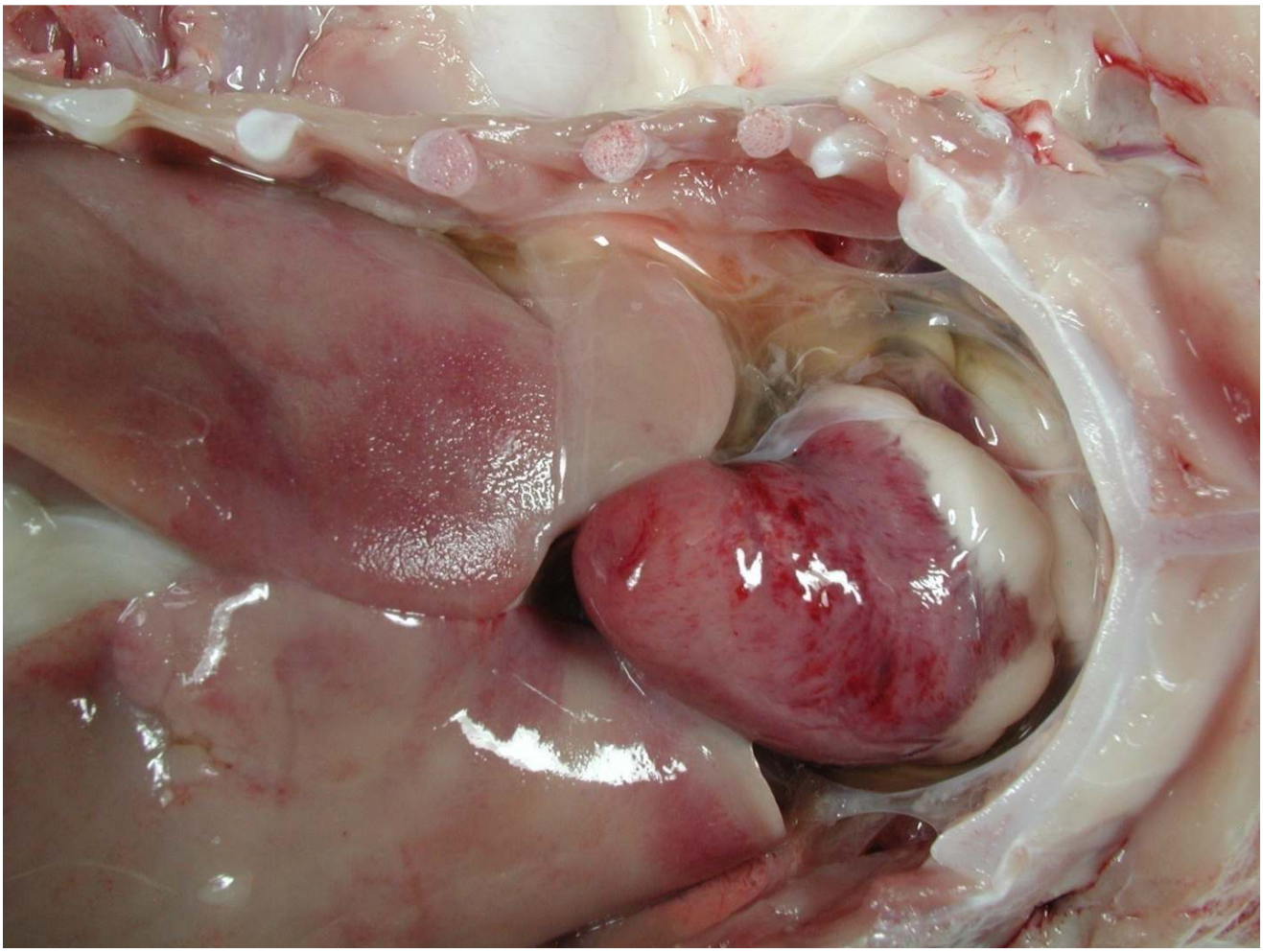

Plate 4.34. Heart: Pekin duck 8 days p.i clade 2.2 HPAI. Diffuse myocardial ecchymotic hemorrhages and myocarditis, diffuse. Liver: diffuse fibrinous perihepatitis.

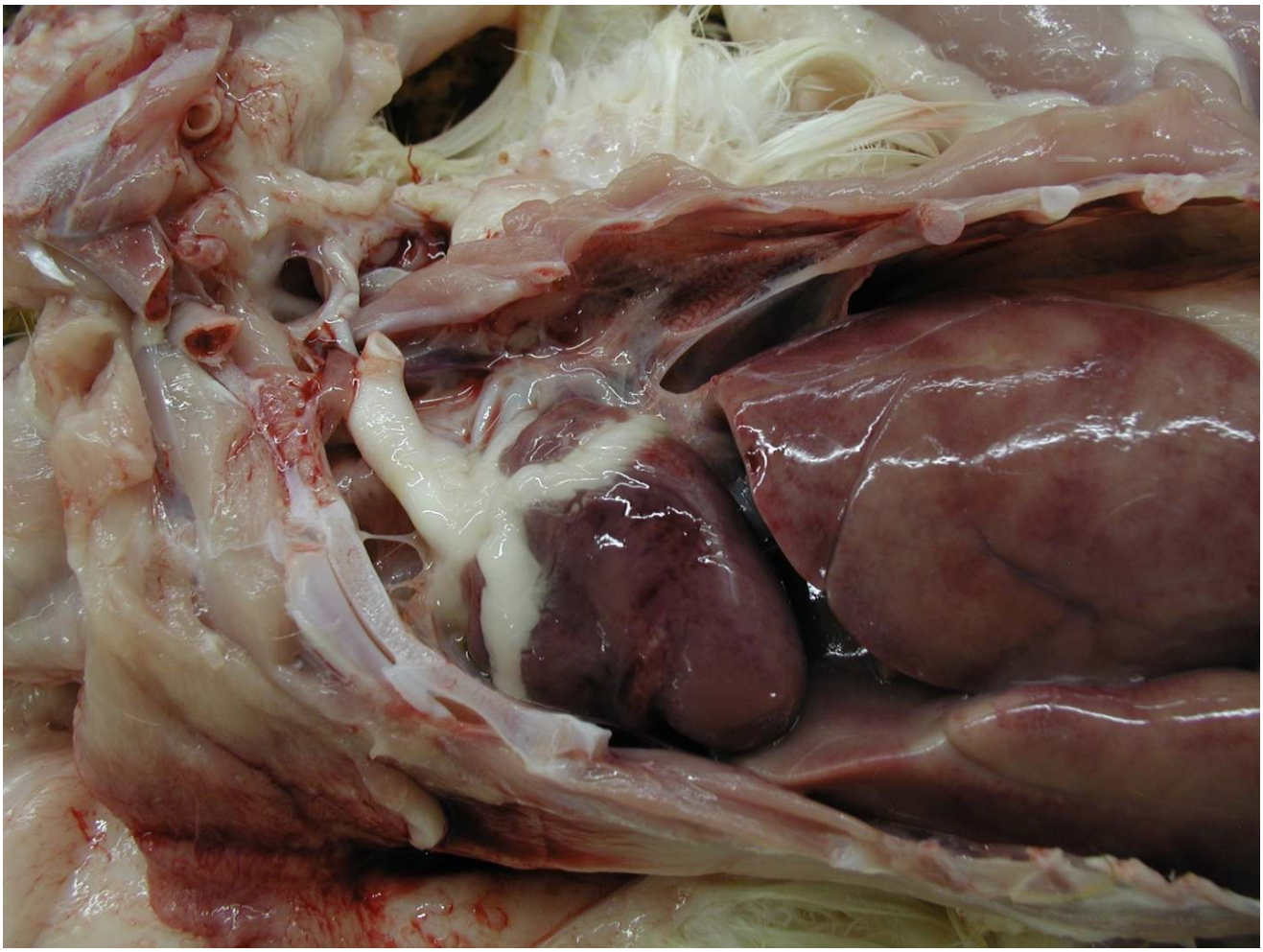

Plate 4.35. Heart: Pekin duck 8 days p.i clade 2.2 HPAI.Focal myocardial ecchymotic hemorrhages. Liver: Hepatic lipidosis, diffuse.

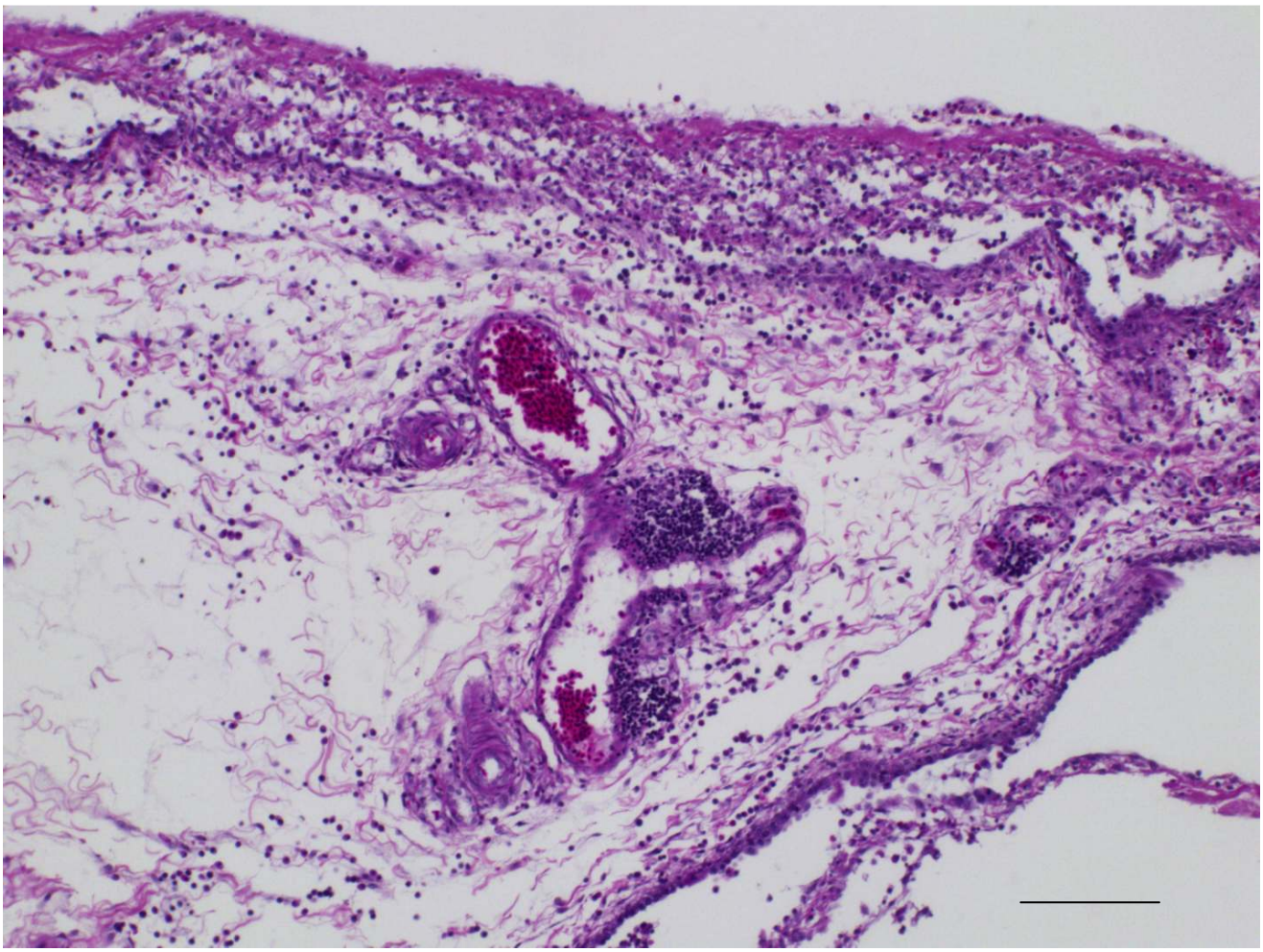

Plate 4.36. Airsac; Pekin duck 4 days p.i clade 2.2 HPAI. Airsacculitis, necrotizing with perivascular lymphocytic cuffing: the mesothelium is diffusely infiltrated by lymphocytes HE. Bar=100µm

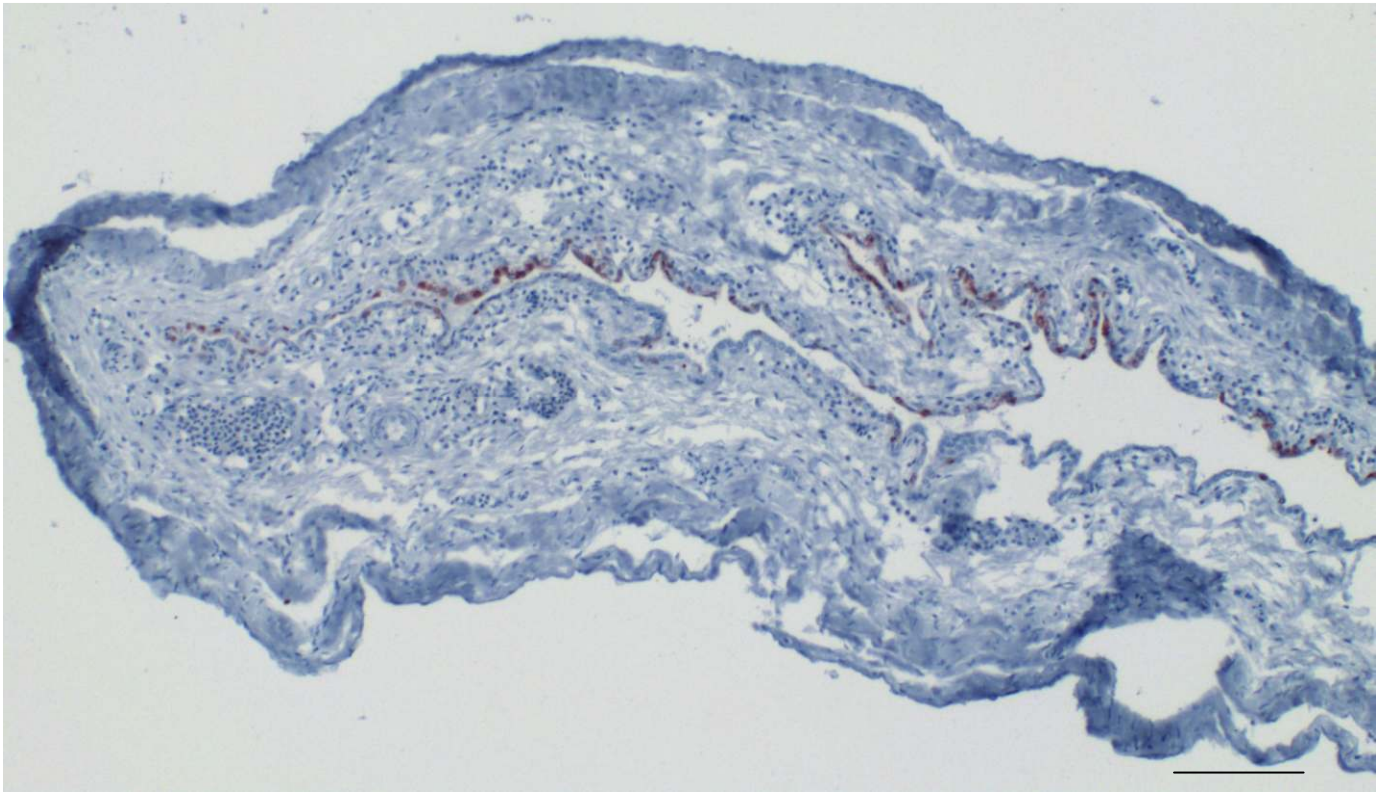

Plate 4.37. Airsac; Pekin duck 4 days p.i clade 2.2 HPAI. Airsacculitis. The mesothelium is diffusely immunoreactive to H5 nuclear protein. Bar=100µm

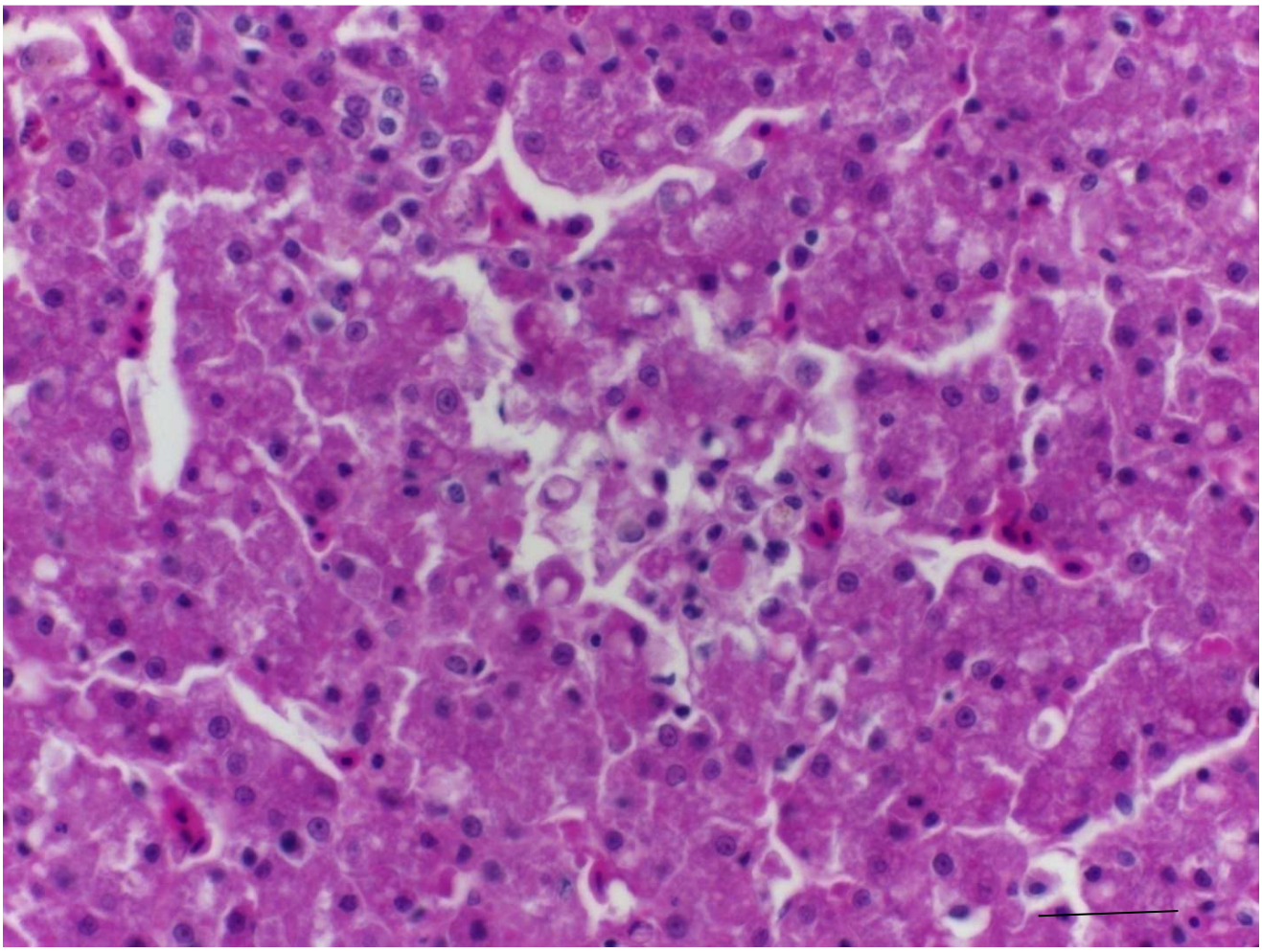

Plate 4.38. Liver; Muscovy duck 3 days p.i clade 2.2 HPAI. Vacuolar degeneration and necrosis of hepatocytes. Bar=100µm.

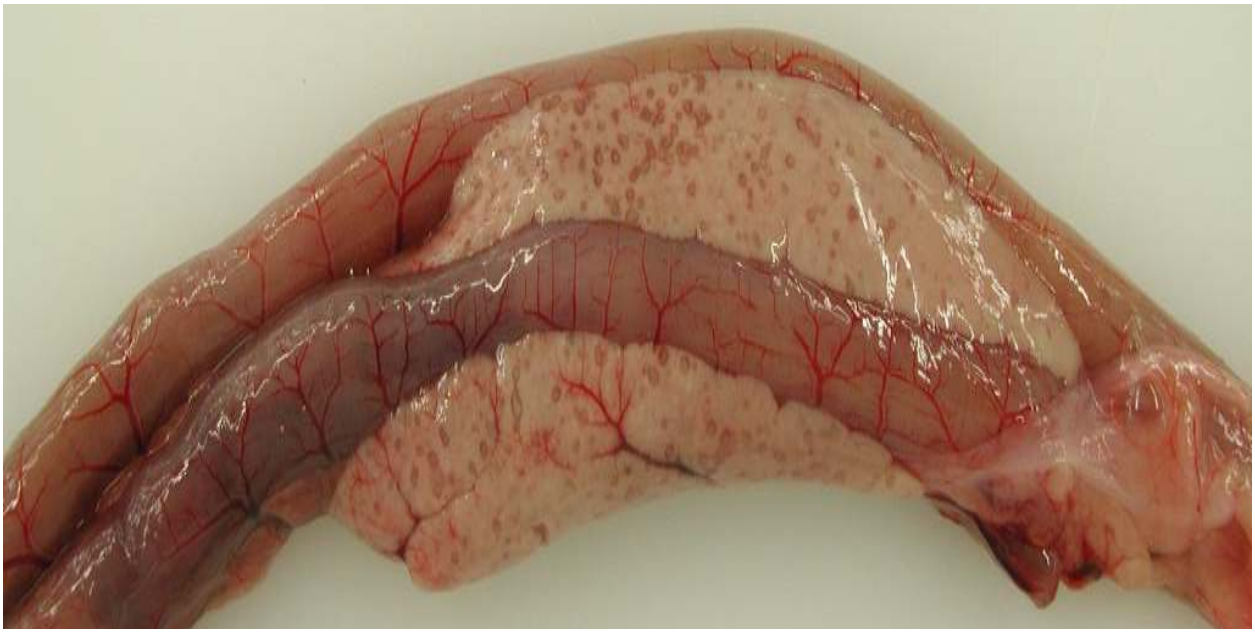

Plate 4.39. Pancreas: Muscovy duck 3 days p.i clade 2.2 HPAI. Necrotic Pancreatitis, multifocal, severe

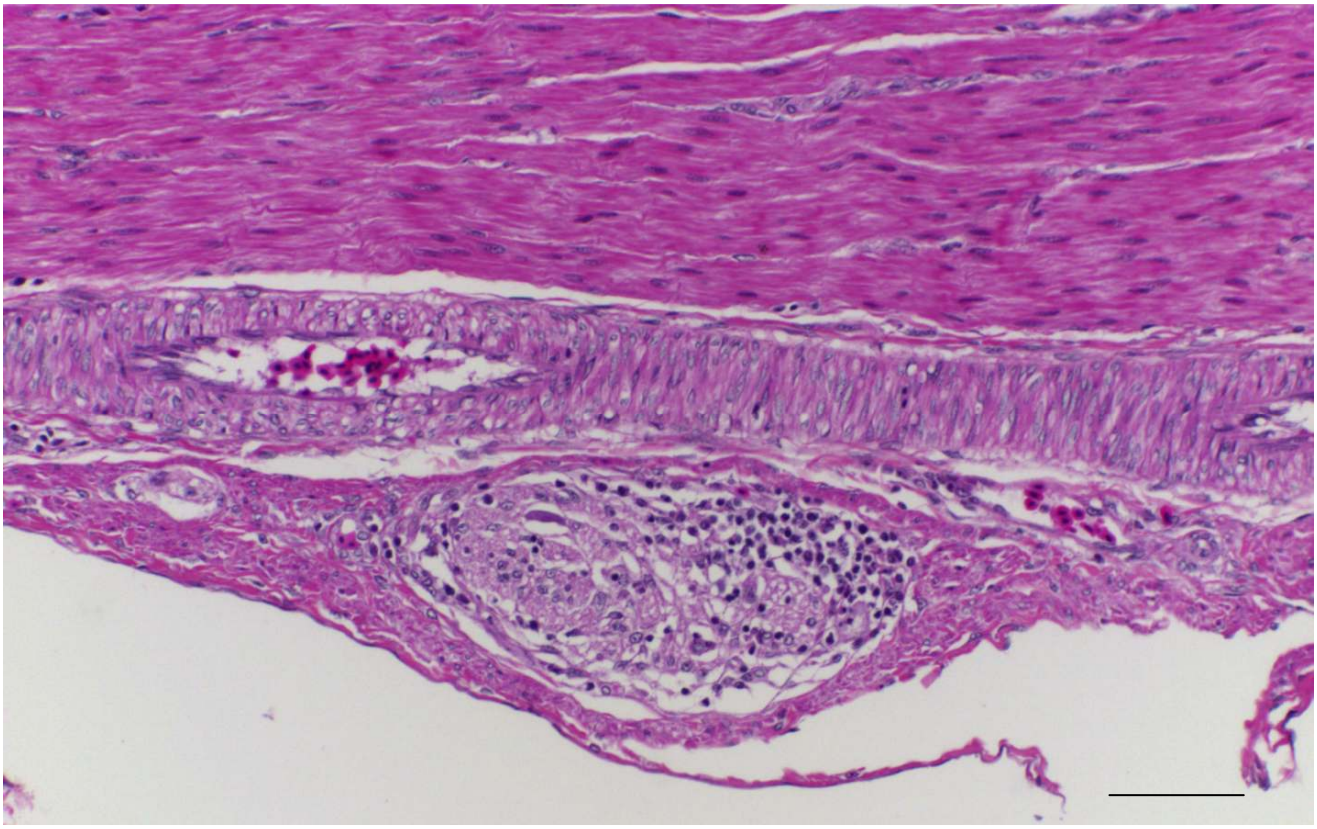

Plate 4.40. Myenteric intramural ganglion-Intestine; Pekin duck 4 days p.i clade 2.2 HPAI. Neuronal and axonal necrosis with diffuse infiltration of mononuclear cells, mainly lymphocytes (ganglioneuritis) HE. Bar=100µm

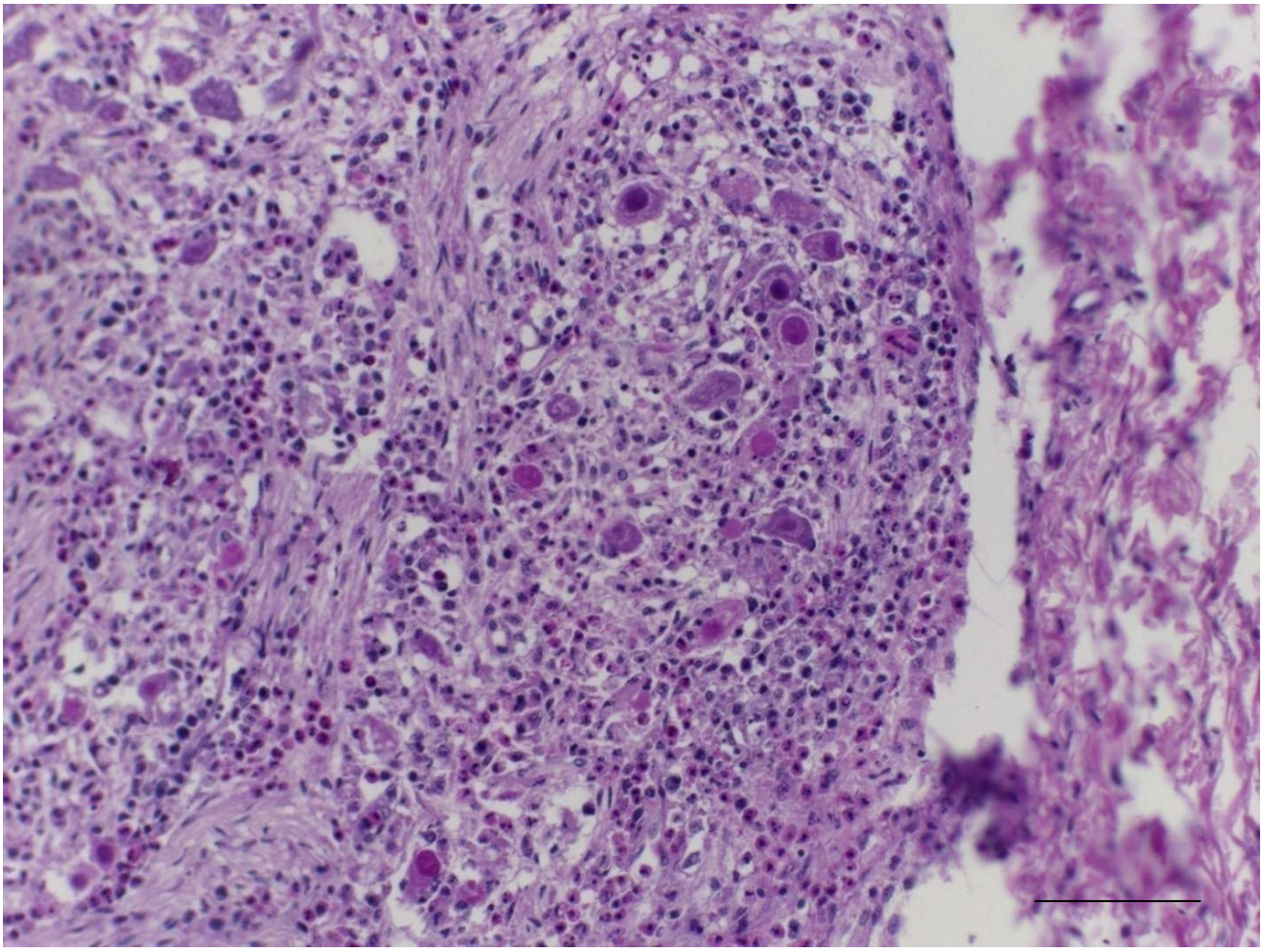

Plate 4.41. Peripheral neural ganglion-duodenum; Pekin duck 4 days p.i clade 2.2 HPAI. Diffuse neuronal necrosis and infiltration of mononuclear cells, mainly lymphocytes HE. Bar=100µm

4.4.2.4 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in Tracheal and Cloacal swabs

Except for the day 0 of infection, 1st, 2nd ,3rd and 4th day post infection (dpi), tracheal swabs for all Muscovy were very positive (high viral genome load) and highest mean RRT-PCR ct-value was at 4dpi (Fig 4.14). While highest mean cloacal RRT-PCR ct-value was at 2dpi (Fig 4.15). Tracheal swabs were more positive with high viral genome load. The tracheal swab RRT-PCR ct-values of Mallard ducks (Fig 4.16) at 3rd and 4th dpi were most positive (mean RRT-PCR ct-value ≤ 30). This also applies to the cloacal swab ct-values (Fig 4.17) as it was most positive at 3rd and 4th dpi (mean RRT-PCR ct-value ≤ 35). 4dpi tracheal swabs of Pekin ducks were most positive except for some individuals at 3dpi (Fig 4.18), having highest viral genome load compared to other days PI. While the cloacal swab ct-value were negative (Fig 4.19) on 0, 1, 6, 7, 8 dpi and most positive on 3dpi.

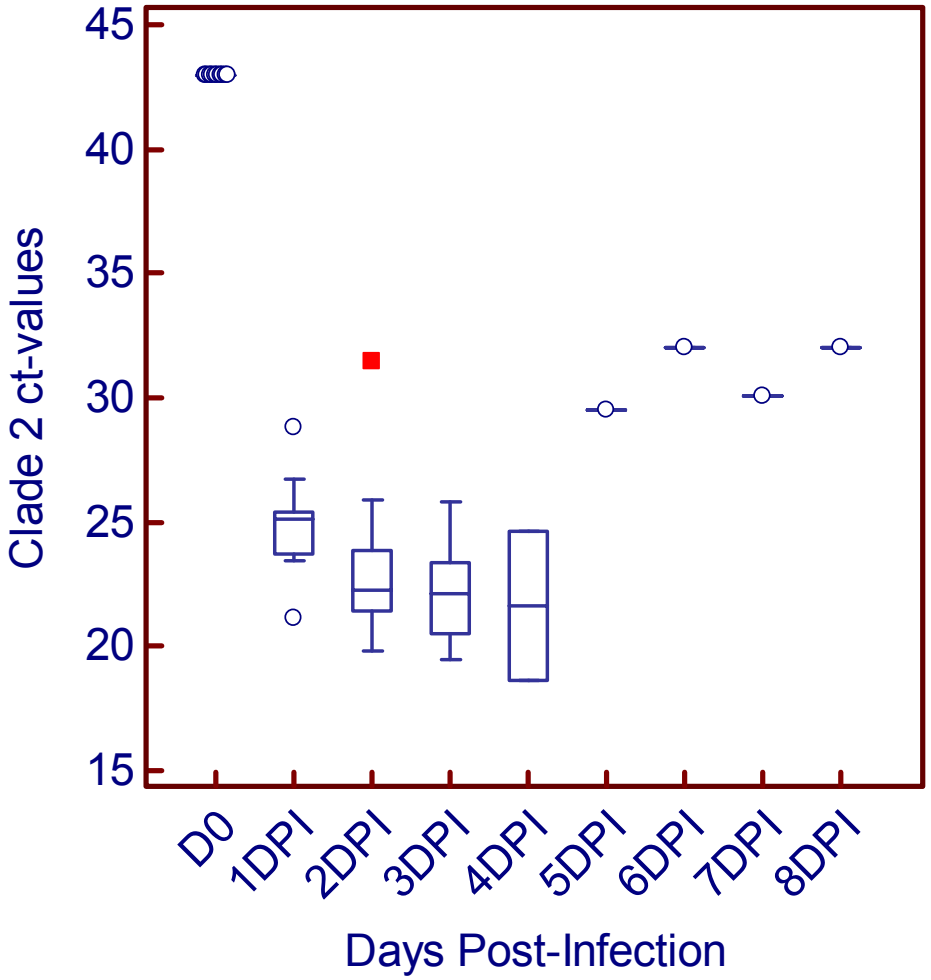

Figure 4.14. Clustered multiple variables graph: Muscovy Tracheal Swab ct-values of Clade 2.2

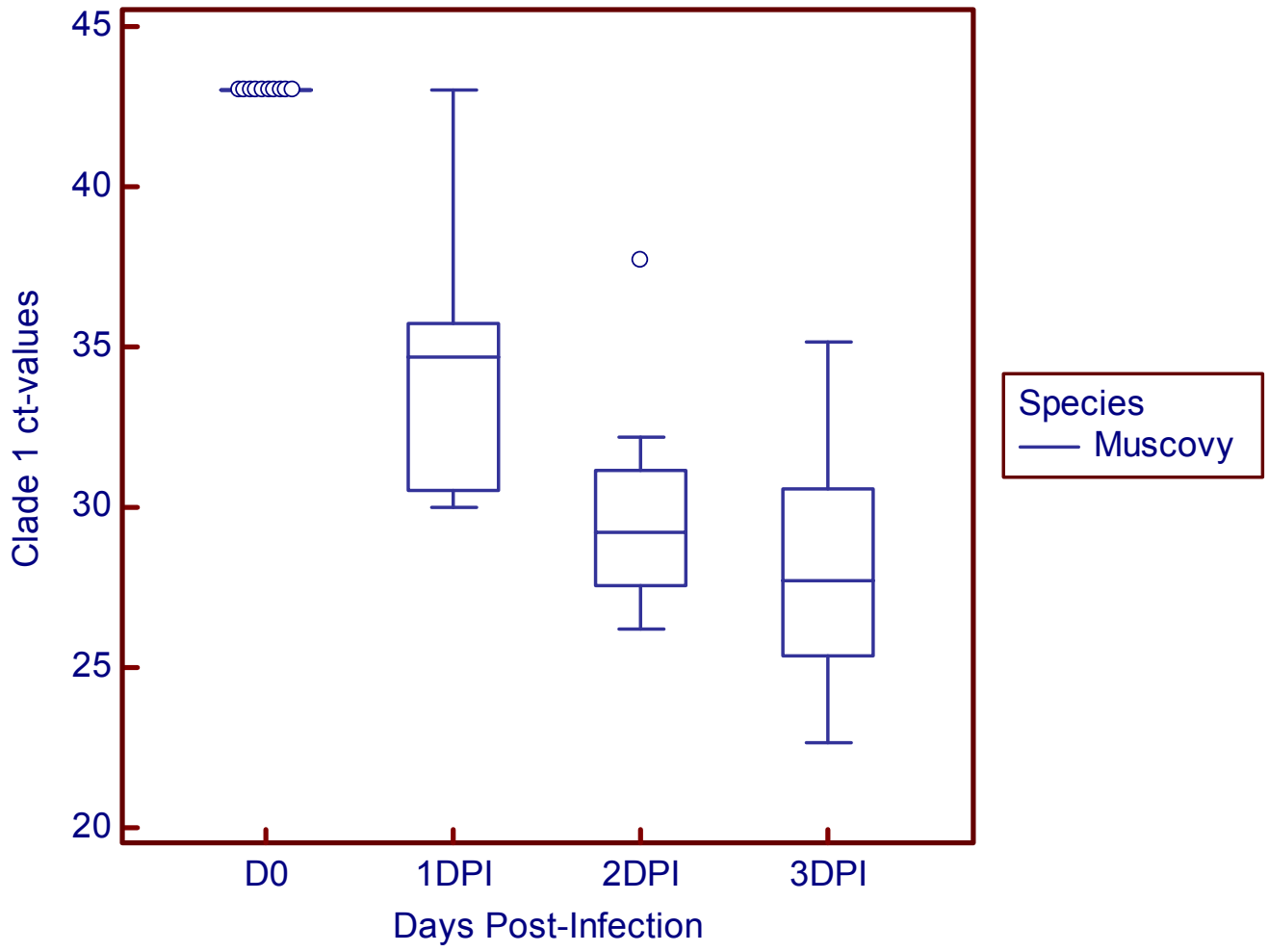

Figure 4.15. Clustered multiple variables graph: Muscovy Cloaca Swab ct-values of Clade 2

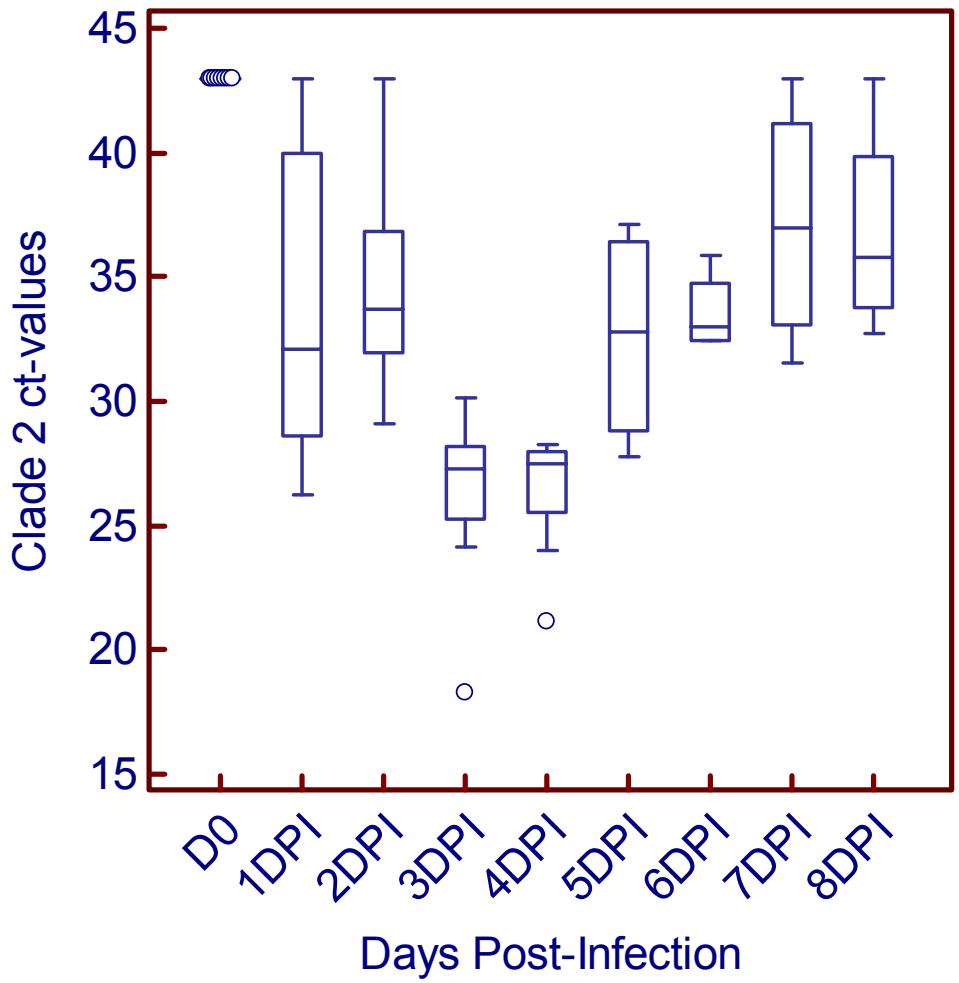

Figure 4.16. Clustered multiple variables graph: Mallard Tracheal Swab ct-values of Clade 2

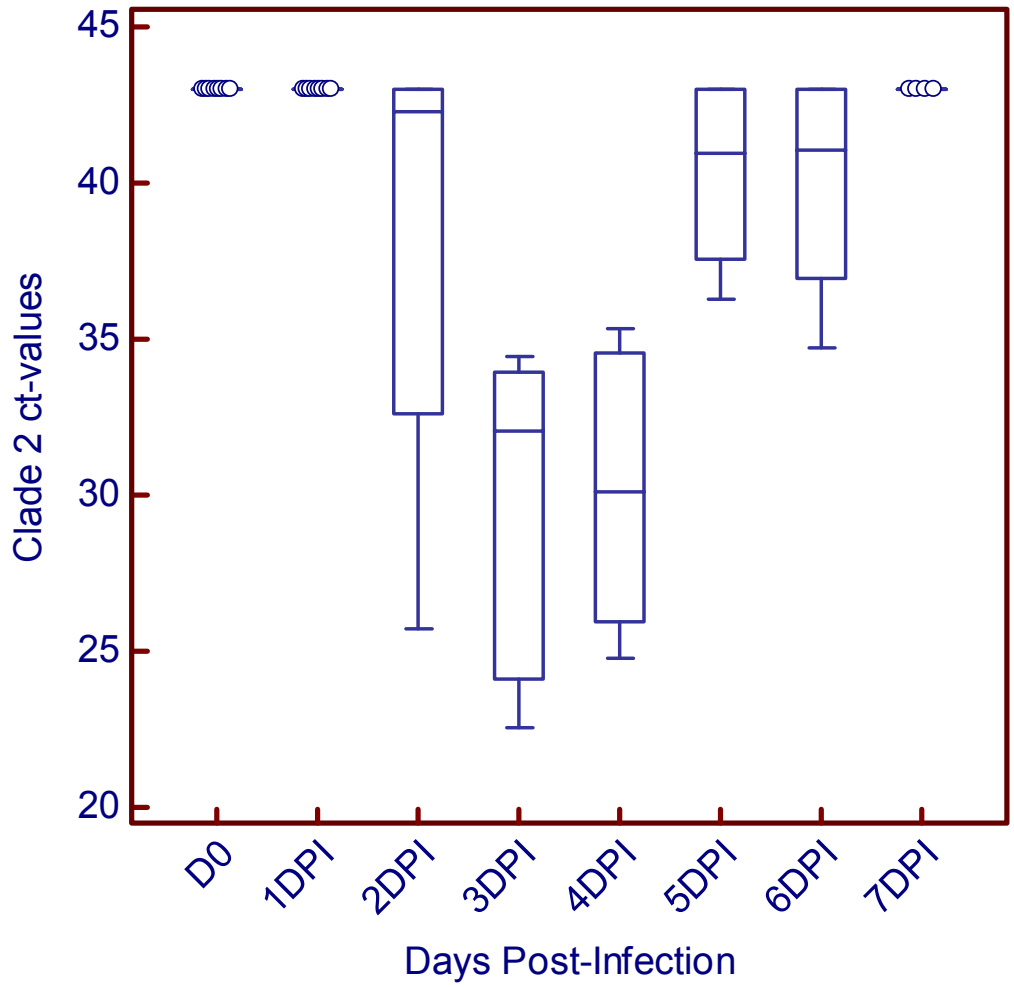

Figure 4.17. Clustered multiple variables graph: Mallard Cloaca Swab ct-values of Clade 2

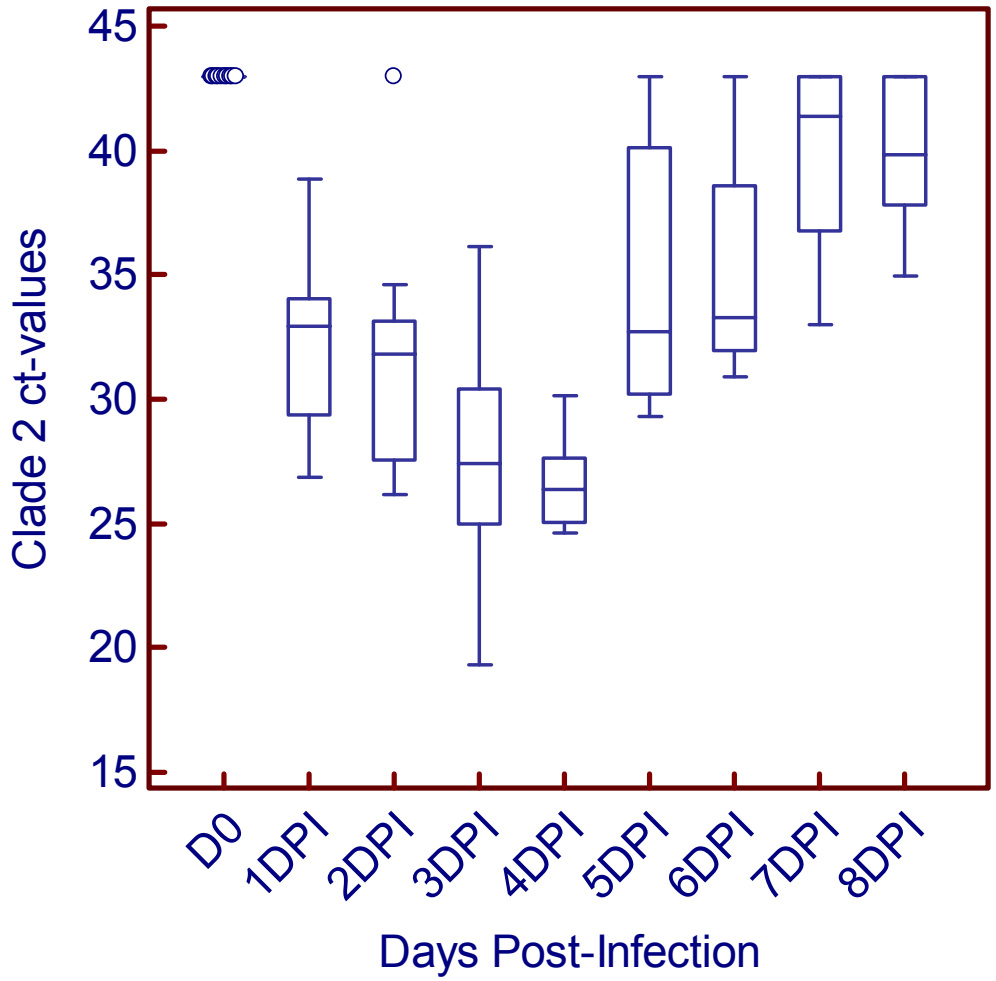

Figure 4.18. Clustered multiple variables graph: Pekin Tracheal Swab ct-values of Clade 2

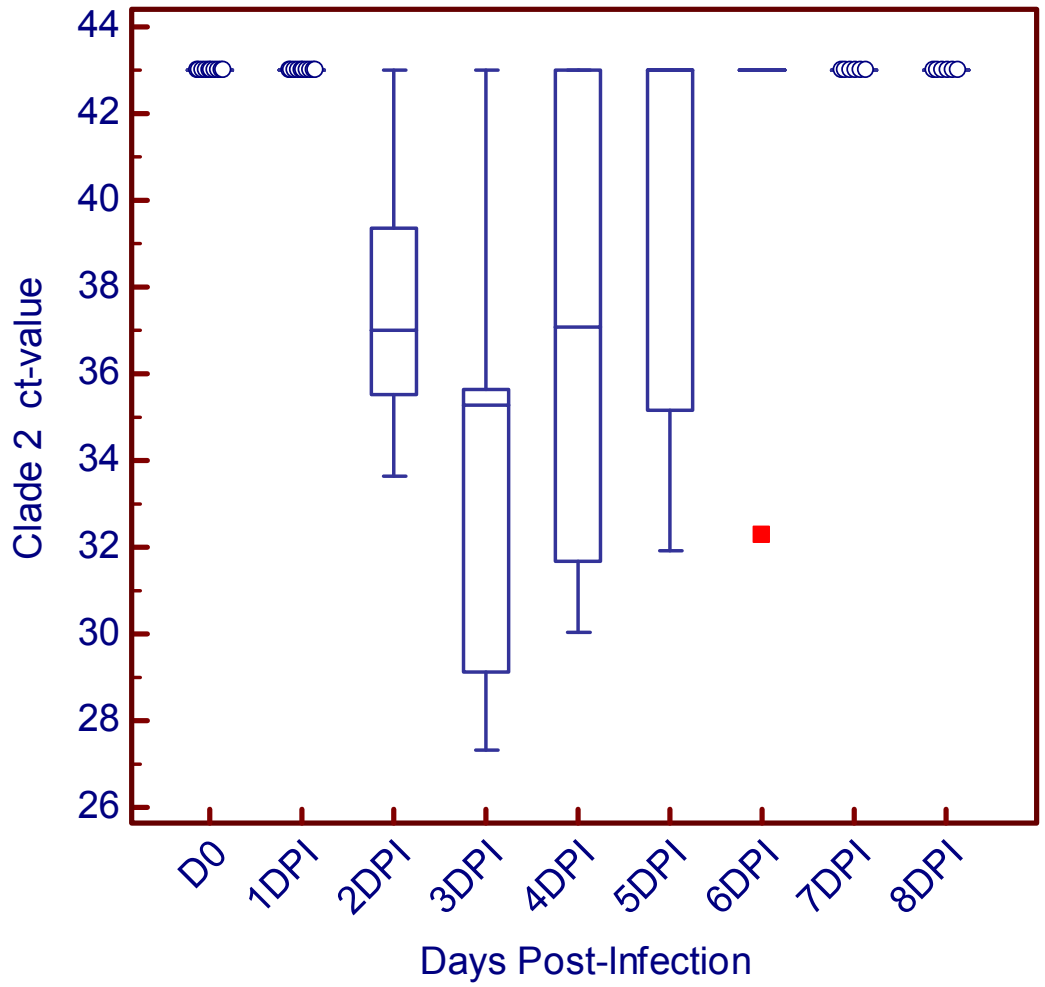

Figure 4.19. Clustered multiple variables graph: Pekin Cloaca Swab ct-values of Clade 2

4.4.2.5 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in tissues

Tissues of Muscovy duck examined at 3dpi were all positive for the virus; with cerebrum, muscle, air sac, nasal cavity, heart, caeca, ovaryand testis having very high ct-values detected by RRT-PCR (Fig 4.20). All tissues of Mallard ducks were positive except for a few pancreas and kidney at 4dpi, with air sac and lung been the most positive tissue samples. Others included cerebrum, nasal cavity, gizzard, ovary and testis. At 8dpi, cerebrum and air sac are still moderately positive (Fig 4.21). All tissues of Pekin ducks examined at 4dpi were positive except for gizzard in some ducks. Nasal cavity, heart and spleen were most positive. Other very positive tissues included air sac, kidney, ovary, testis and muscle. The RRT-PCR ct-value of Pekin duck examined at 8dpi varied widely (Fig 4.22) but nasal cavity and air sac were still moderately positive.

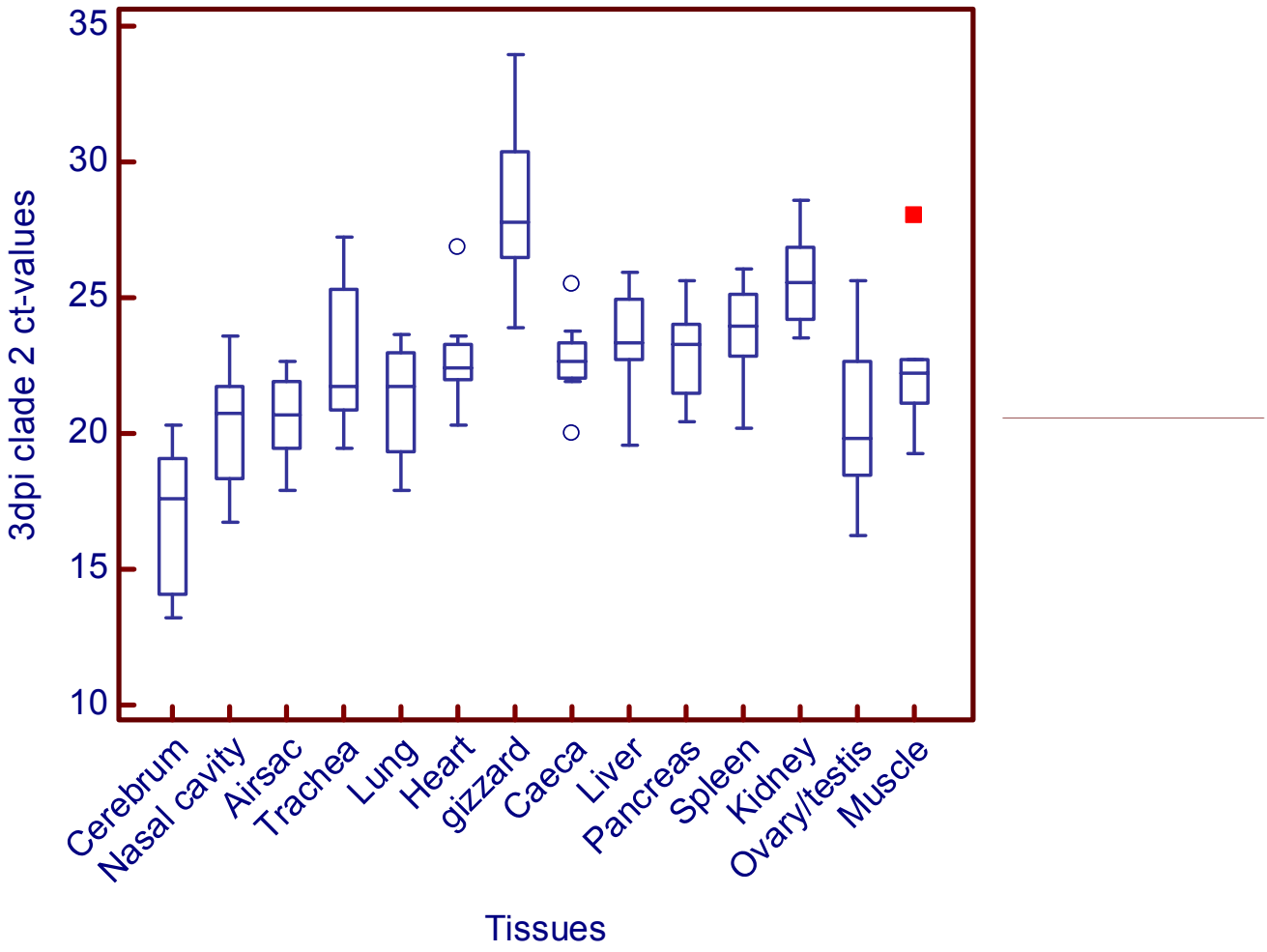

Figure 4.20. Clustered multiple variables graph: Muscovy Tissue ct-values of Clade 2 3DPI

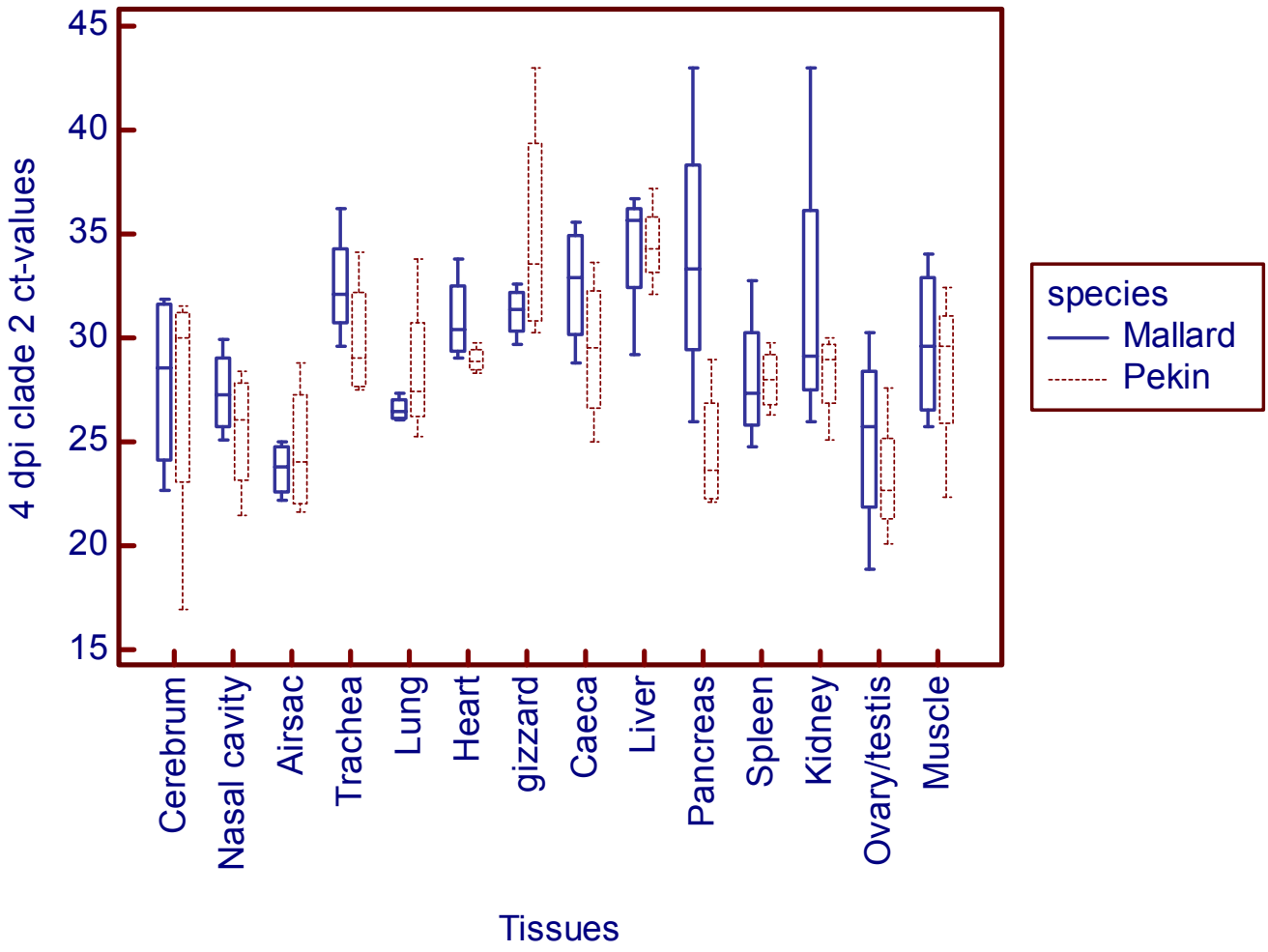

Figure 4.21 Clustered multiple variables graph: Mallard and Pekin Tissue ct-values of Clade 2 4DPI

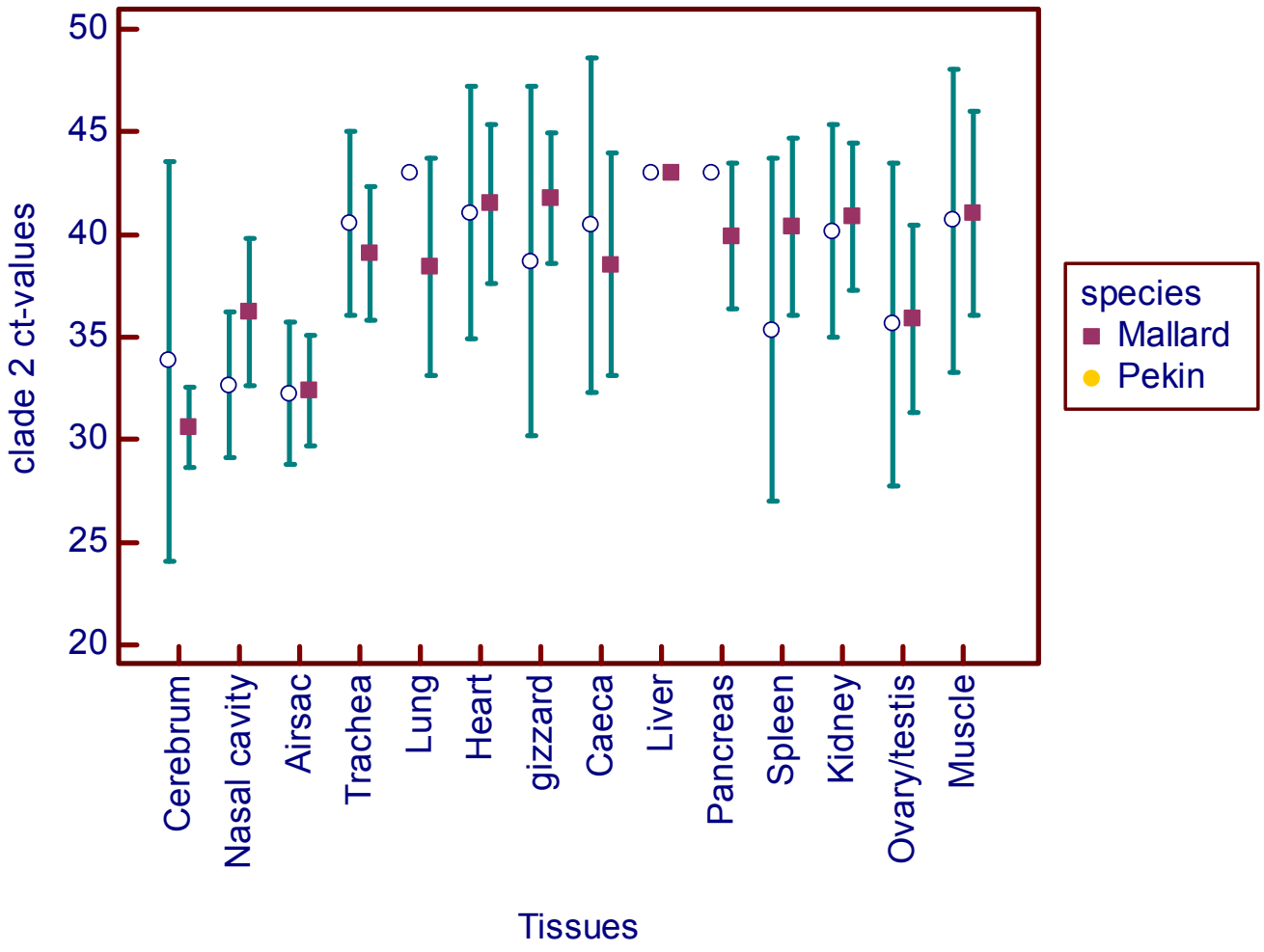

Figure 4.22. Clustered multiple variables graph: Mallard and Pekin Tissue ct-values of Clade 2 8DPI

4.4.3 COMPARATIVE ANALYSIS OF TISSUE, TRACHEAL AND CLOACAL SWABS OF *Influenza A/duck/Vietnam/TG24-01/05* (clade 1) and *Influenza A/Cygnus cygnus/Germany/R65/2006* (clade 2.2) H5N1 VIRUS INFECTION IN PEKIN, MUSCOVY AND MALLARD DUCKS.

4.4.3.1 Tracheal and Cloacal Swabs Analysis 1st Day Post-Infection

When the mean tracheal and cloacal ct-values were compared between the duck species, i.e Muscovy, Mallard and Pekin in the two virus groups at 1 d.p.i, by a pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011. It was evident at 1 d.p.i, there was a significant difference (Bonferroni corrected $P<0.0001$) in the mean tracheal ct-values between the Muscovy ducks compared to Mallard ducks, and between Muscovy ducks compared to Pekin ducks, put together irrespective of virus type. Also, there was a significant difference (Bonferroni corrected $P<0.0001$) in the mean cloacal swab ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 compared with infection with clade 2.2, but there was no significant difference (Bonferroni corrected $P=0.196$) in the mean cloacal ct-values between the Muscovy ducks compaired to Mallard ducks, and between Muscovy ducks compared to Pekin ducks 1 d.p.i. The graphs below showed that the 1dpi tracheal RRT-PCR ct-values for Muscovy ducks were the most positive in both clade 1 and 2.2 viruses and that clade 1 infected Muscovy ducks had the most viral genome load in this experiment. This implies that apart from the high susceptibility of Muscovy ducks to the H5N1virus, clade 1 was more pathogenic and hence replicated more in Muscovy ducks than clade 2.2 (Figs 4.23 and 4.24).

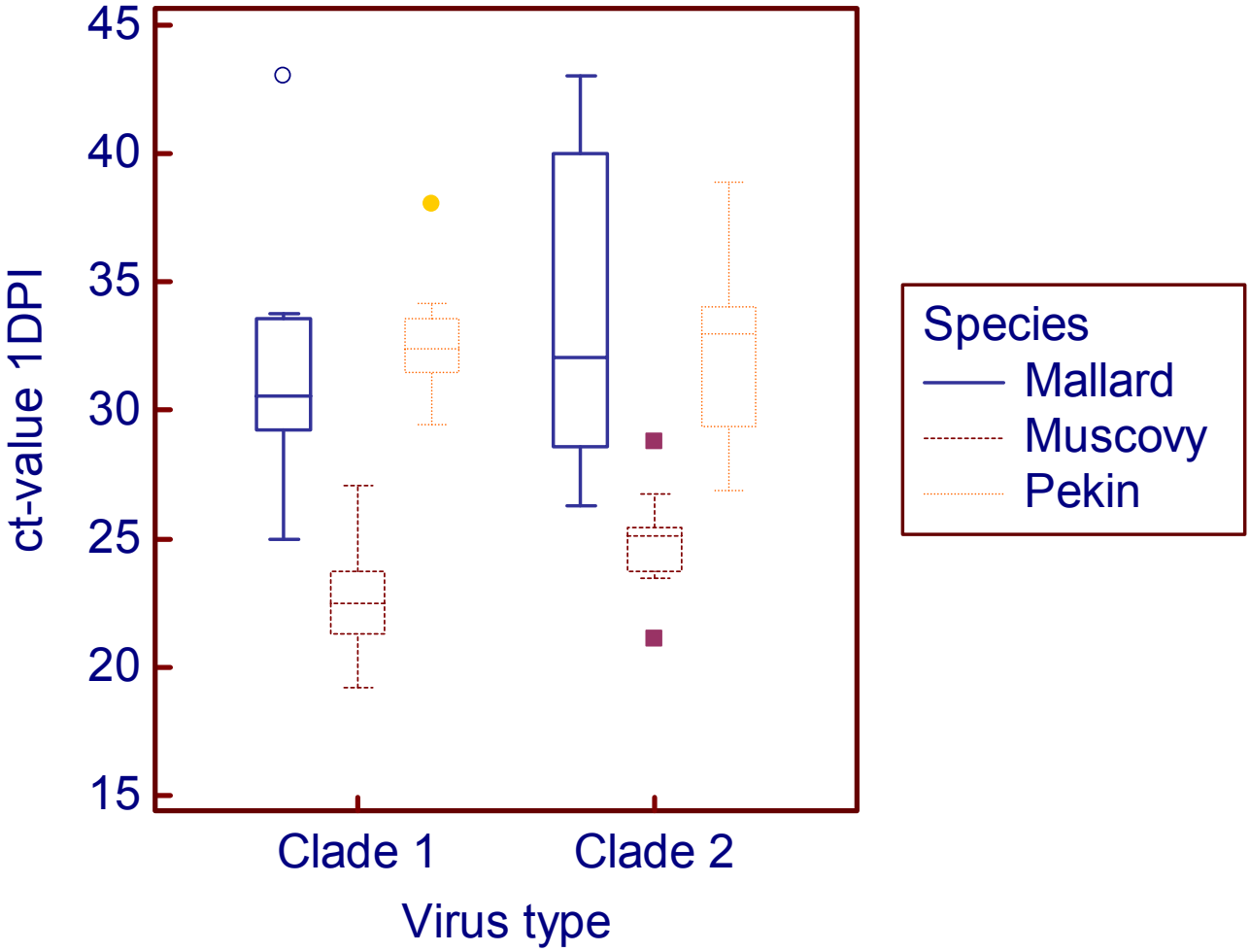

Figure 4.23 Clustered multiple variables graph: Tracheal Swab ct-values of the three types of ducks infected with Clade 1 and 2 1DPI

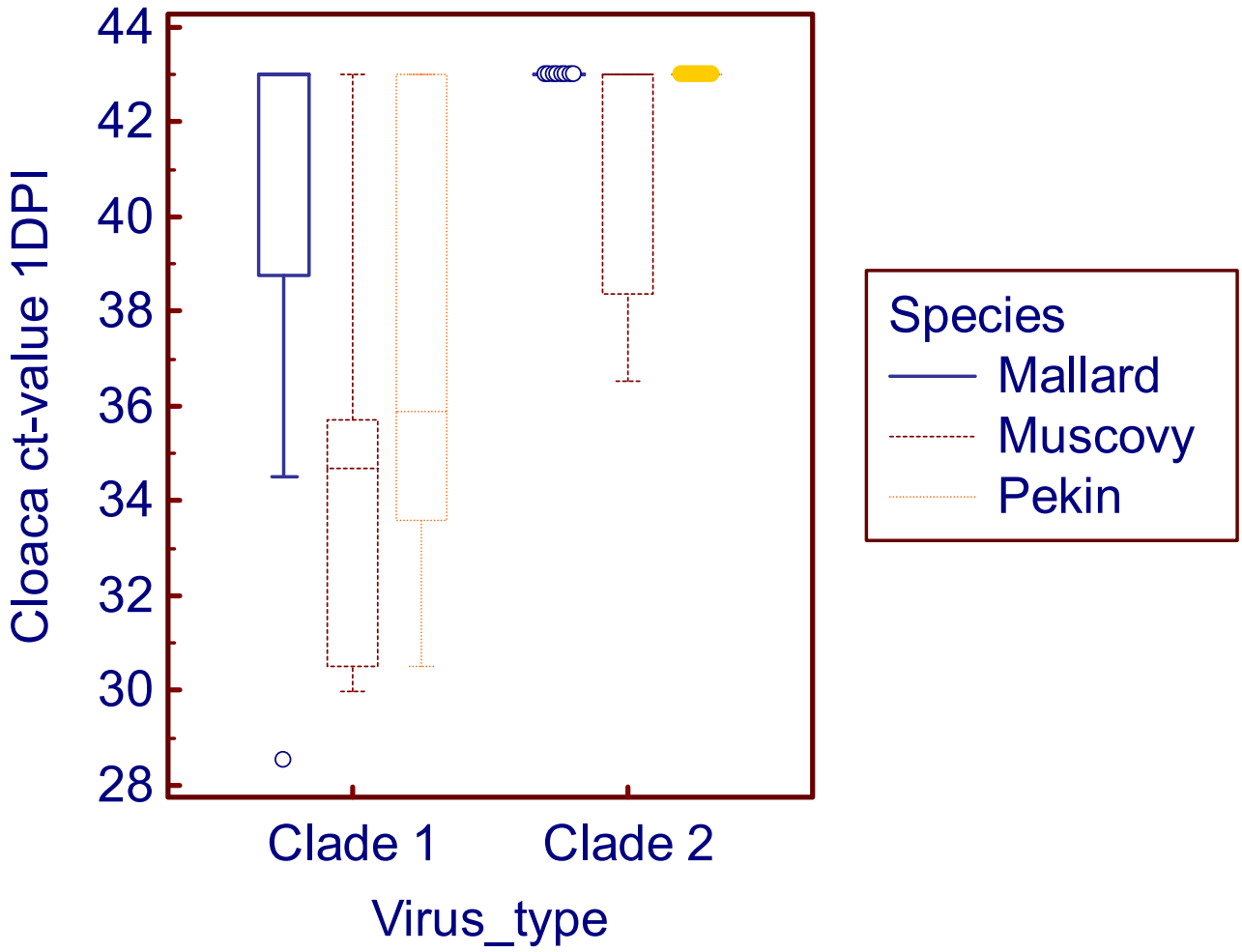

Figure 4.24. Clustered multiple variables graph: Cloaca Swab ct-values of the three types of ducks infected with Clade 1 and 2 1DPI

4.4.3.2 Tracheal and Cloacal Swabs Analysis 2nd Day Post-Infection

A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, showed that at 2 d.p.i, there was significant difference (Bonferroni corrected P=0.0002) in the mean tracheal ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 compared with infecting ducks with clade 2.2. There was a significant difference (Bonferroni corrected P<0.0001) in the mean tracheal ct-values between the Muscovy ducks compaired to Mallard ducks, and between Muscovy ducks compared to Pekin ducks (Bonferroni corrected P<0.0001), put together irrespective of virus type. There was a significant difference (Bonferroni corrected P=0.036) in the mean cloacal ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 compared with infection with clade 2.2, and there was also a significant difference (Bonferroni corrected P<0.0001) in the mean cloacal ct-values between the Muscovy ducks compared to Mallard ducks and between Muscovy ducks compared to Pekin ducks (Bonferroni corrected P<0.01) 2 d.p.i. The graphs below showed that the 2dpi tracheal RRT-PCR ct-values for Muscovy ducks were the most positive in both clade 1 and 2.2 viruses and that clade 1 infected Muscovy ducks had the most viral genome load in this experiment. This implies that apart from the high susceptibility of Muscovy ducks to the H5N1virus, clade 1 was more pathogenic and hence replicated more in Muscovy ducks than clade 2.2 (Figs 4.25 and 4.26).

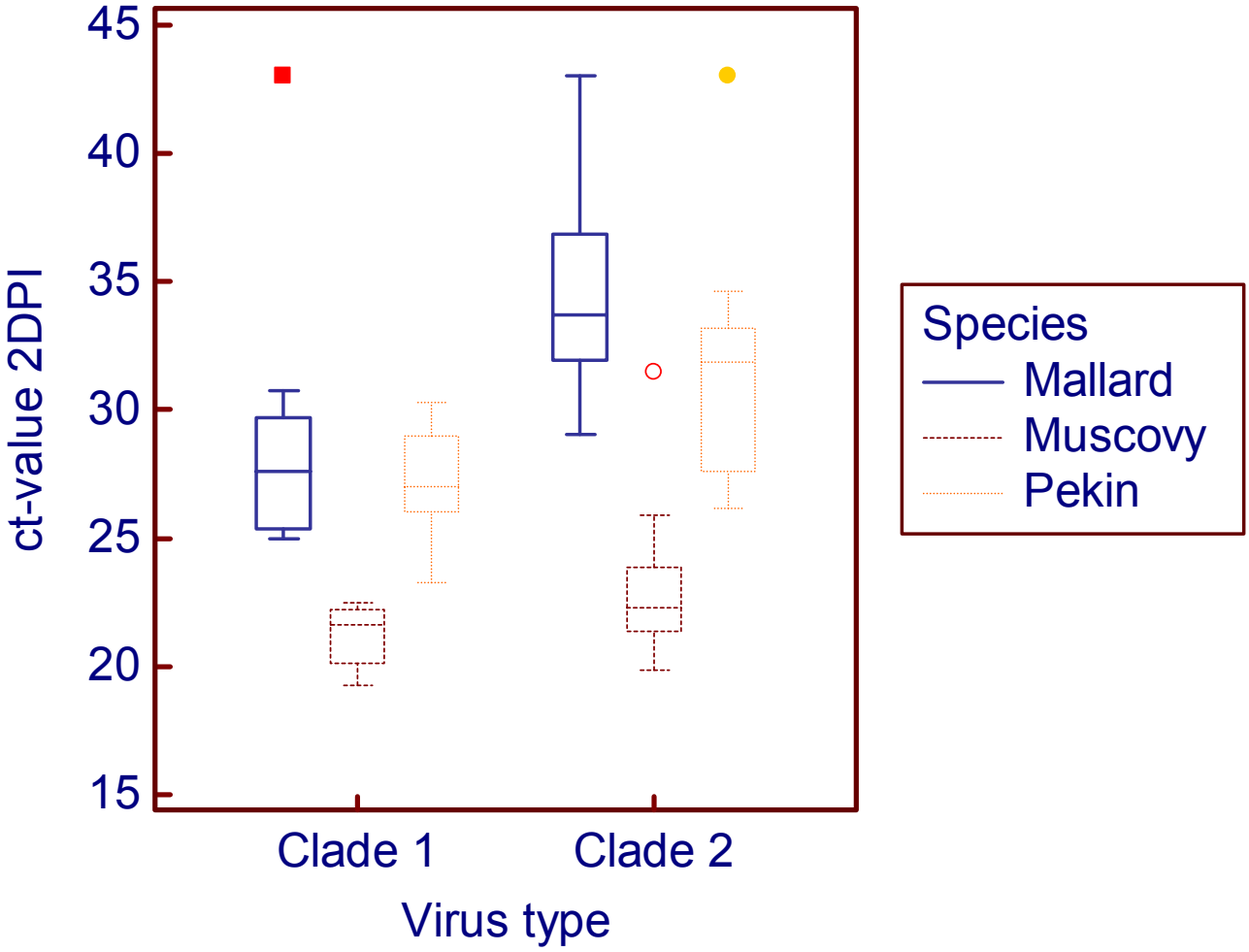

Figure 4.25. Clustered multiple variables graph: Trachea Swab ct-values of the three types of ducks infected with Clade 1 and 2.2, 2DPI

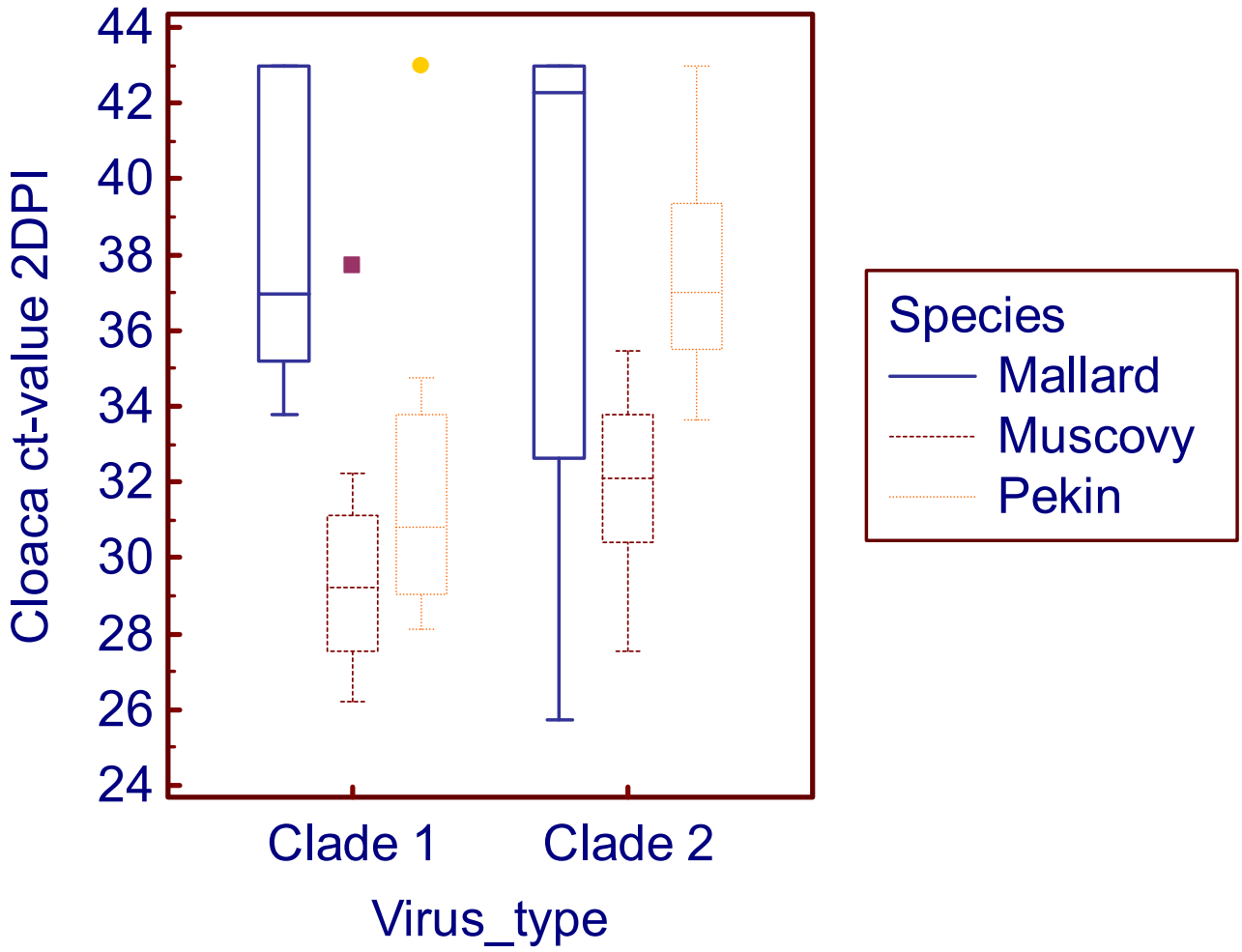

Figure 4.26 Clustered multiple variables graph: Cloaca Swab ct-values of the three types of ducks infected with Clade 1 and 2 2DPI

4.4.3.3 Tracheal and Cloacal Swabs Analysis 3rd Day Post-Infection

A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, showed that at 3 d.p.i, there was no significant difference (Bonferroni corrected P=0.21) in the mean tracheal ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 compared with infecting ducks with clade 2.2.There was a significant difference (Bonferroni corrected P=0.0002) in the mean tracheal ct-values between the Muscovy ducks compaired to Mallard ducks, and between Muscovy ducks compared to Pekin ducks (Bonferroni corrected P=0.0001), put together irrespective of virus type (Fig 4.27). There was no significant difference (Bonferroni corrected P=0.21) in the mean cloacal ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 or clade 2.2. But there was a significant difference (Bonferroni corrected P<0.0016) in the mean cloacal ct-values between the Muscovy ducks compared to Mallard ducks and between Muscovy ducks compared to Pekin ducks (Bonferroni corrected P<0.002) 3 d.p.i. The graphs below showed that the 3dpi tracheal RRT-PCR ct-values for Muscovy ducks were the most positive in both clade 1 and 2.2 viruses and that clade 1 infected Muscovy ducks had the most viral genome load in this experiment. This implies that apart from the high susceptibility of Muscovy ducks to these H5N1virus, clade 1 was more pathogenic and hence replicated successfully in Muscovy ducks than clade 2.2 (Figs 4.27 and 4.28).

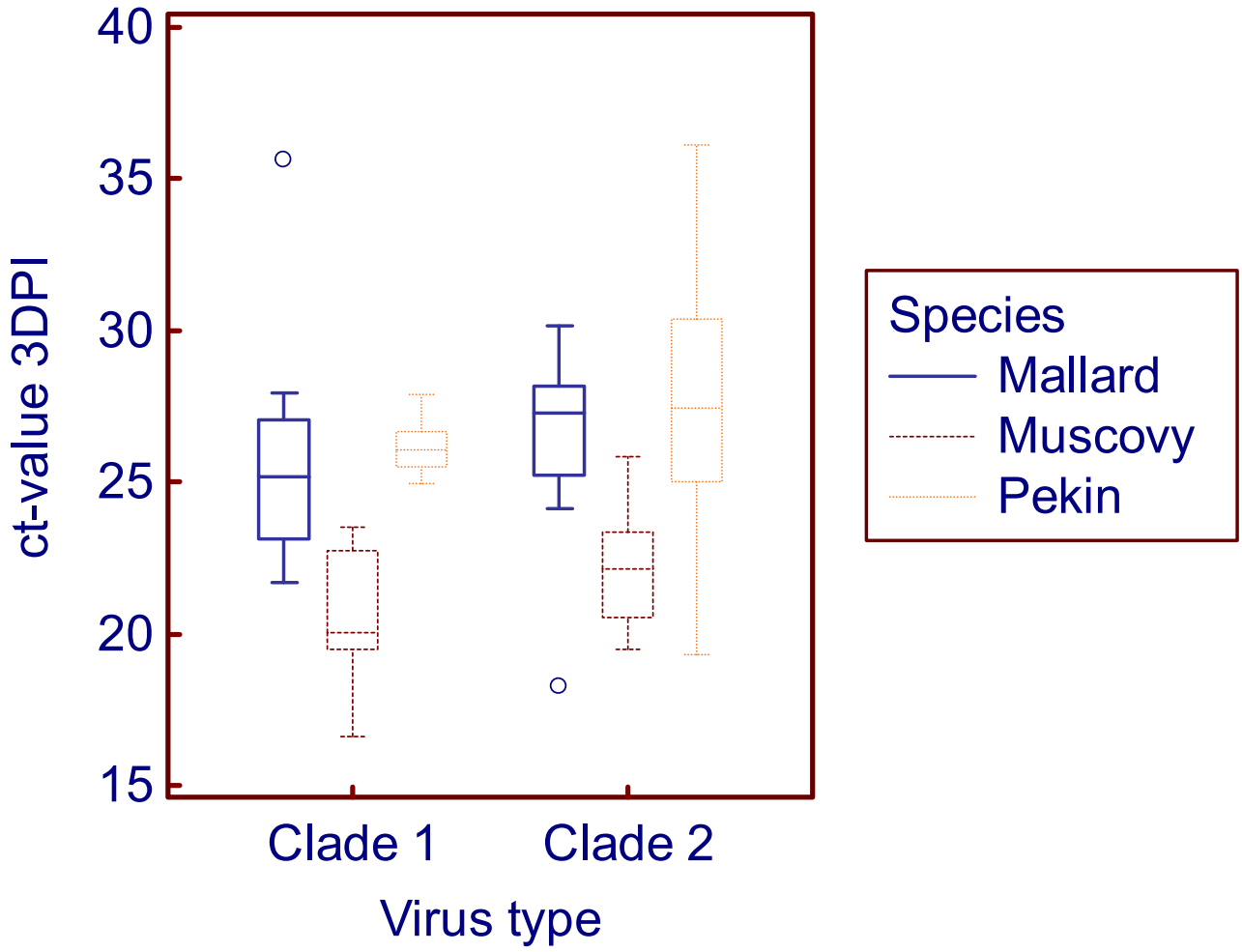

Figure 4.27. Clustered multiple variables graph: Trachea Swab ct-values of the three types of ducks infected with Clade 1 and 2 3DPI

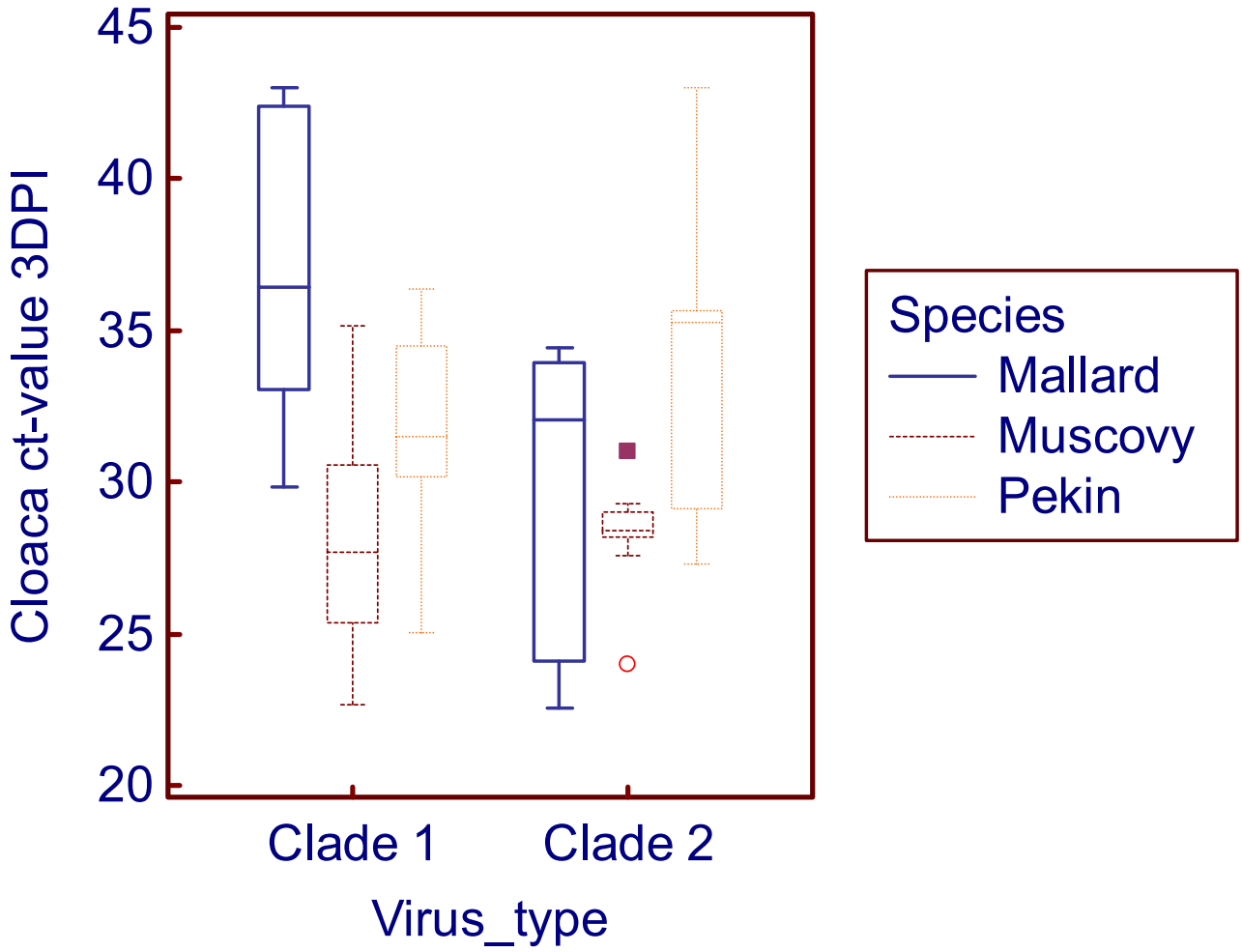

Figure 4.28 Clustered multiple variables graph: Cloaca Swab ct-values of the three types of ducks infected with Clade 1 and 2 3DPI

4.4.3.4 Tracheal and Cloacal Swabs Analysis 4th, 5th, 6th, 7th and 8th Day Post-Infection

In the Mallard and Pekin ducks remaining at 4th, 5th, 6th and 8th dpi, mean tracheal and cloacal swab ct-values for all ducks in both virus groups varied from weakly positivity cloacal swabs, to negative cloacal swabs. It is worth knowing that from 6th and 8th d.p.i, in clade 2 virus group, cloaca swabs were negative in Mallard and Pekin ducks. Also on 8th d.p.i cloaca swabs were negative in the remaining Mallard and Pekin ducks in the clade 1 and clade 2.2 infected groups. A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, showed that there is no significant difference (Bonferroni corrected $P>0.05$) in the mean tracheal and cloacal ct-values between infecting ducks (Muscovy, Mallard and Pekin put together for each virus type respectively) with clade 1 compared with infecting ducks with clade 2.2, and also there was no significant difference (Bonferroni corrected $P>0.05$) in the mean tracheal and cloacal ct-values between the Muscovy ducks compaired to Mallard ducks, and between Muscovy ducks compared to Pekin ducks, put together irrespective of virus type.

4.4.3.5 Tissue Analysis for Muscovy ducks 3 d.p.i

At 3 d.p.i for the two viruses, clade 1 and clade 2.2, all tissues (cerebrum, nasal cavity, airsac, trachea, lung, heart, gizzard, caeca, liver, pancreas, spleen, kidney, ovary/testis and muscle), were moderately to strongly positive by real time RRT-PCR with mean ct-value range 16-28 (Fig 4.29). In clade 1 group, H5N1 viral genome load detection by RRT-PCR was very high (mean ct-value ≤ 20) in the cerebrum, nasal cavity, trachea, lung, heart, spleen, ovary/testis and muscle. While in clade 2.2 group, H5N1 viral genome load detection by RRT-PCR was moderate to very high (mean ct- value ≤ 21) in the cerebrum, nasal cavity, airsac, lung and ovary/testis. The graph below summarizes the findings.

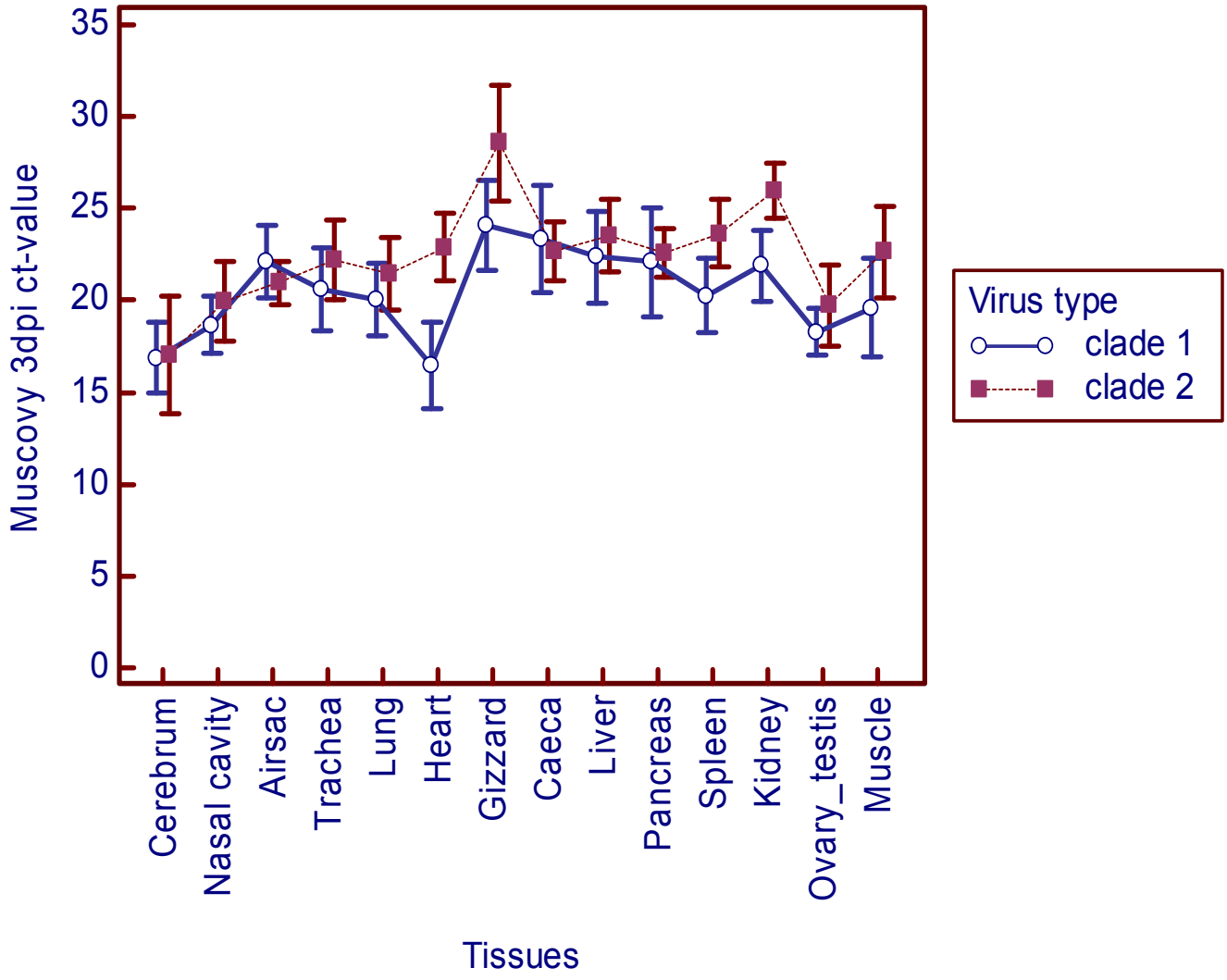

Figure 4.29. Clustered multiple variables graph: Muscovy ducks tissue RRT-PCR ct-values of Clade 1 and 2 3DPI

4.4.3.6 Tissue Analysis for Pekin and Mallard ducks 4 d.p.i

At 4 d.p.i for the two viruses, clade 1 and clade 2.2, all tissues (cerebrum, nasal cavity, airsac, trachea, lung, heart, gizzard, caeca, liver, pancreas, spleen, kidney, ovary/testis and muscle), were mildly to moderately positive by real time RT-PCR with mean ct-value range 21-35 in Pekin ducks (Fig 4.30) and 20-34 in Mallard ducks (Fig.4. 31). In clade 1 group, H5N1 viral genome load detection by RT-PCR was moderately high (mean ct- value $\leq$ 23) in the cerebrum, nasal cavity, airsac, heart and ovary/testis in Mallard ducks and in the cerebrum, nasal cavity, airsac, heart, pancreas and ovary/testis in Pekin ducks (Fig 4.30) . While in clade 2.2 group, H5N1 viral genome load detection by RT-PCR was moderate to mildly positive (mean ct- value $\leq$ 27) in the cerebrum, nasal cavity, airsac, lung and ovary/testis in Mallard ducks and in the cerebrum, nasal cavity, airsac and ovary/testis of Pekin ducks.

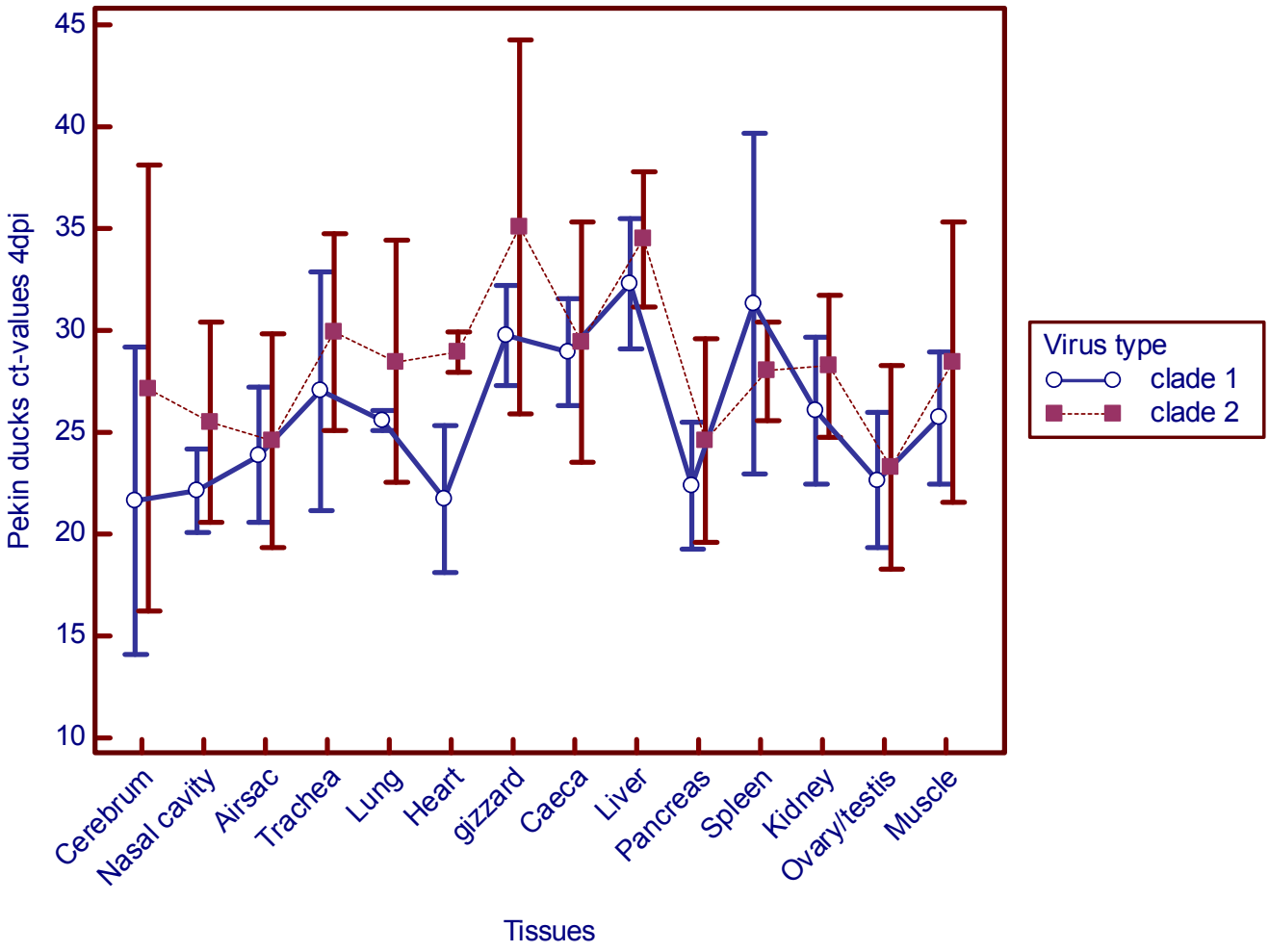

Figure 4.30. Clustered multiple variables graph: Pekin duck tissue ct-values of Clade 1 and 2 4DPI

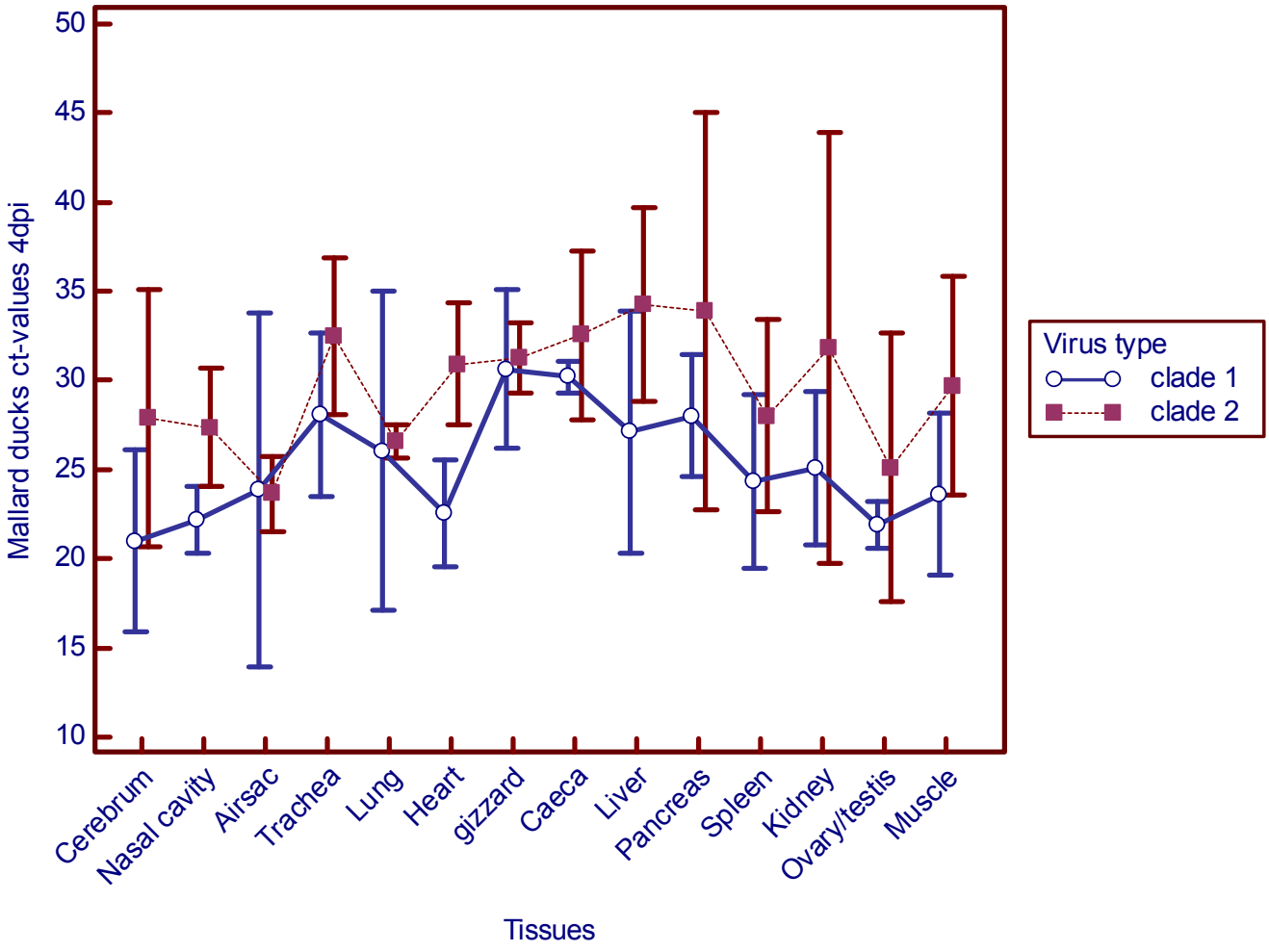

Figure 4.31. Clustered multiple variables graph: Mallard duck tissue ct-values of Clade 1 and 2 4DPI

4.4.3.7 Tissue Analysis for Mallard and Pekin ducks 8 d.p.i

At 8 d.p.i, (with only 13 ducks left: In clade 1, 2 Mallard ducks; and in clade 2, 6 Pekin, 4 Mallard and 1 Muscovy) for the two viruses, clade 1 and clade 2.2, all tissues (cerebrum, nasal cavity, airsac, trachea, lung, heart, gizzard, caeca, liver, pancreas, spleen, kidney, ovary/testis and muscle), were mildly positive and a lot were negative by real time RT-PCR (Fig 4.32). In clade 1 group, H5N1 viral genome load detection by RT-PCR was moderately high (mean ct- value ≤ 25) in the cerebrum and ovary/testis in Mallard ducks and weak in the cerebrum, nasal cavity, airsac, spleen and ovary/testis in the Mallard ducks of clade 2, mean ct- value ≤ 35. In Pekin ducks in the clade 2 group at 8 d.p.i, only the cerebrum, Airsac and Ovary/Testis had mean ct-values of ≤ 35.

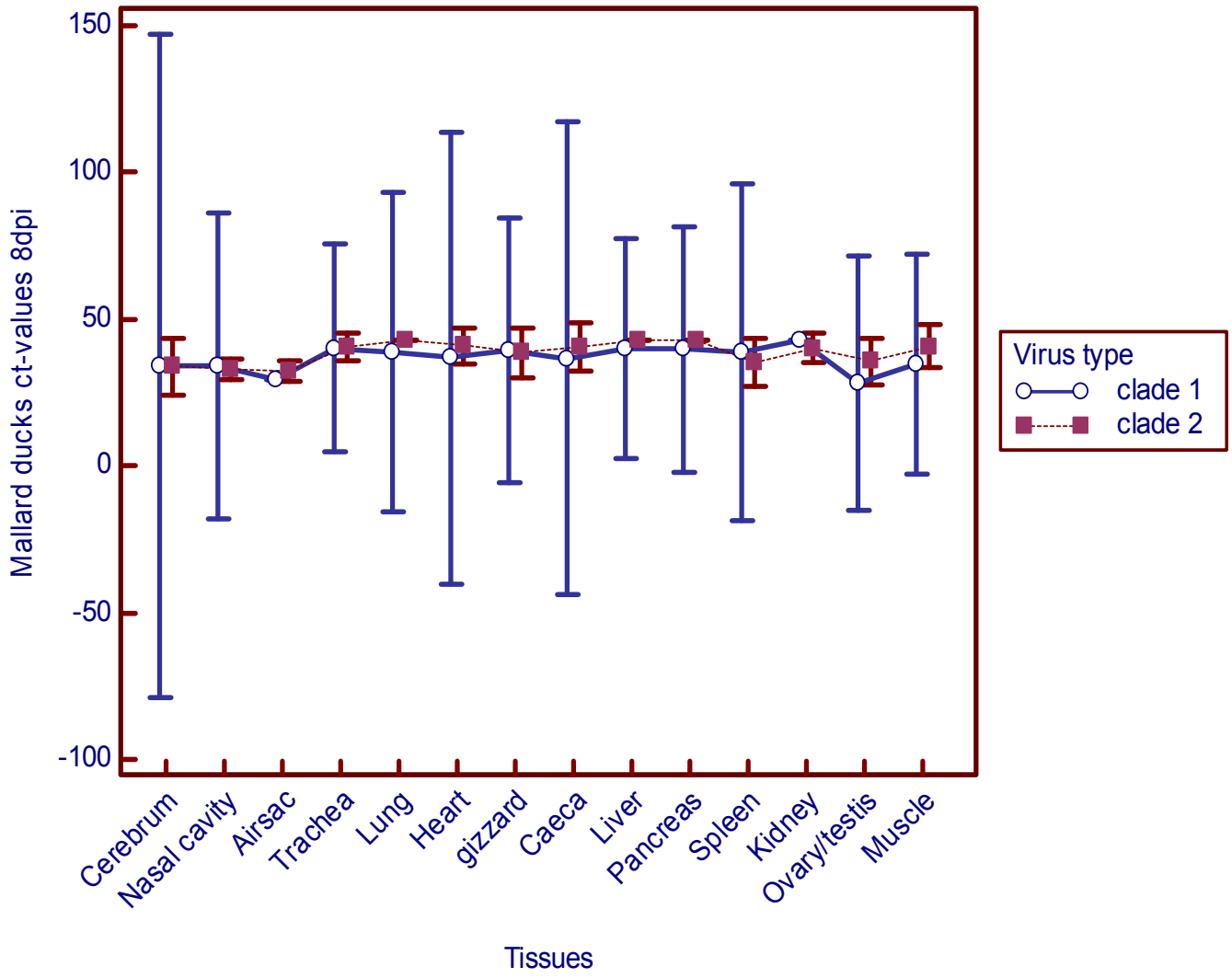

Figure 4.32. Clustered multiple variables graph: Mallard duck tissue ct-values of Clade 1 and 2 8DPI

4.4.3.8 Comparative Tissue Virus Isolation and RRT-PCR for Muscovy, Mallard and Pekin ducks

Clade 1and Clade 2.2 virus re- isolation in 10-day-old embryonated eggs done on one gram each of the brain, lung, and intestine (terminal colon and cloaca) of infected ducks and the viral genome load detection by RRT-PCR, showed that virus isolation and genome detection were highest and most successful in Muscovy ducks in both virus groups (Fig 4.33). It was very successful and higher in Pekin and Mallard ducks infected with clade 1 virus and was least successful in clade 2.2 infected Pekin and Mallard ducks. Log of tissue virus isolation and RRT-PCR for Muscovy, Mallard and Pekin (Fig. 4.33), showed that, though viral genome detection were higher, virus isolation was more successful in Muscovy ducks than in Pekin and Mallard ducks which had low virus titres in the tissues.

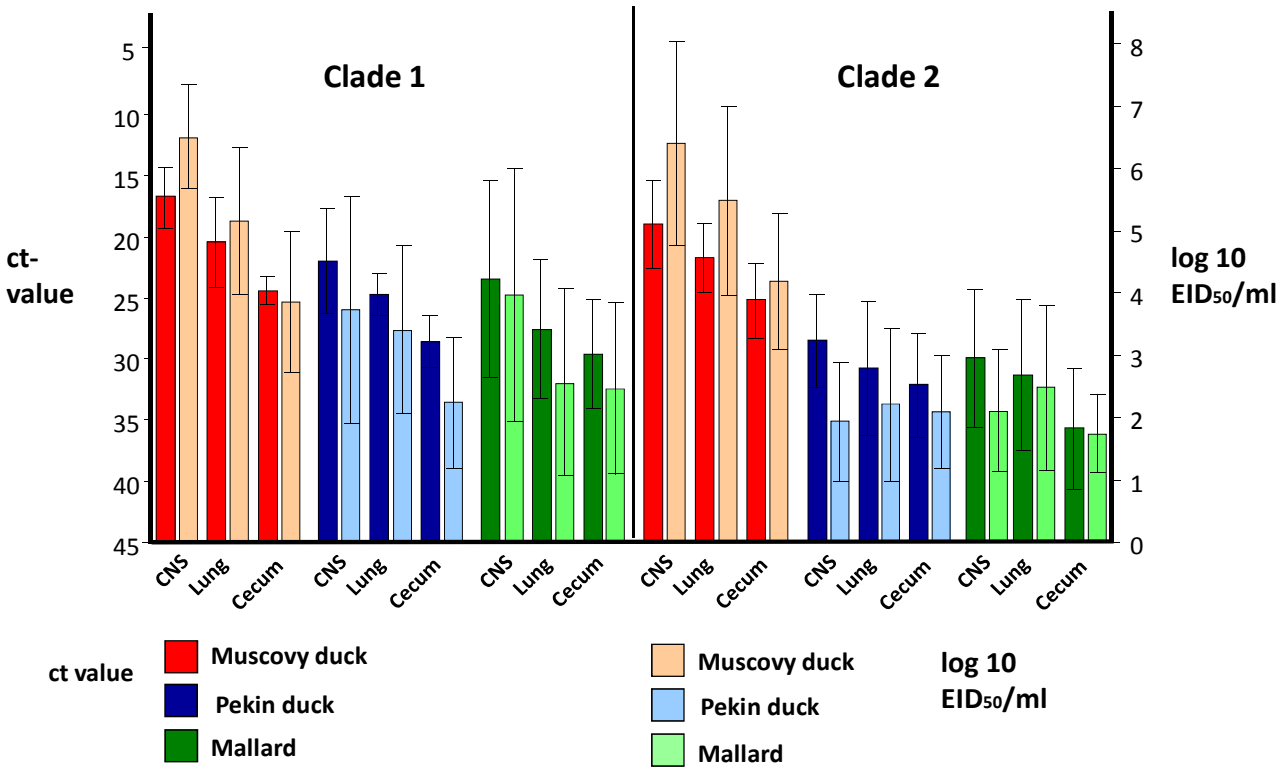

Figure 4.33. Comparison of RRT-PCR HPAI viral genome load and virus isolation in Muscovy, Mallard and Pekin

4.4.4 *Influenza A/duck/Vietnam/TG24-01/05* of clade 1 subtype H5N1 virus exposure in Chickens

4.4.4.1Clinical signs

Up to 100% morbidity and mortality was seen starting at 2days post- exposure (d.p.e) until 5d.p.e in the in-contact 3- weeks old white leghorn Lohmann breed domestic chickens (Galus domesticus) exposed to HPAI A/duck/Vietnam/TG24-01/05 of clade 1 subtype H5N1 virus infected Pekin, Mallard and Muscovy ducks.

4.4.4.2 Gross Pathology

Gross lesions were few and only evident in chickens that died 4^{th} and 5^{th} d.p.e, and these are mainly associated with the cardiovascular-myocardial petechial to ecchymotic haemorrhages and gastrointestinal (yolksac) systems,

4.4.4.3 Histopathology and Immunohistochemistry

while histopathologic lesions were consistent in all chickens and mainly in the comb, keratinocytes necrosis, cerebrum, cerebellum and mid brain gliosis, thymic, splenic and Bursaof Fabricius necrosis with additional lymphoid depletion in the Bursa of Fabricius and spleen. Tracheal and atria epithelia necrosis, while the GIT lesion was mainly villi and crypt necrosis in duodenum, jejunum, ileum, caeca and rectum. The Liver, pancreas and kidney were not spared as hepatocytes, acinar cells and tubular epithelium were necrotic. Vessels of the comb showed heterophilic vasculitis and cardiomyocytes and mucosal epithelium of the proventriculus were necrotic with ulceration in one proventriculus. Immunoreativity was seen in all the cells mentioned and in addition, haderian gland of the eye and peripheral nerve ganglia in the ileum. At 4days p.e chicken, immunoreactivity was also seen in the interstitium of the seminiferous tubules, endothelia cells in the ileum and caeca blood vessels and in the duodenal and jejuna enterocytes. In the 5DPE chicken (Mallard group), feather follicular necrosis, and parabronchial epithelia necrosis was seen and additional immunoractivity in the esophageal and jejunal intramural ganglia. The following plates 4.42- 4.53 shows the lesions encountered in these chickens.

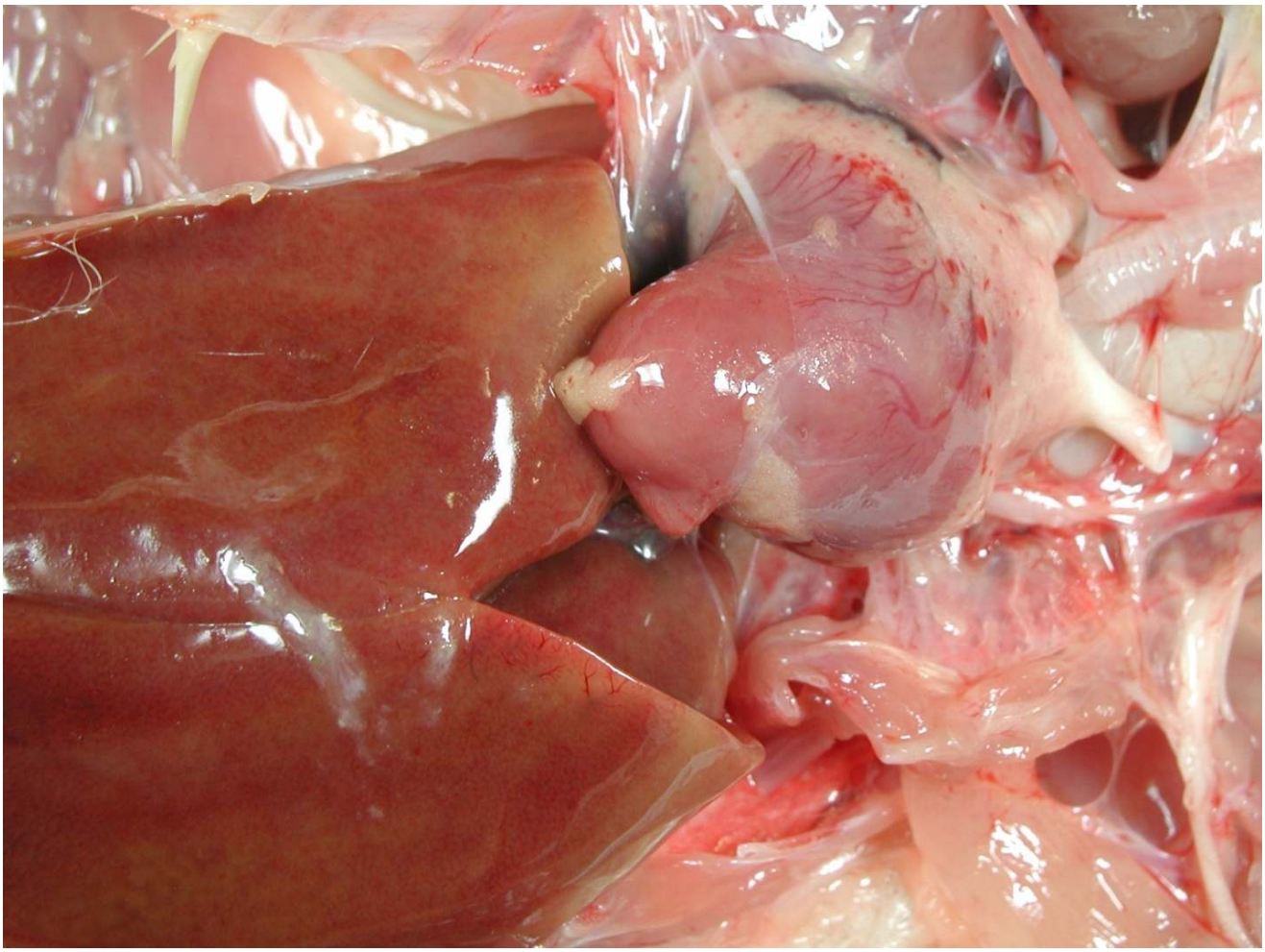

Plate 4.42. A. Heart: Chicken, experimental exposure, 4 days p.e clade 1 HPAI. Diffuse myocardial petechial to ecchymotic hemorrhages with cloudy pericardium. B. Liver: hepatic lipidosis diffuse.

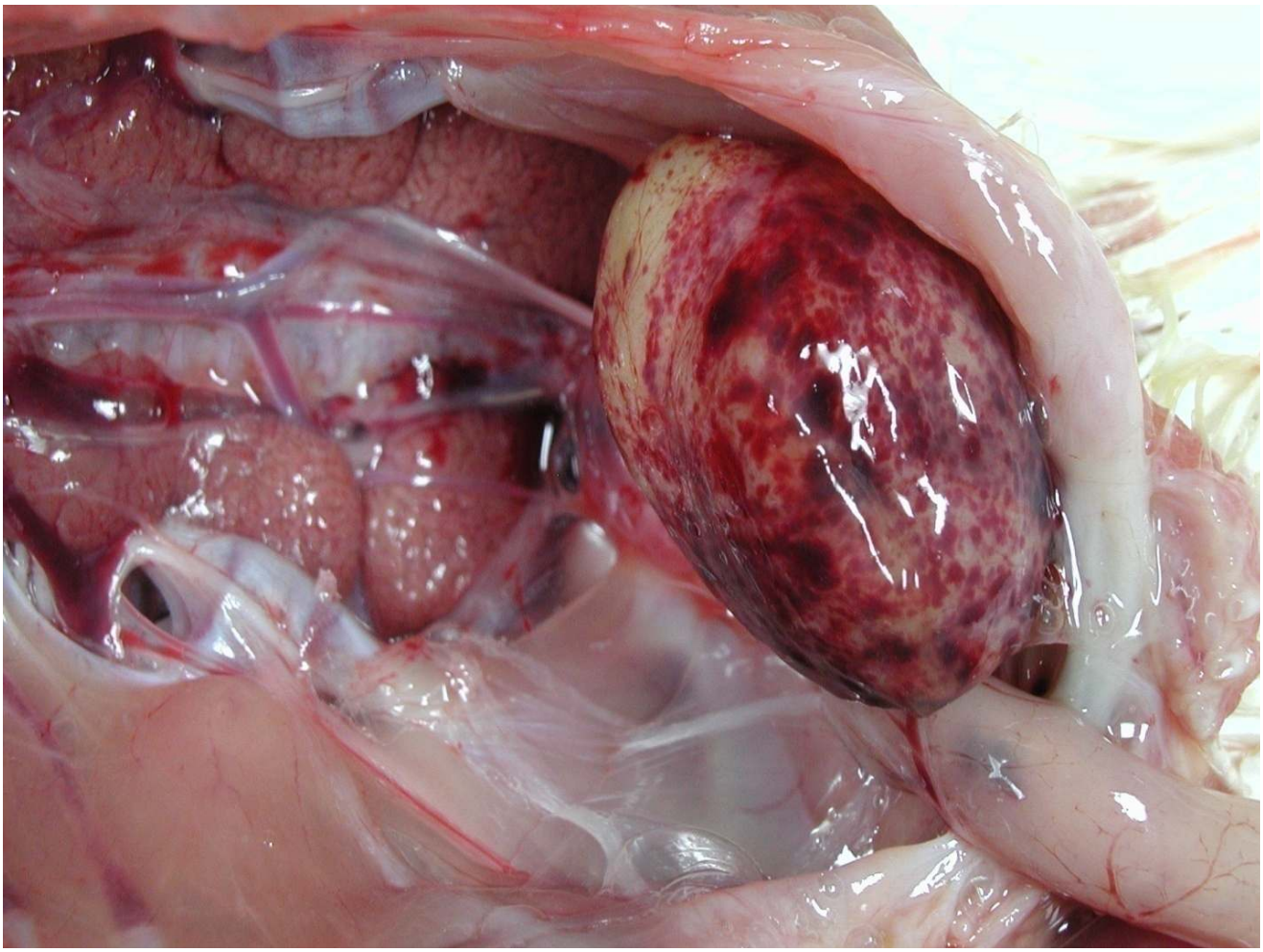

Plate 4.43. York sac: Chicken, experimental exposure, 4 days p.e clade 1 HPAI. Severe diffuse hemorrhagic yolk sac and cloudy serous membranes.

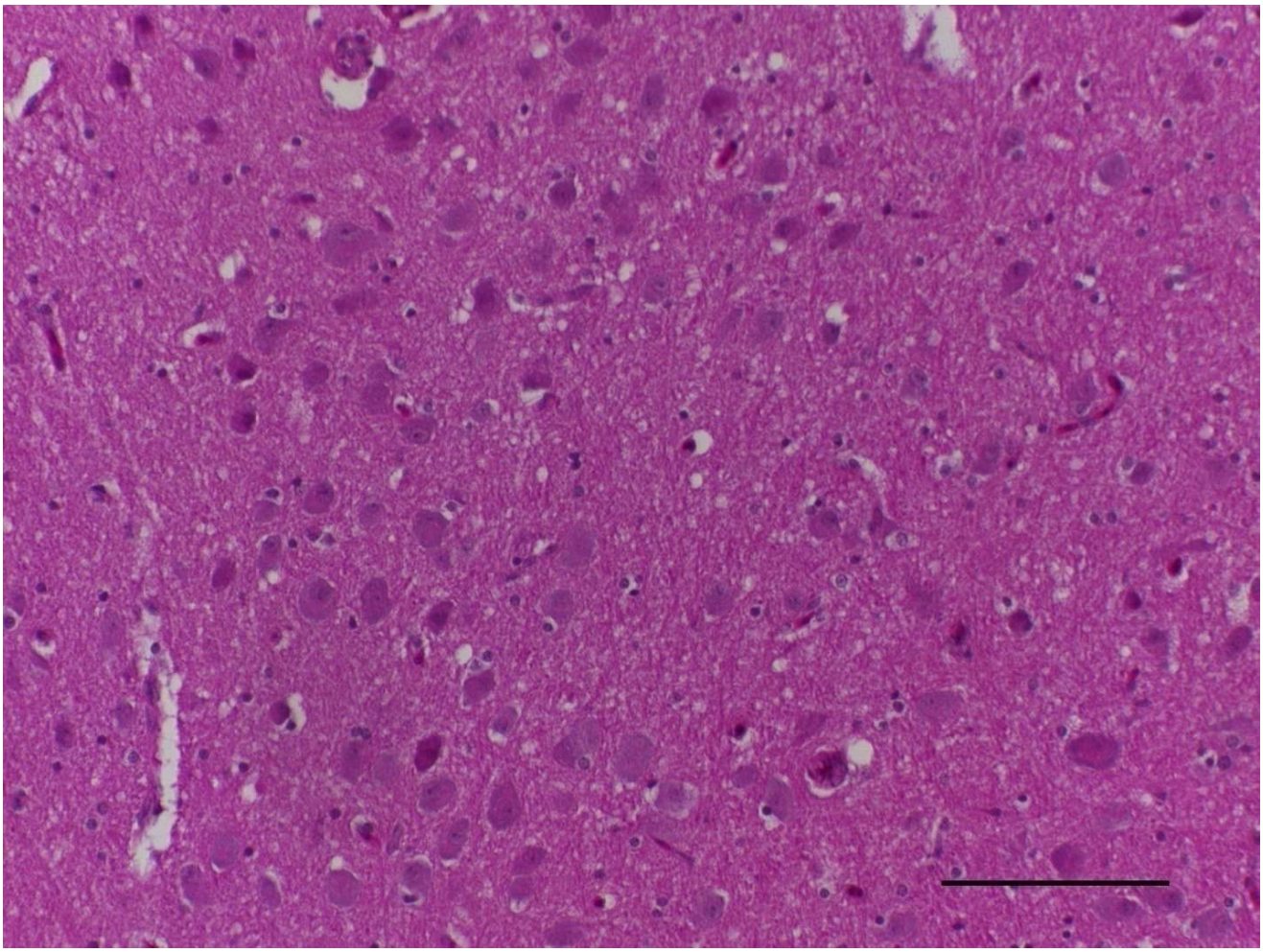

Plate 4.44. Cerebrum; Chicken clade 1 HPAI 4 days p.e. neuronal necrosis. HE. Bar=100µm

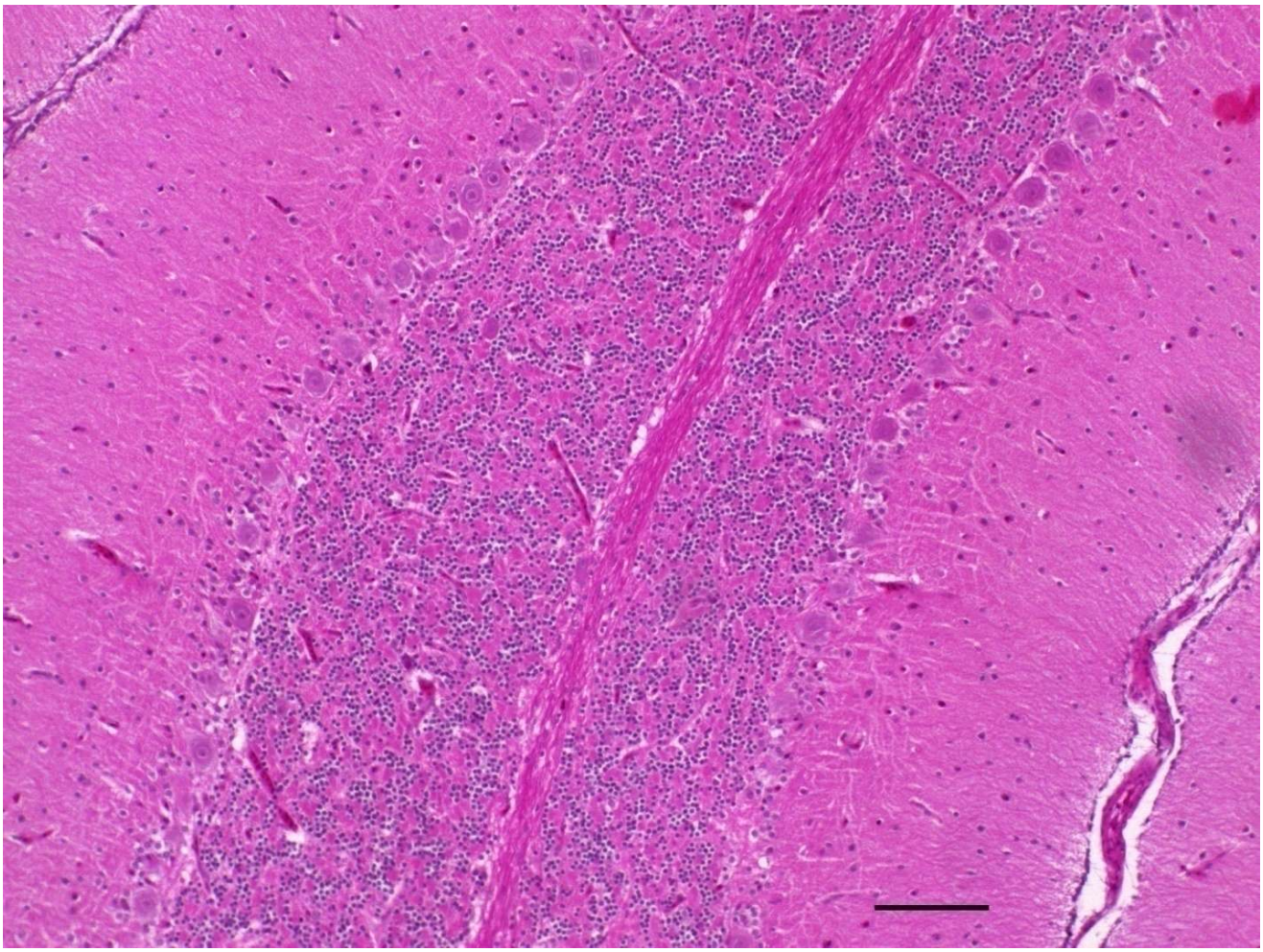

Plate 4.45. Cerebellum; Chicken clade 1 HPAI 2 days p.e. Purkinje cells necrosis, diffuse. HE. Bar=100µm

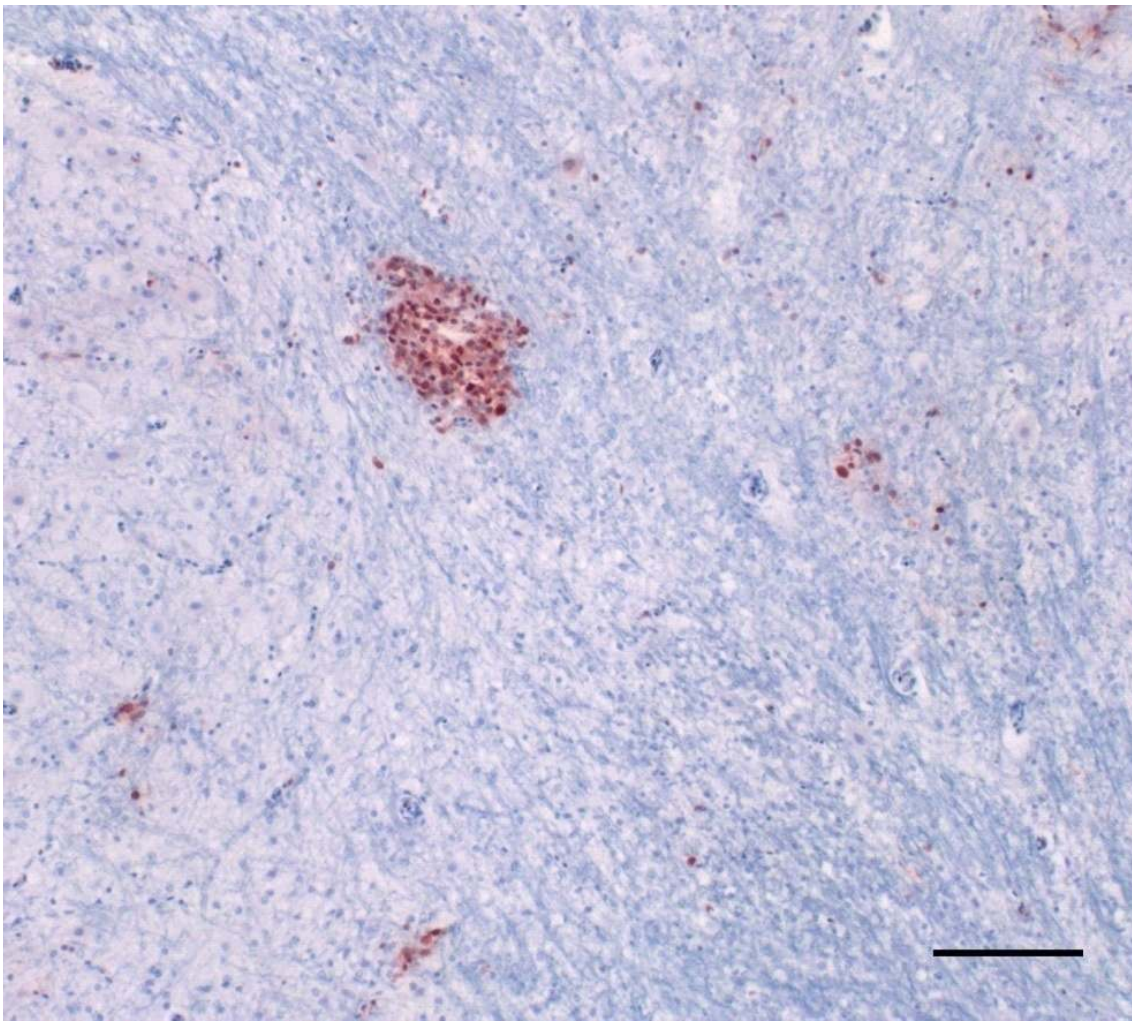

Plate 4.46. Mid Brain; Chicken clade 1 HPAI 4 days p.e. Neurons and nerve ganglia, are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

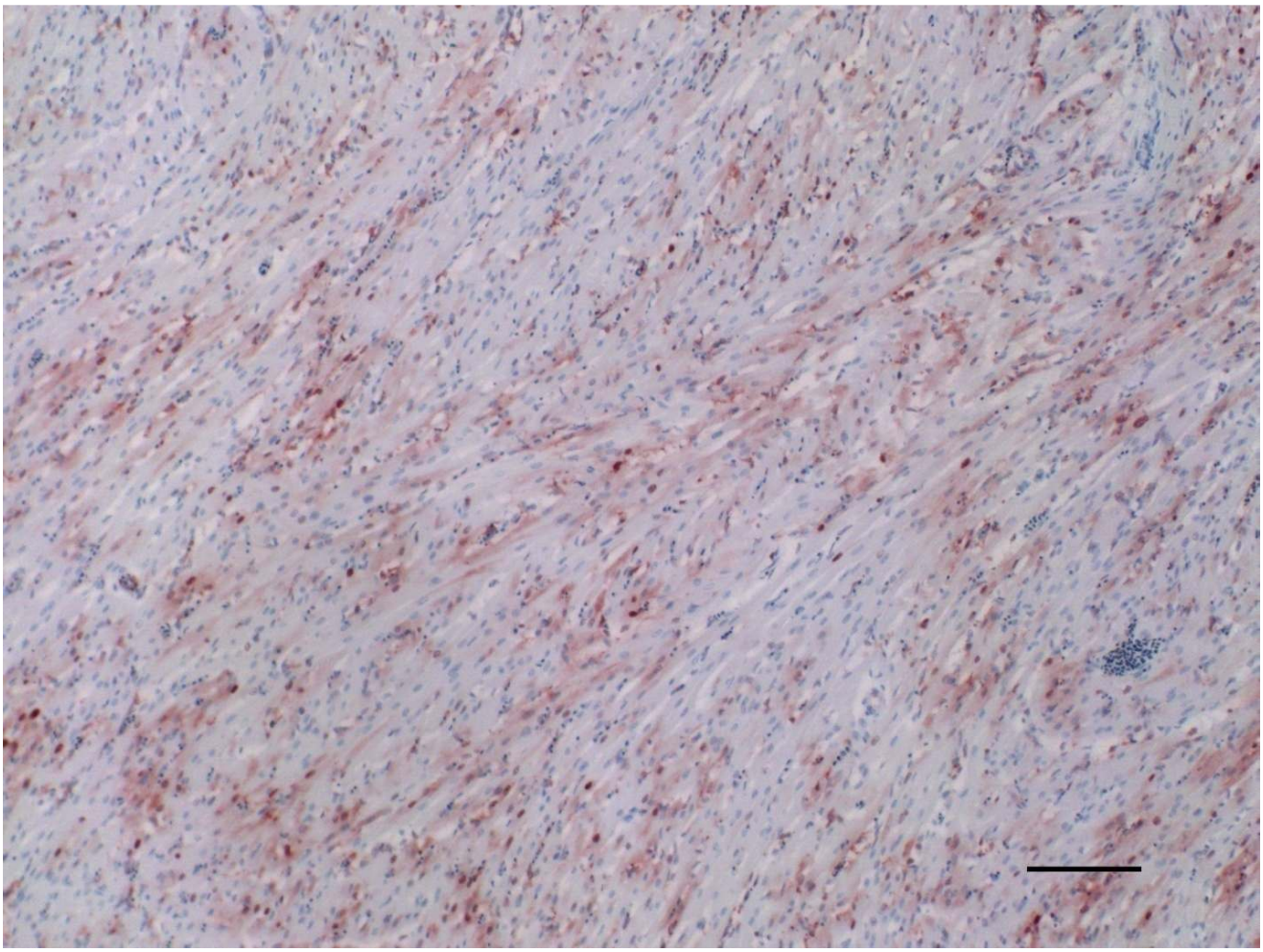

Plate 4.47. Heart; Chicken clade 1 HPAI 4 days p.e. Myocarditis, severe diffuse. Cardiomyocytes and endothelia cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm.

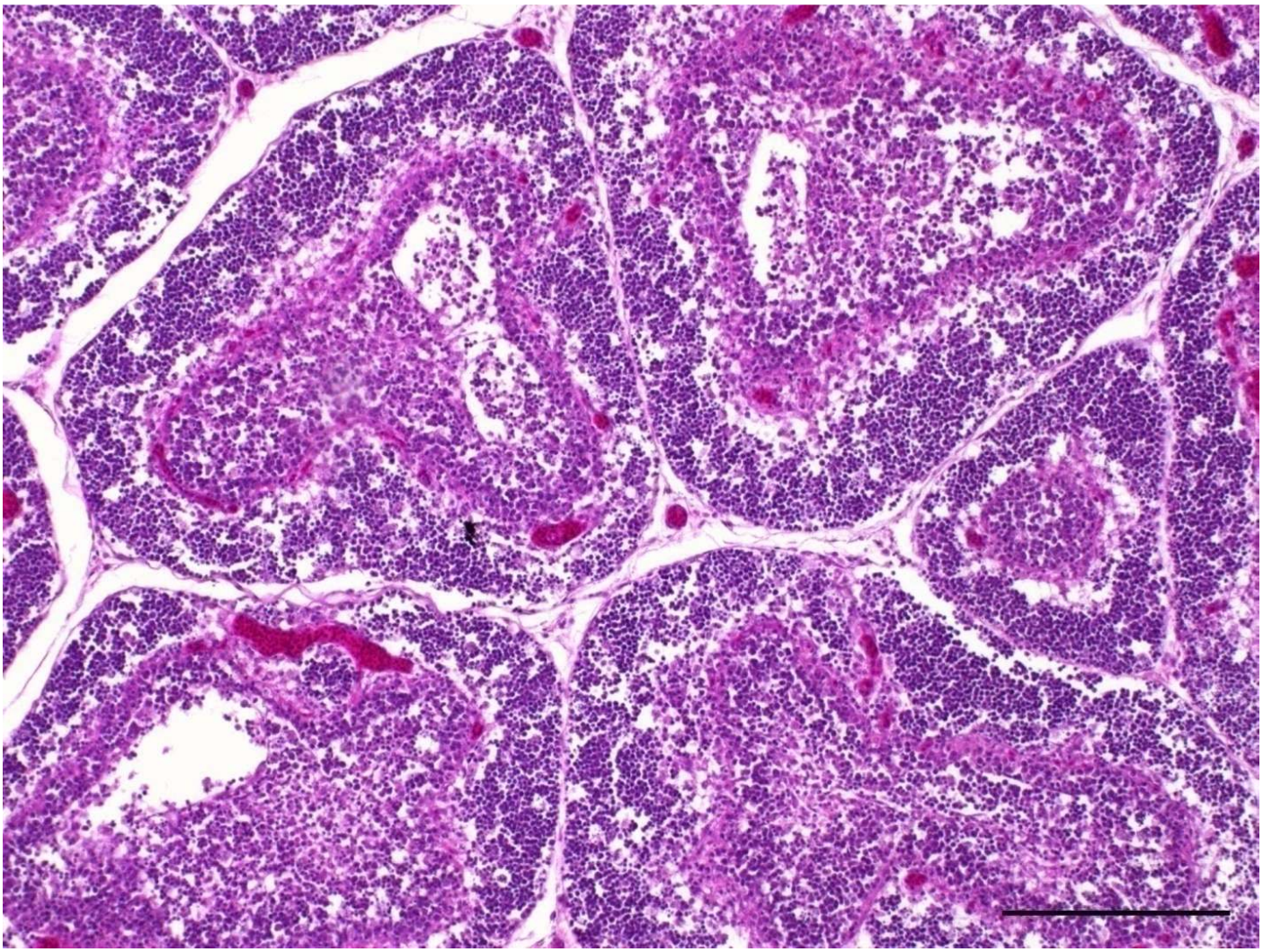

Plate 4.48. Bursa of Fabricius-lymphoid follicles; Chicken clade 1 HPAI 4 days p.e. Follicular lymphocytes are necrotic and depleted with hyperplastic follicular epithelium. Severe vascular congestion HE. Bar=100µm

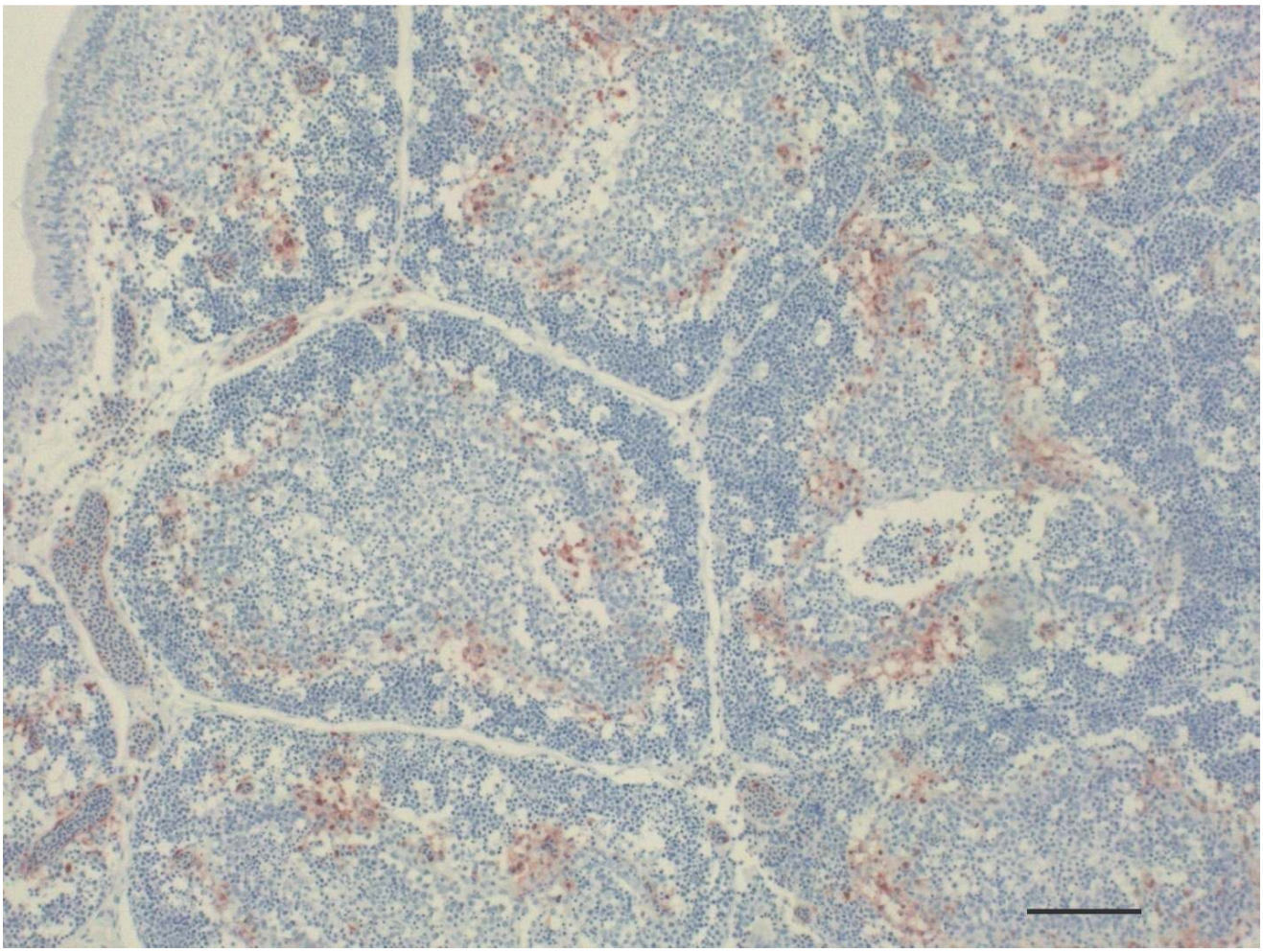

Plate 4.49. Bursa of Fabricius; Chicken clade 1 HPAI 4 days p.e. Follicular mononuclear cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

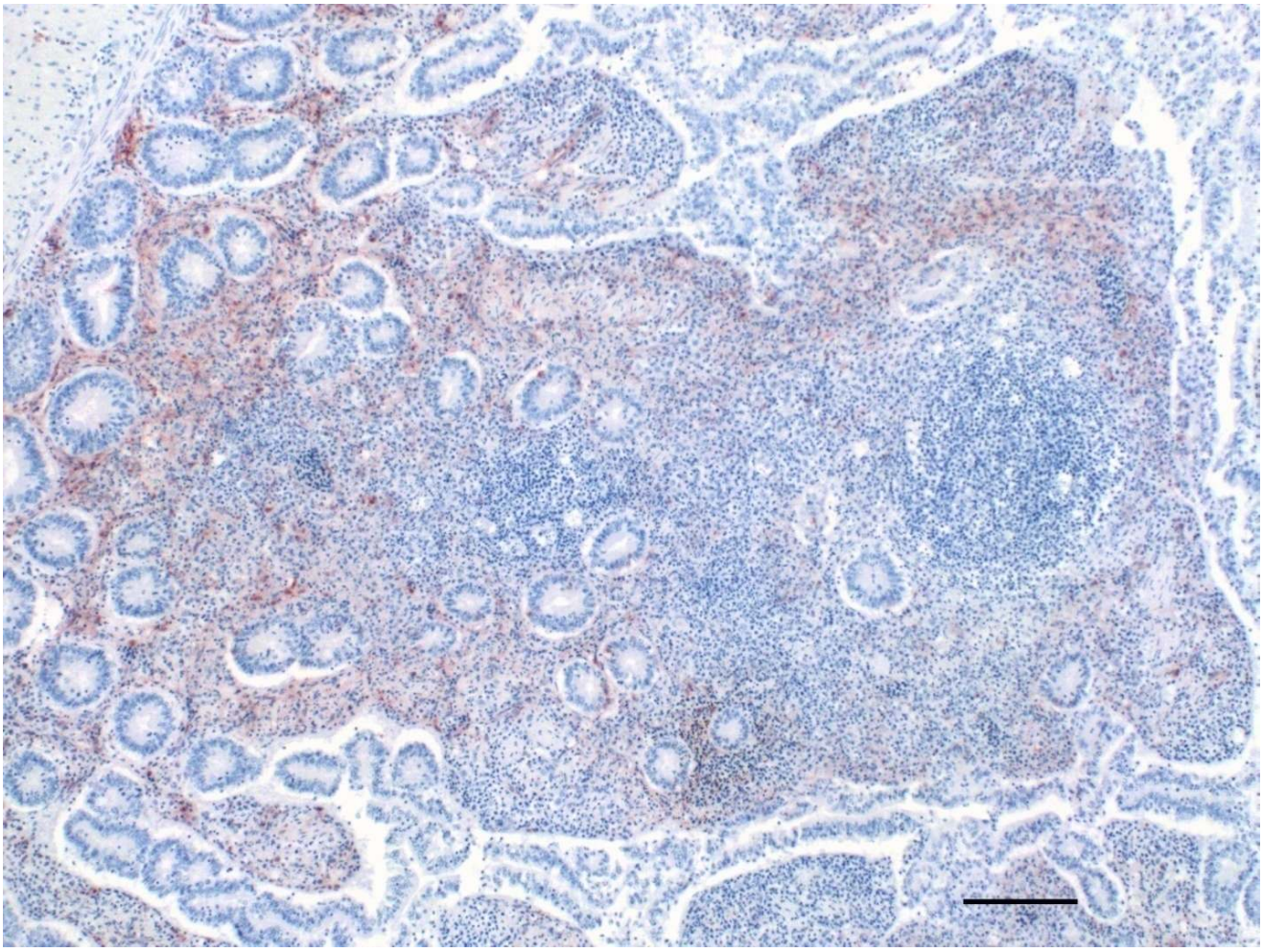

Plate 4.50. Caeca tonsil; Chicken clade 1 HPAI. Perifollicular and interstitial cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

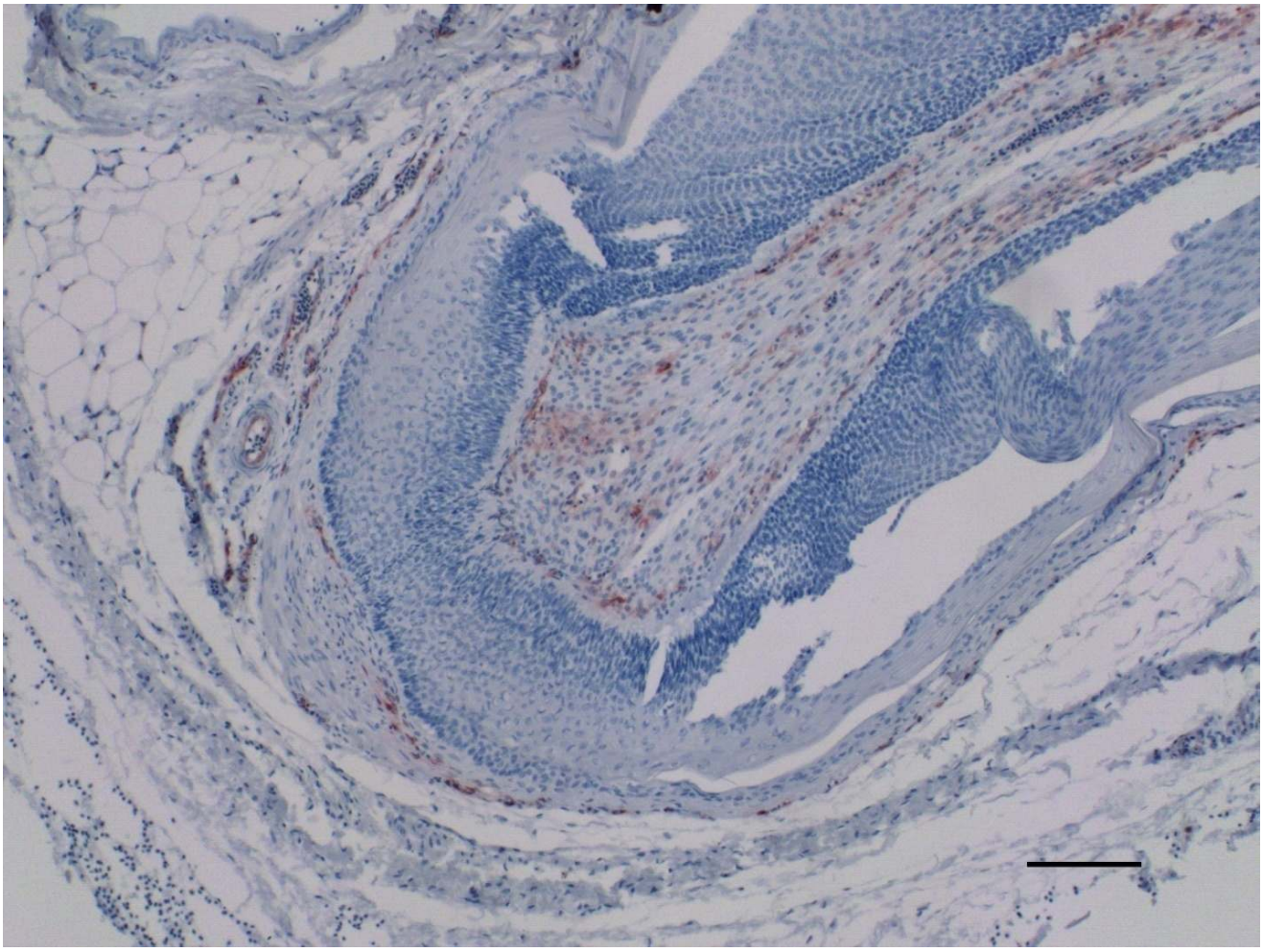

Plate 4.51. Feather follicle- Skin; Chicken clade 1 HPAI 4 days p.e. Folliculitis with cellular inflammatory infiltrates. Follicular keratinocytes are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

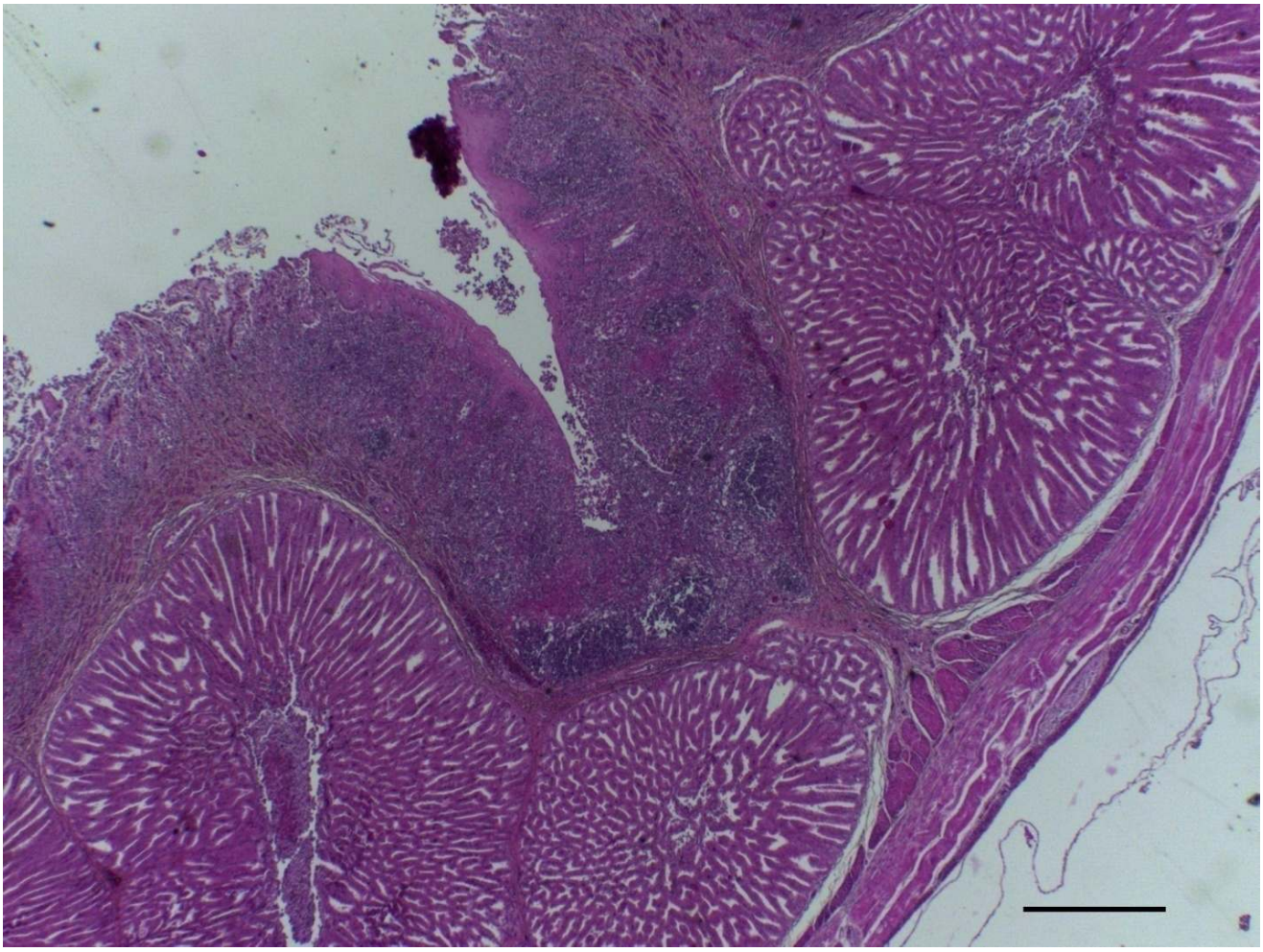

Plate 4.52. Proventriculus; Chicken clade 1 HPAI 4 days p.e. Stratified epithelial erosion and ulceration with multifocal to coalescing lymphoplasmacytic necrotic proventriculitis. HE. Bar=100µm

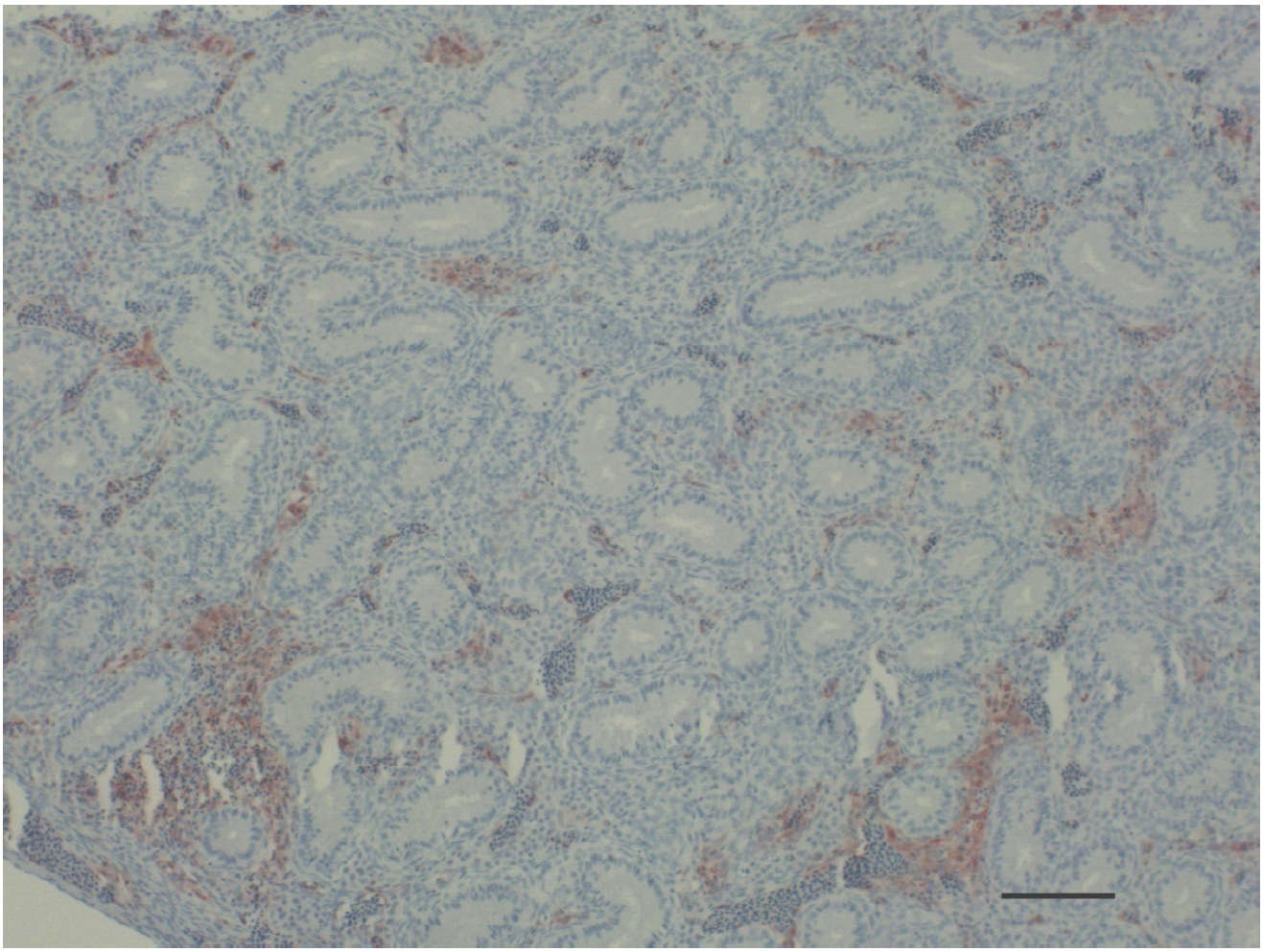

Plate 4.53 . Testis; Chicken clade 1 HPAI 4 days p.e. Intertubular connective tissue cells and vascular endothelia cells are multifocally and strongly Intranuclearlly immunoreactive to H5 nuclear protein. HE. Bar=100µm

4.4.4.4 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in Tracheal and Cloacal Swabs

Tracheal virus detection by real-time RT-PCR was first observed in chicken 3 (Muscovy group) at 1d.p.e, while at 4d.p.e the chickens in the Pekin duck group were negative having been positive 2 and 3DPE. Also chicken 3 (Muscovy group), and chicken 5 (Mallard group) at 1d.p.e had positive real-time RT-PCR cloacal swab, while the cloacal swab in chicken 1 & 2 (Pekin group) were negative at 4 d.p.e having been positive at 2d.p.i (chicken 1) and 3 d.p.e. (Fig 4.34-4.35 illustrates the RRT-PCR ct-values)

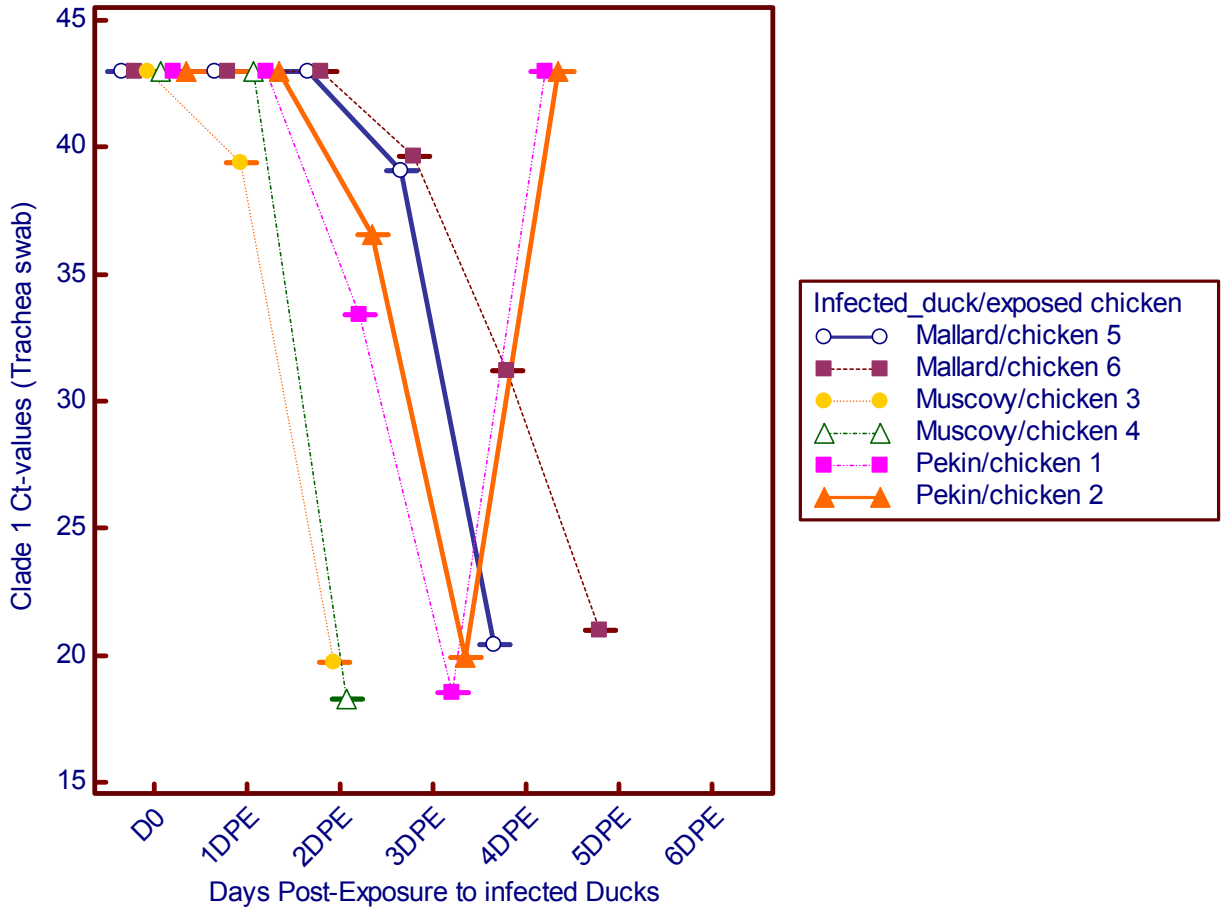

Figure 4.34. Clustered multiple variables graph: Tracheal Swab ct-values of chickens exposed to Clade 1 virus.

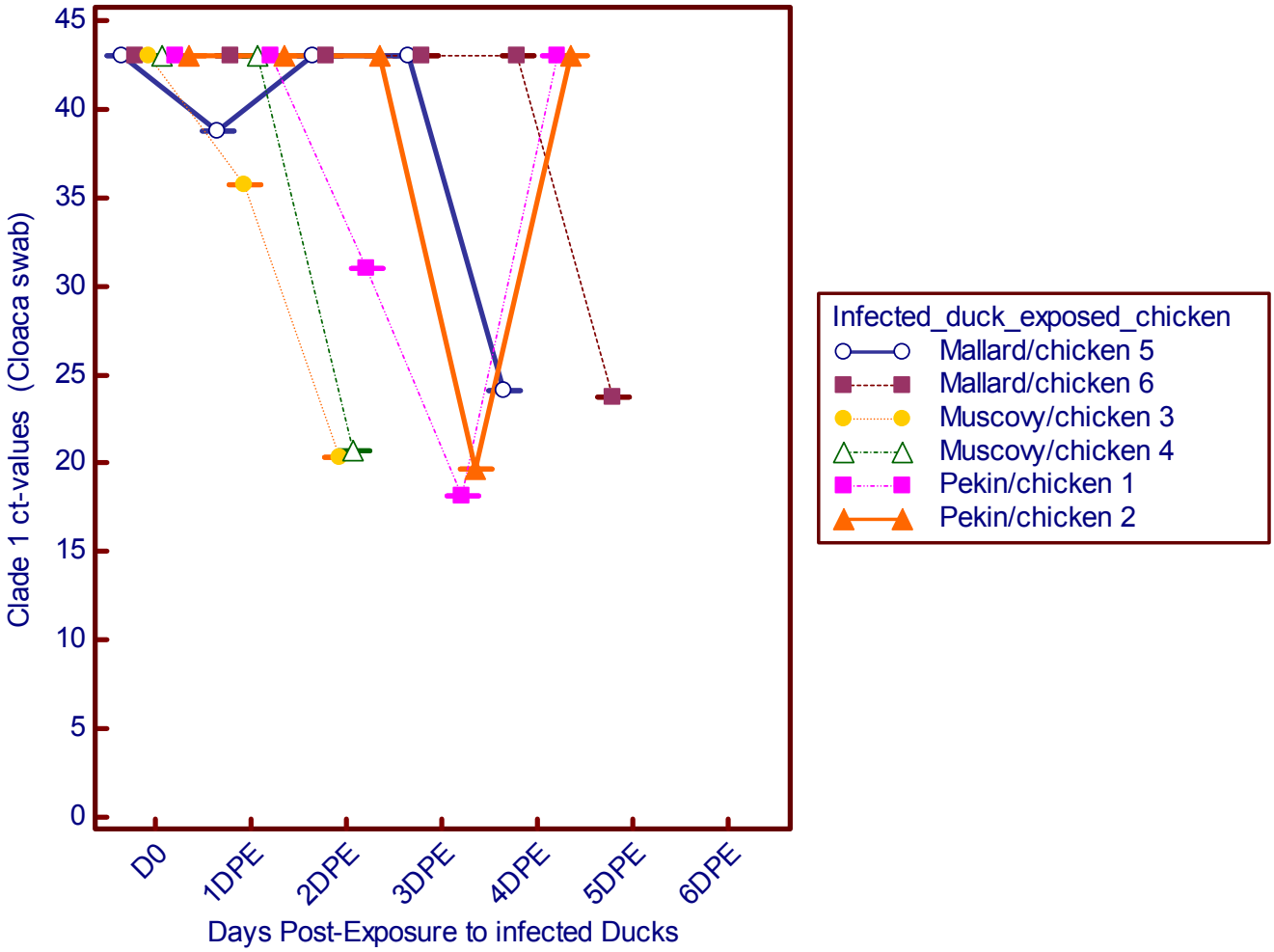

Figure 4.35. Clustered multiple variables graph: Cloacal Swab ct-values of chickens exposed to Clade 1 virus

4.4.5 *Influenza A/Cygnus cygnus/Germany/R65/2006* of clade 2.2 subtype H5N1 virus exposure in Chickens

4.4.5.1Clinical signs

Up to 100% morbidity and mortality was seen starting at 2dpe until 4dpe in in-contact 3- weeks old white leghorn Lohmann breed domestic chickens (Galus domesticus) exposed to Highly Pathogenic Avian Influenza *A/Cygnus cygnus/Germany/R65/2006* of clade 2.2 subtype H5N1 virus infected Pekin, Mallard and Muscovy ducks.

4.4.5.2 Gross Pathology

Gross lesions of cardiovascular system (myocardial petechial haemorrhage) and pancreas were seen in 80% of the carcasses examined while respiratory (pulmonary edema) and hemorrhagic lesions of the GIT were observed in 40%.

4.4.5.3 Histopathology and Immunohistochemistry

Histopathologic lesions were not seen in the chickens that died 2 & 3 d.p.e, but immunoractivity was seen in Keratinocytes in the Comb, cerebral and cerebellar neurons, lymphocytes in thymus, Bursa of Fabricius and spleen, cardiomyocytes, atria and endothelia cells. The esophageal endothelia cells, the hepatocytes, pancreatic acinar cells and renal tubular epithelia cells also had immunoreactivity. In 4 d.p.e chickens, cerebral and cerebellar gliosis, purkinje cell necrosis, depletion of the inner nuclear/ganglion layer of the eye, thymic and splenic necrosis and lymphoid depletion, also seen in rectal GALT. Tracheal and atria epithelia necrosis, while the GIT lesion was mainly villi and crypt necrosis in duodenum, jejunum, ileum, caeca and rectum in addition to proventricular and ventricular epithelia necrosis and vasculitis. The Liver, pancreas and kidney were not spared as hepatocytes, pancreatic acinar cells and renal tubular epithelium were necrotic. Immunoreativity was seen in all the cells mentioned and in addition, Bursa of Fabricius follicular lymphocytes, endothelia cells of the eye, esophageal and proventricular endothelia cells, myocytes in jejunum, caeca laminar propria, rectal endothelia cells and skeletal muscle myocytes. Plates 4.54 -4.61 showed the lesions observed in these chickens**.**

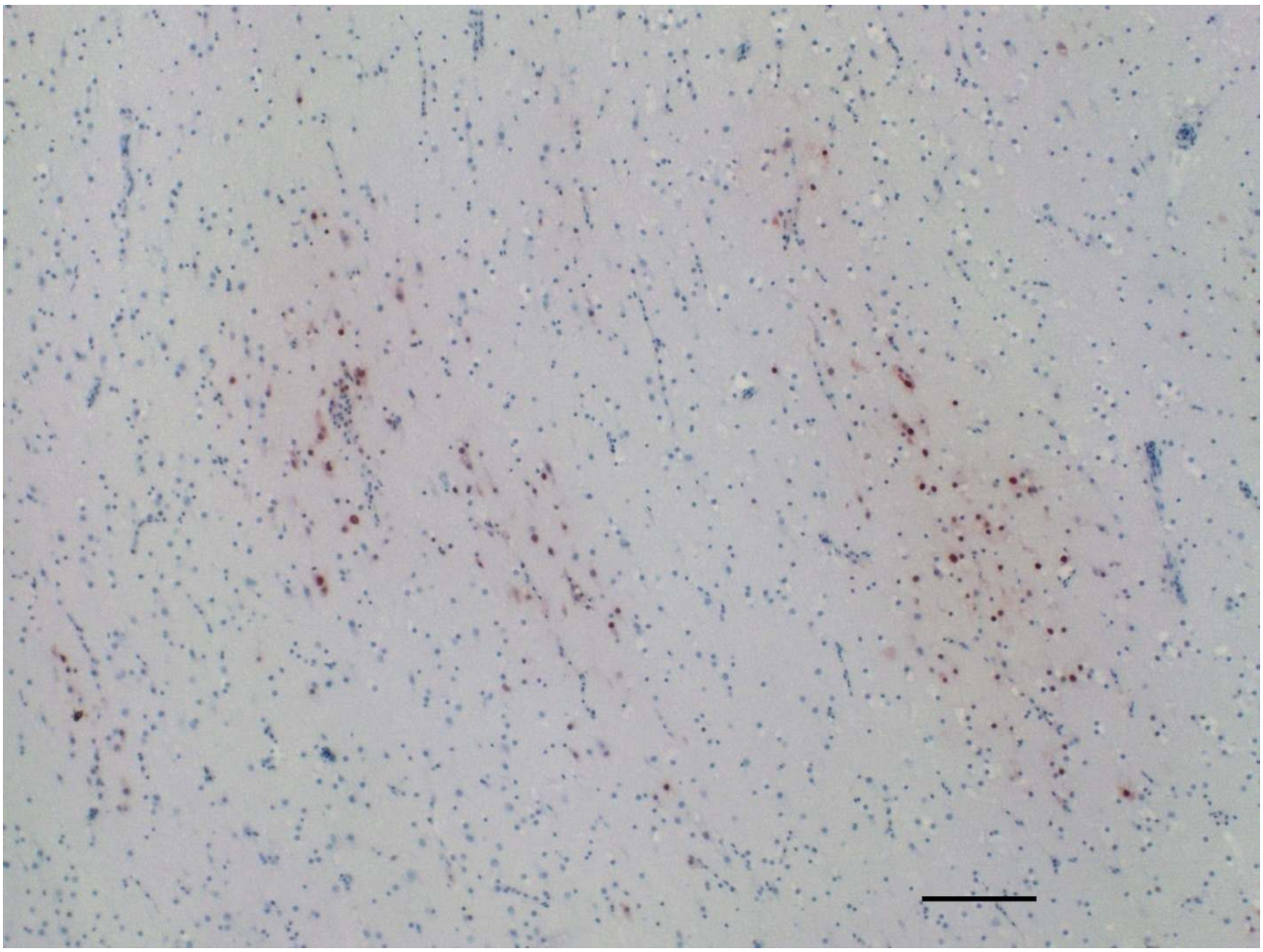

Plate 4.54. Cerebrum; Chicken clade 2.2 HPAI 4 days p.e. Neurons and glia cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

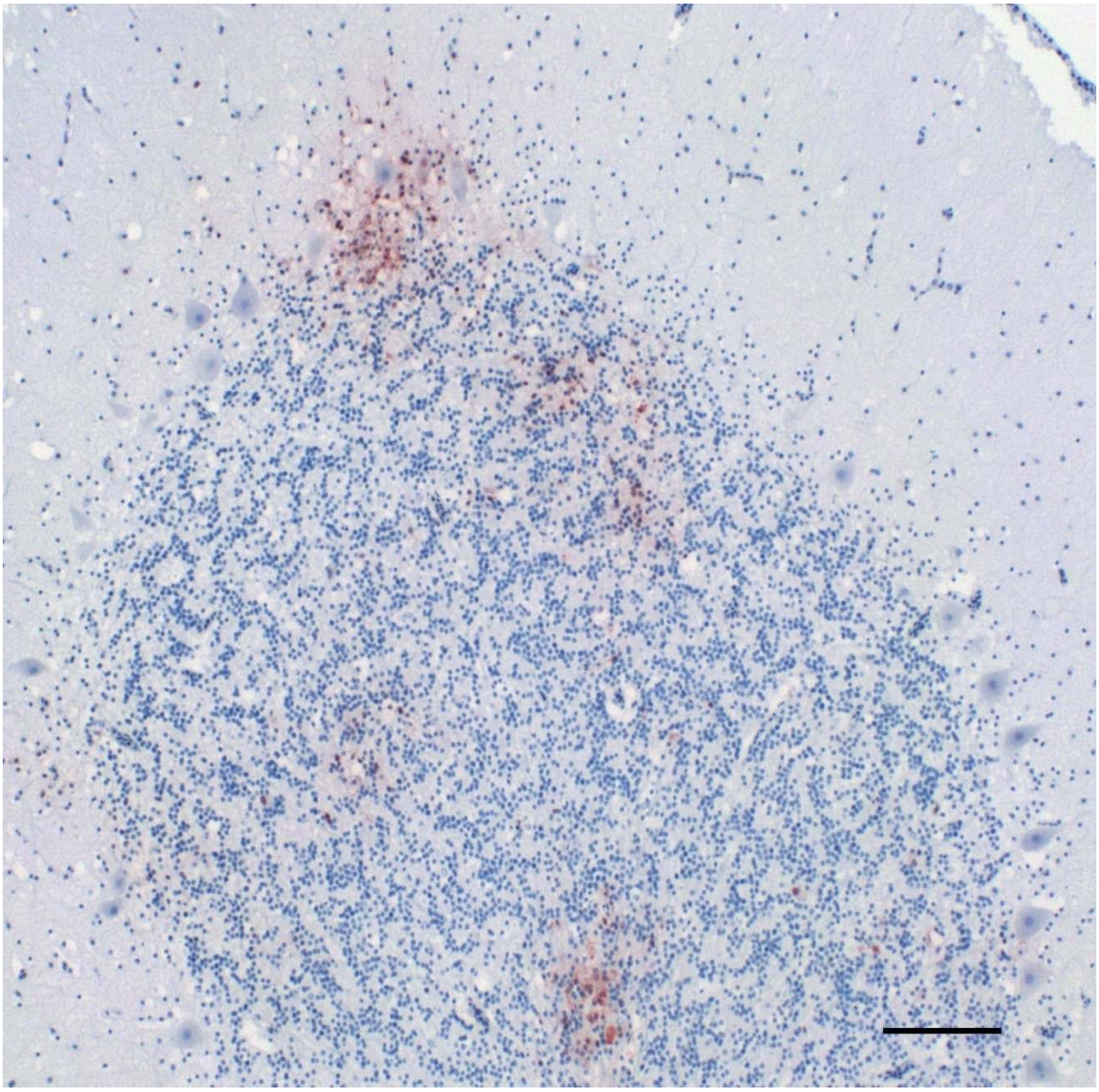

Plate 4.55. Cerebellum; Chicken clade 2.2 HPAI 4 days p.e. Neuronal cell bodies and purkinje cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

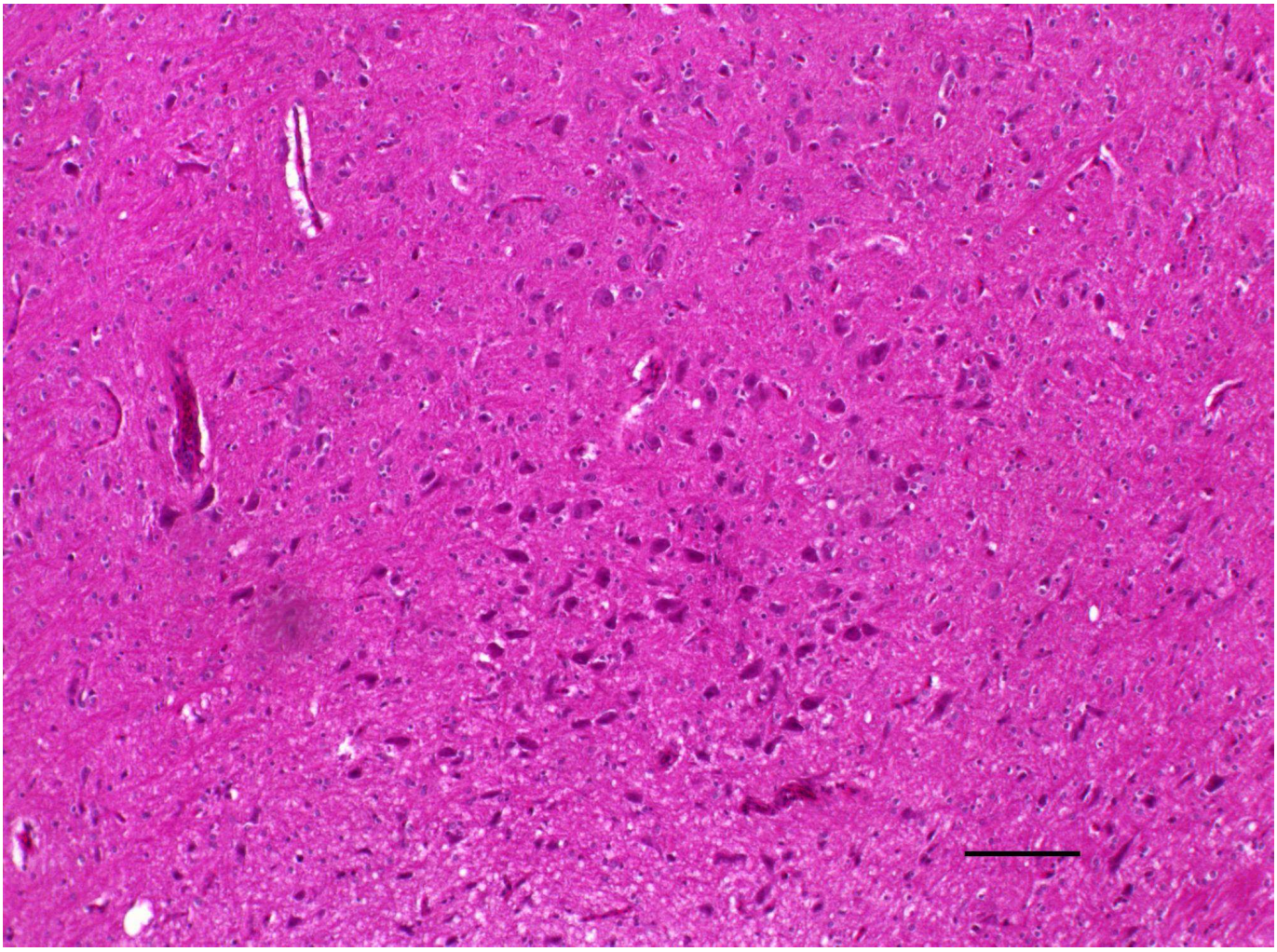

Plate 4.56. Cerebrum; Chicken clade 2.2 HPAI 4 days p.e. Encephalitis, severe diffuse with neuronophagia and glia nodule formation. HE. Bar=100µm

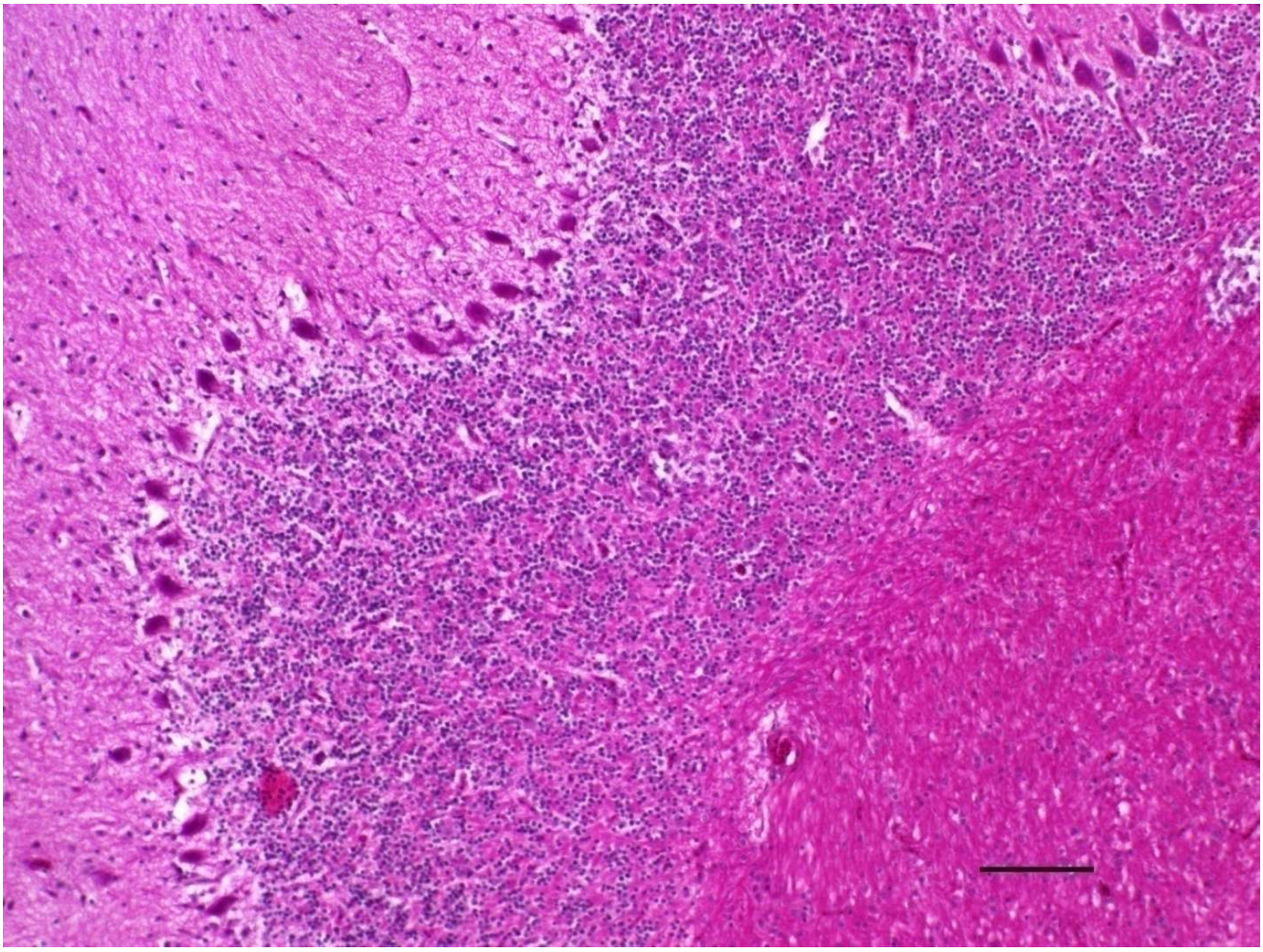

Plate 4.57. Cerebellum; Chicken clade 2.2 HPAI 4 days p.e. Encephalitis, molecular layer, diffuse, severe, with purkinje cells necrosis. HE. Bar=100µm

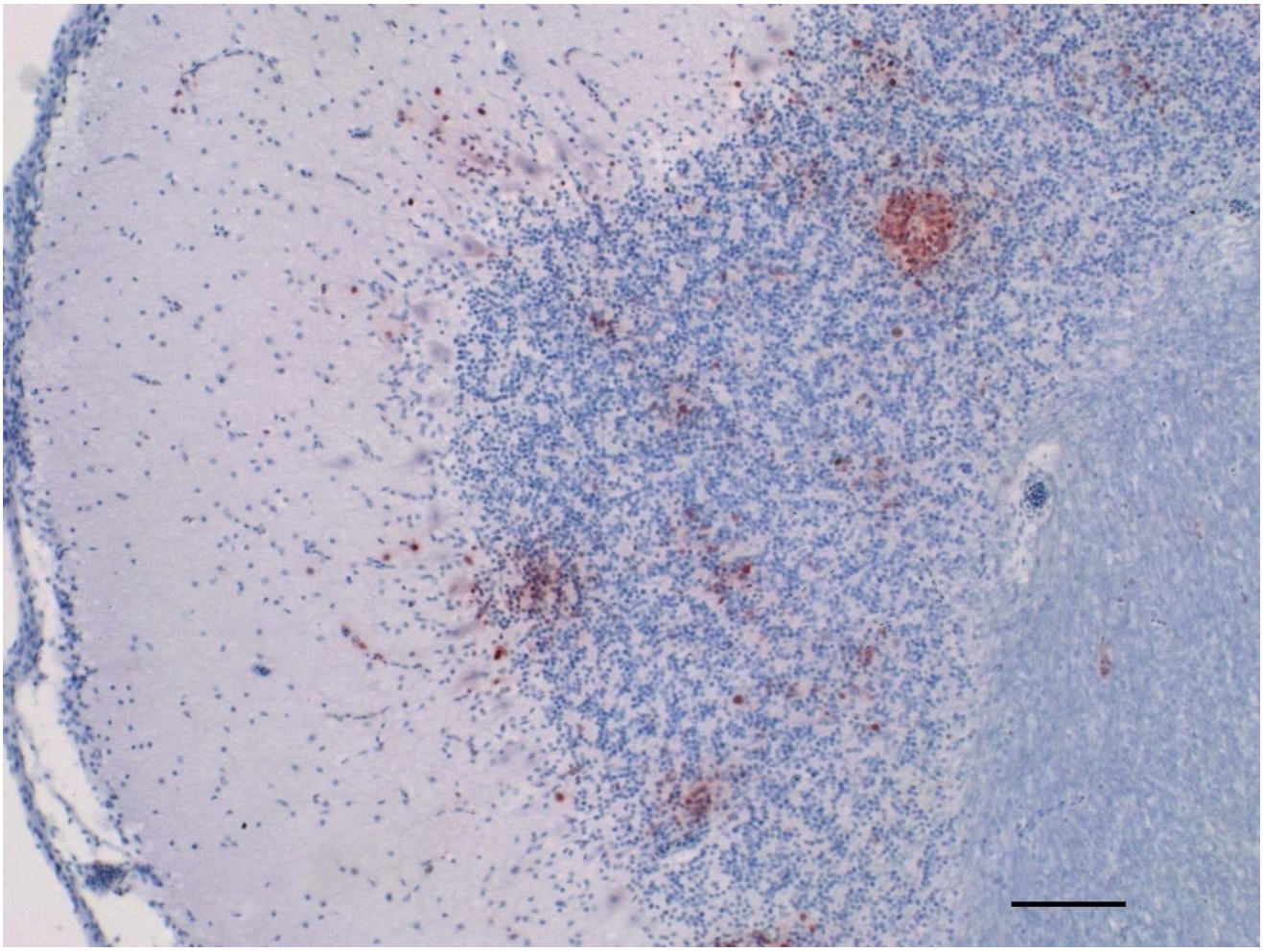

Plate 4.58. Cerebellum; Chicken clade 2.2 HPAI 4 days p.e.Granular cell layer and purkinje cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

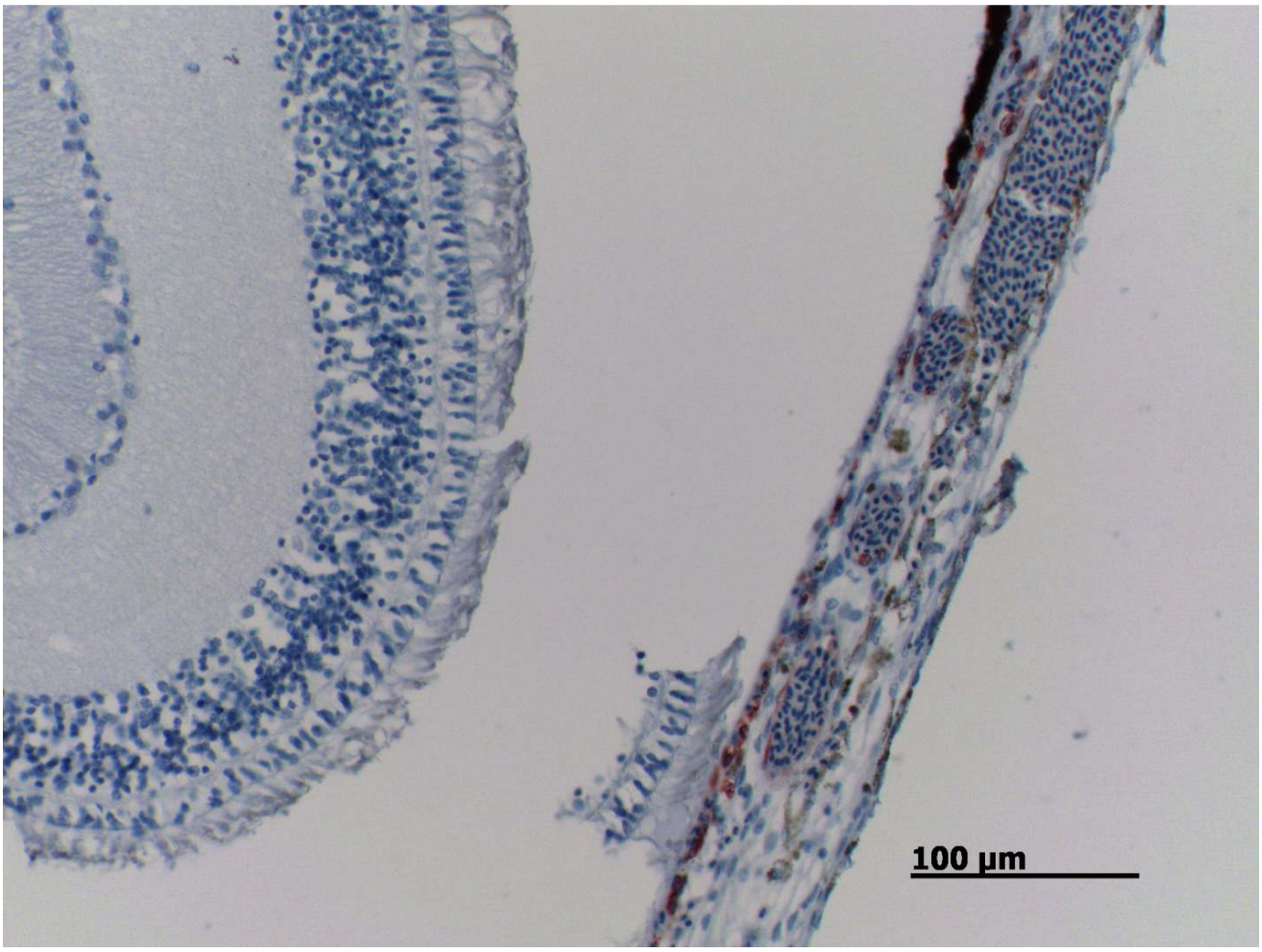

Plate 4.59. Eye- Retina; Chicken clade 2.2 HPAI 4 days p.e. Retinopathy of outer cell layer and Vascular congestion. Vascular endothelia cells are diffusely and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

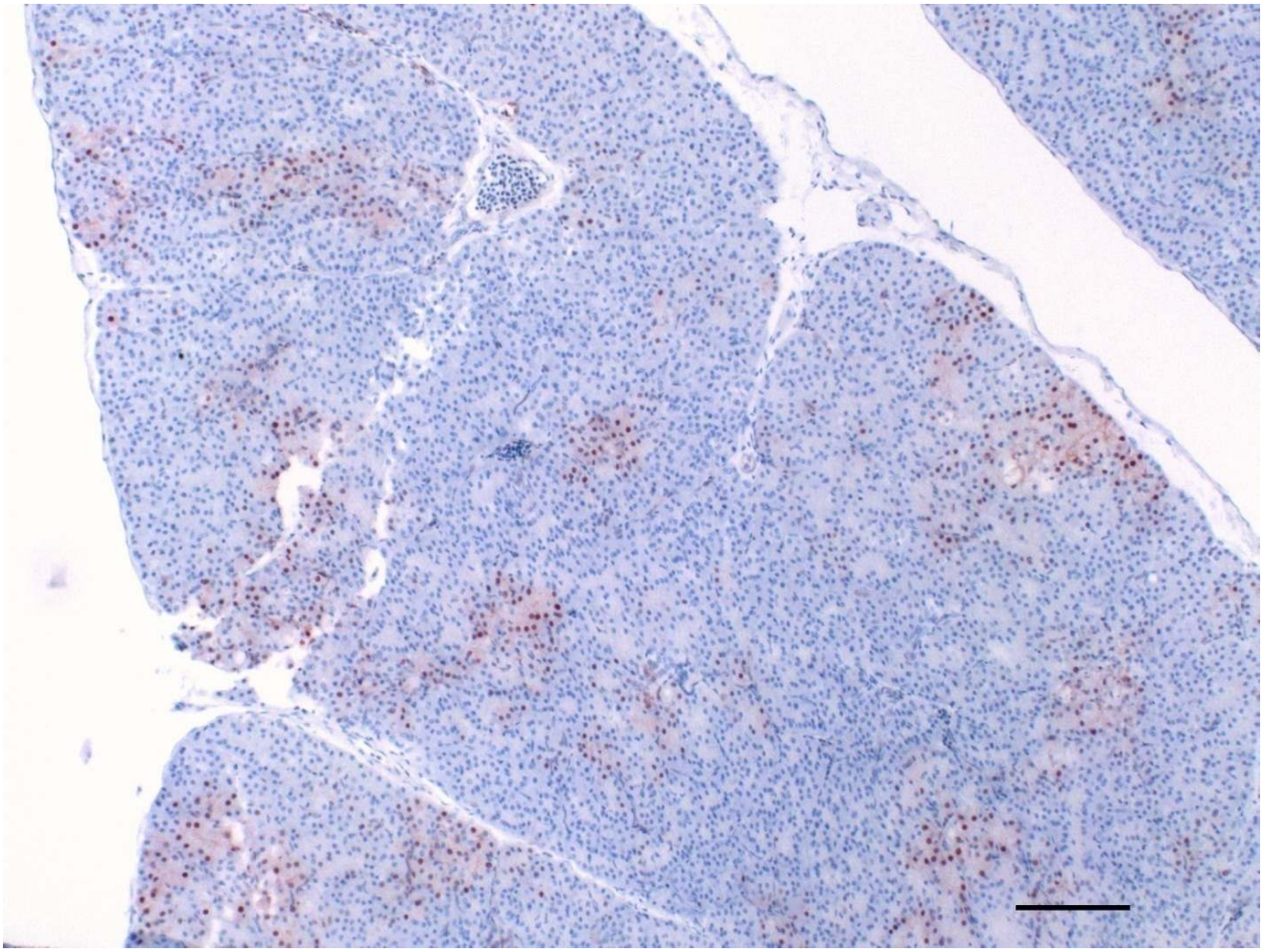

Plate 4.60. Thymus; Chicken clade 2.2 HPAI 4 days p.e. Cortical and medullar cell necrosis, with cells multifocally and strongly intranuclearlly immunoreactive to H5 nuclear protein. Bar=100µm.

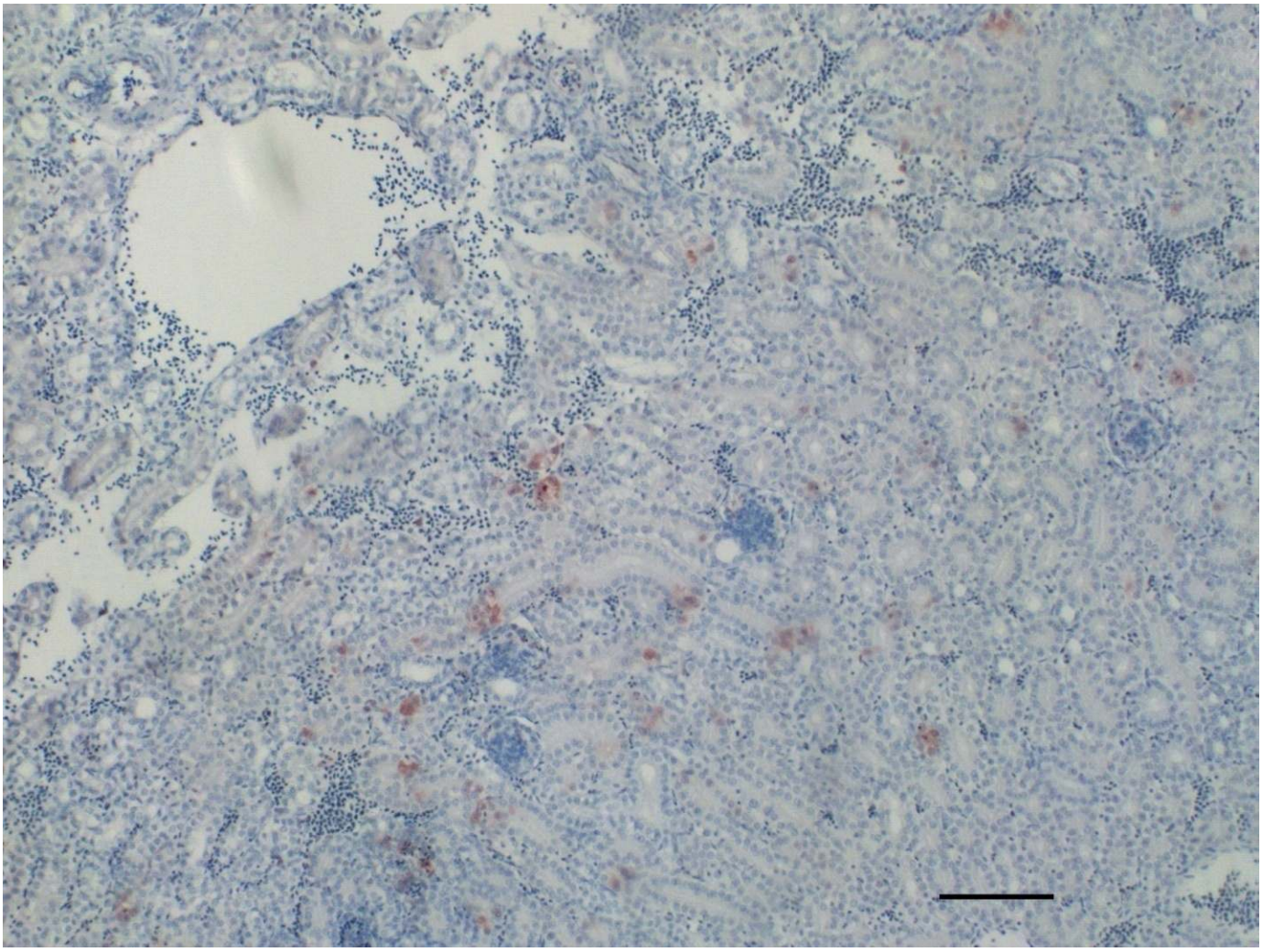

Plate 4.61. Kidney; Chicken clade 2.2 HPAI 4 days p.e. Tubular epithelial cells are multifocally and strongly intranuclearly immunoreactive to H5 nuclear protein. Bar=100µm

4.4.5.4 Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) HPAI Detection in Tracheal and Cloacal Swabs

Daily tracheal and cloacal swabs of all exposed chickens subjected to real-time RT-PCR, revealed that all tracheal swabs from all chickens except the two chickens in the Muscovy group were negative for the H5N1 virus by 24hrs p.e. At 2DPE, all except one chicken exposed to mallard ducks was positive. At 3DPE, only one chicken with the Muscovy ducks was alive. Three of the chickens died while one was sacrificed at 4DPE. Cloacal swabs were positive in one Muscovy and Pekin groups chickens respectively 24hrs p.e. Two chickens in the Pekin group had no detectible virus 2DPE. While at 3days p.e, one in the Mallard group showed no detectable virus. Figures 4.36-4.37 showed the RRT-PCR ct values

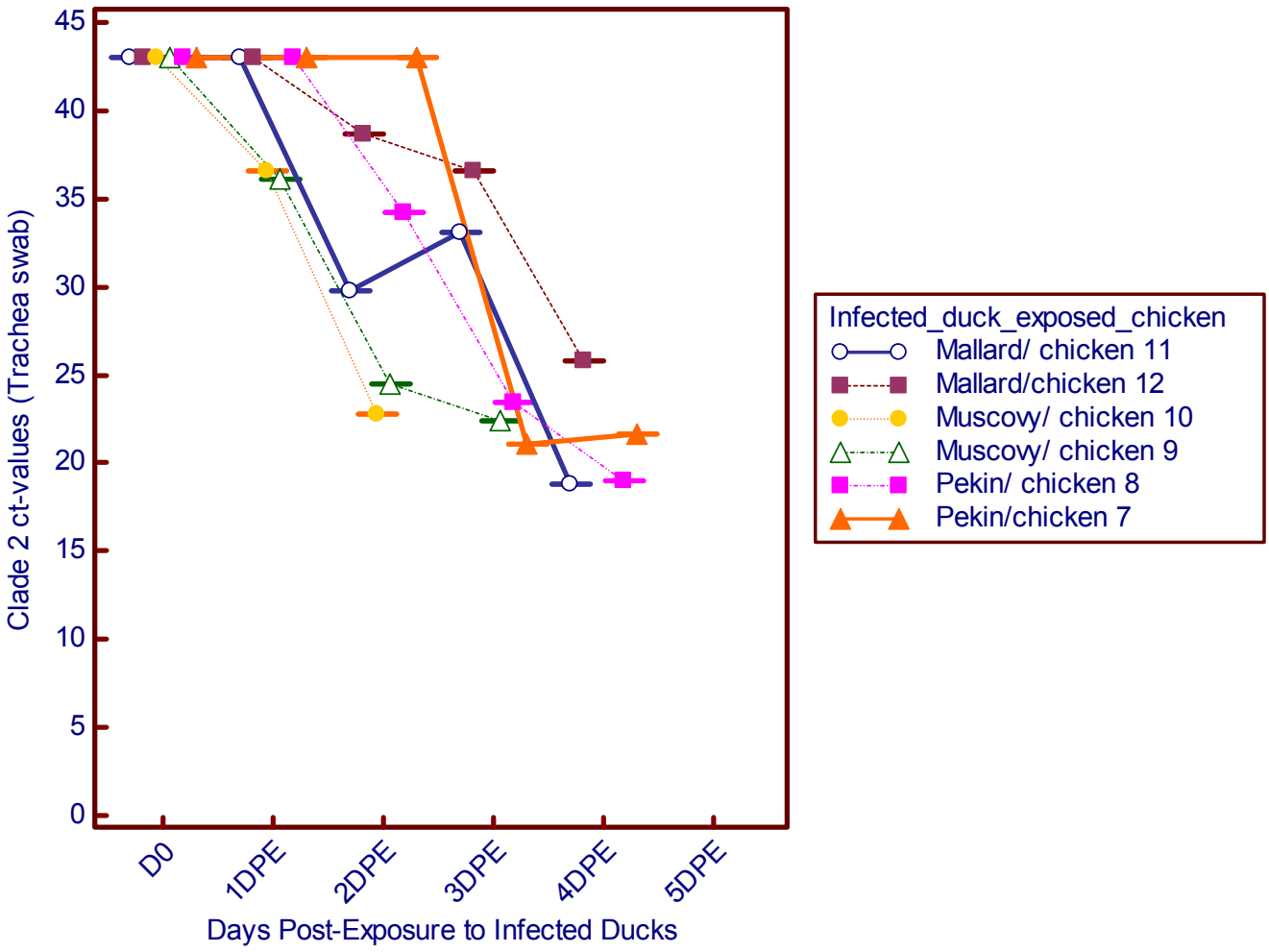

Figure 4.36. Clustered multiple variables graph: Tracheal Swab RRT-PCR ct-values of chickens exposed to Clade 2.2 virus

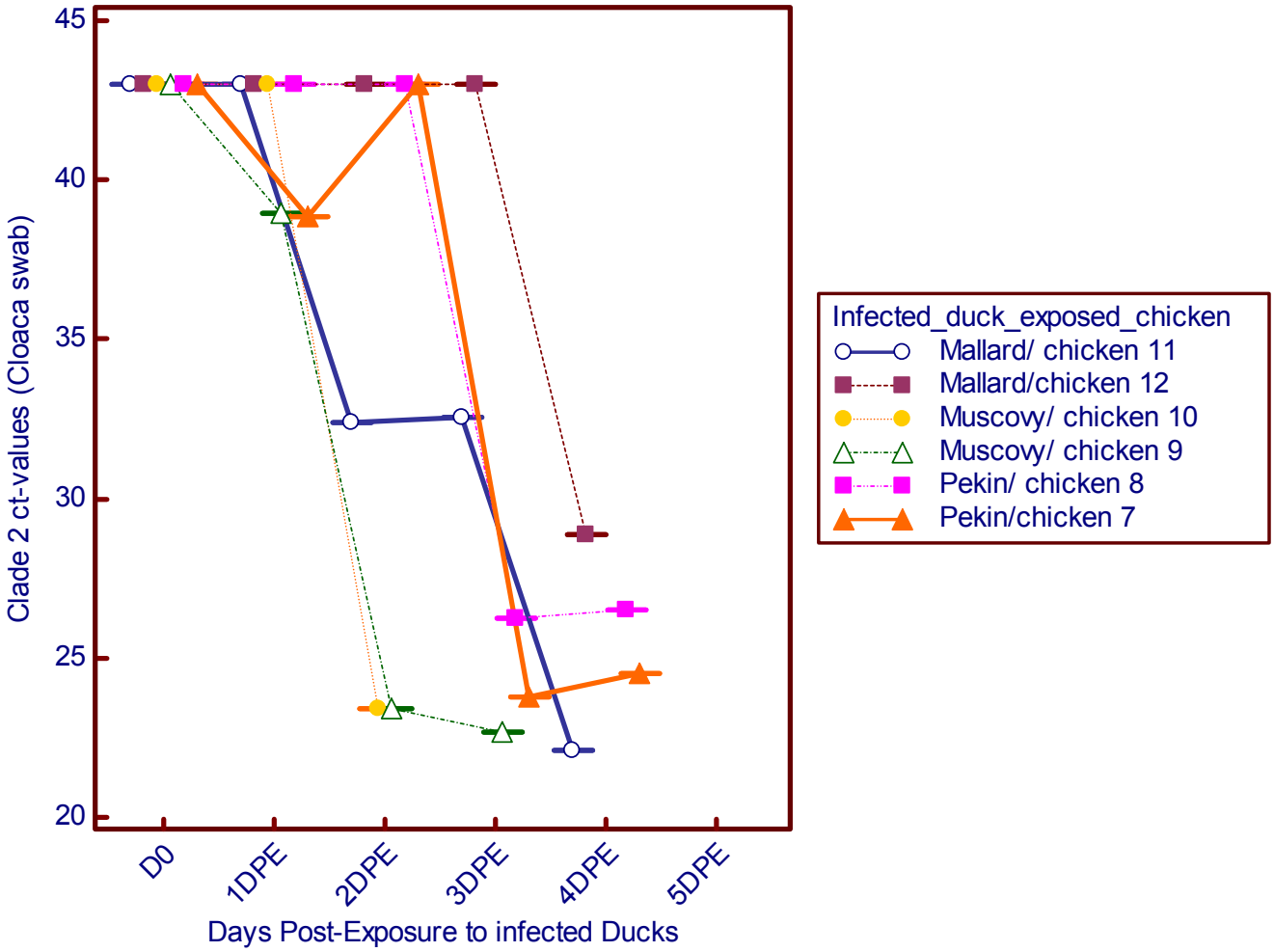

Figure 4.37. Clustered multiple variables graph: Cloacal Swab RRT-PCR ct-values of chickens exposed to Clade 2.2 virus

4.4.6 Comparative rRT-PCR Virus Load of *Influenza A/duck/Vietnam/TG24-01/05* of clade 1 and *Influenza A/Cygnus cygnus/Germany/R65/2006* of clade 2.2 subtypes H5N1 virus exposure in chickens.

4.4.6.1 Tracheal and Cloacal Analysis 2nd Day Post-Exposure (d.p.e)

When the mean tracheal and cloacal ct-values were compared between the chickens exposed to the duck species, i.e Muscovy, Mallard and Pekin in the two virus groups at 2 d.p.e, it was found that the mean tracheal swab ct-values for chickens in the Muscovy ducks group was the only slightly positive mean ct-value in the clade 2.2 virus groups (Fig 4.38). The mean cloacal swab ct-values was the only slightly positive mean ct-value in the clade 1 virus groups (Fig 4.39). A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, showed that at 2 d.p.e, there was a significant difference (Bonferroni corrected P=0.004) in the mean tracheal ct-values of the chickens exposed to infected Muscovy ducks when compared with the chickens exposed to infected Mallard ducks. There was also a significant difference (Bonferroni corrected P=0.004) in the mean tracheal ct-values of the chickens exposed to infected Muscovy ducks when compared with those exposed to infected Pekin ducks. There was also a significant difference (Bonferroni corrected P=0.03) in the mean tracheal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 at 2 d.p.e irrespective of the duck species of exposure. This result did not apply with mean cloacal ct-values as no significant difference was seen in either duck species of exposure or exposure virus type.

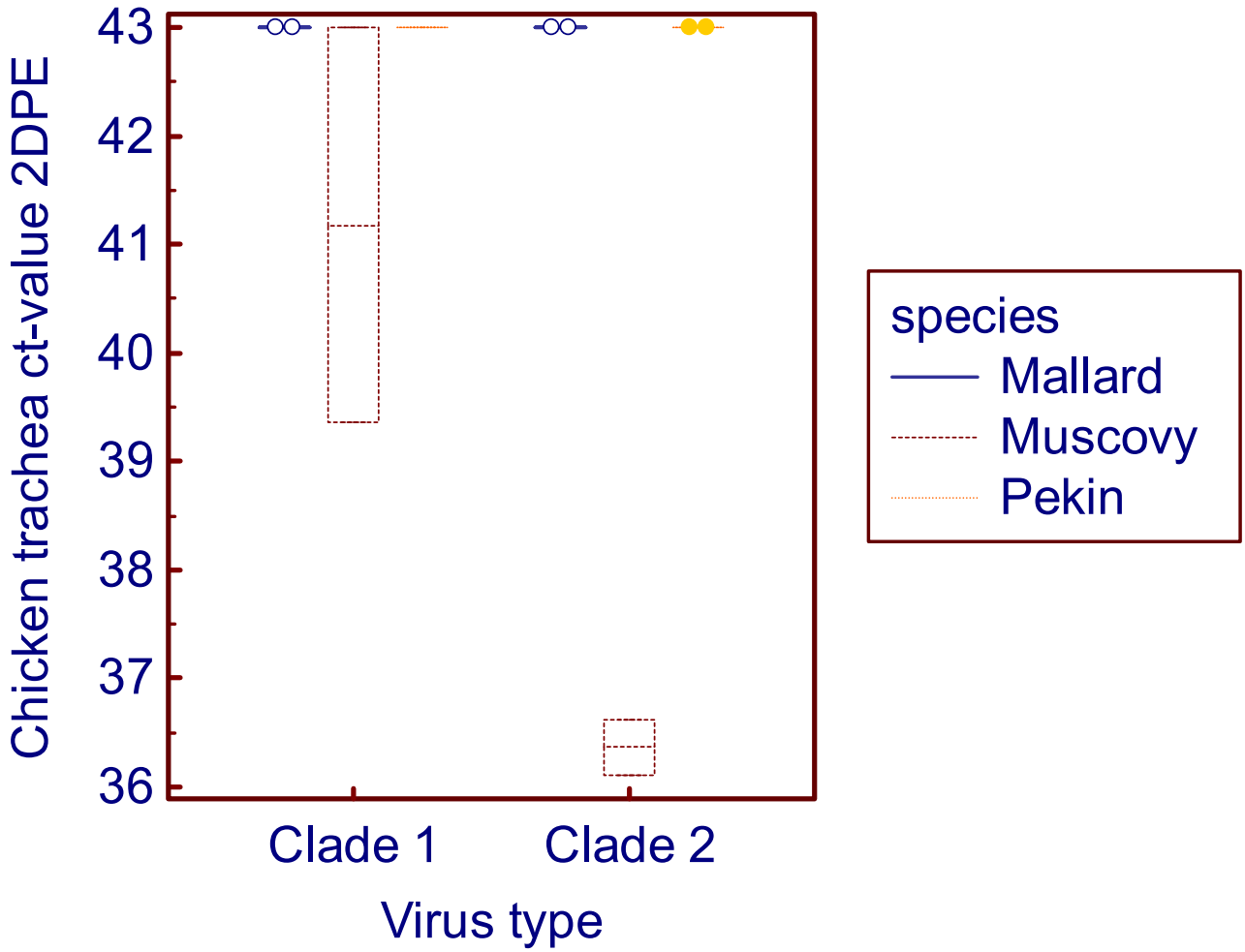

Figure 4.38. Clustered multiple variables graph: Tracheal Swab ct-values of chickens exposed to Clade 1 and 2.2 virus 2DPE

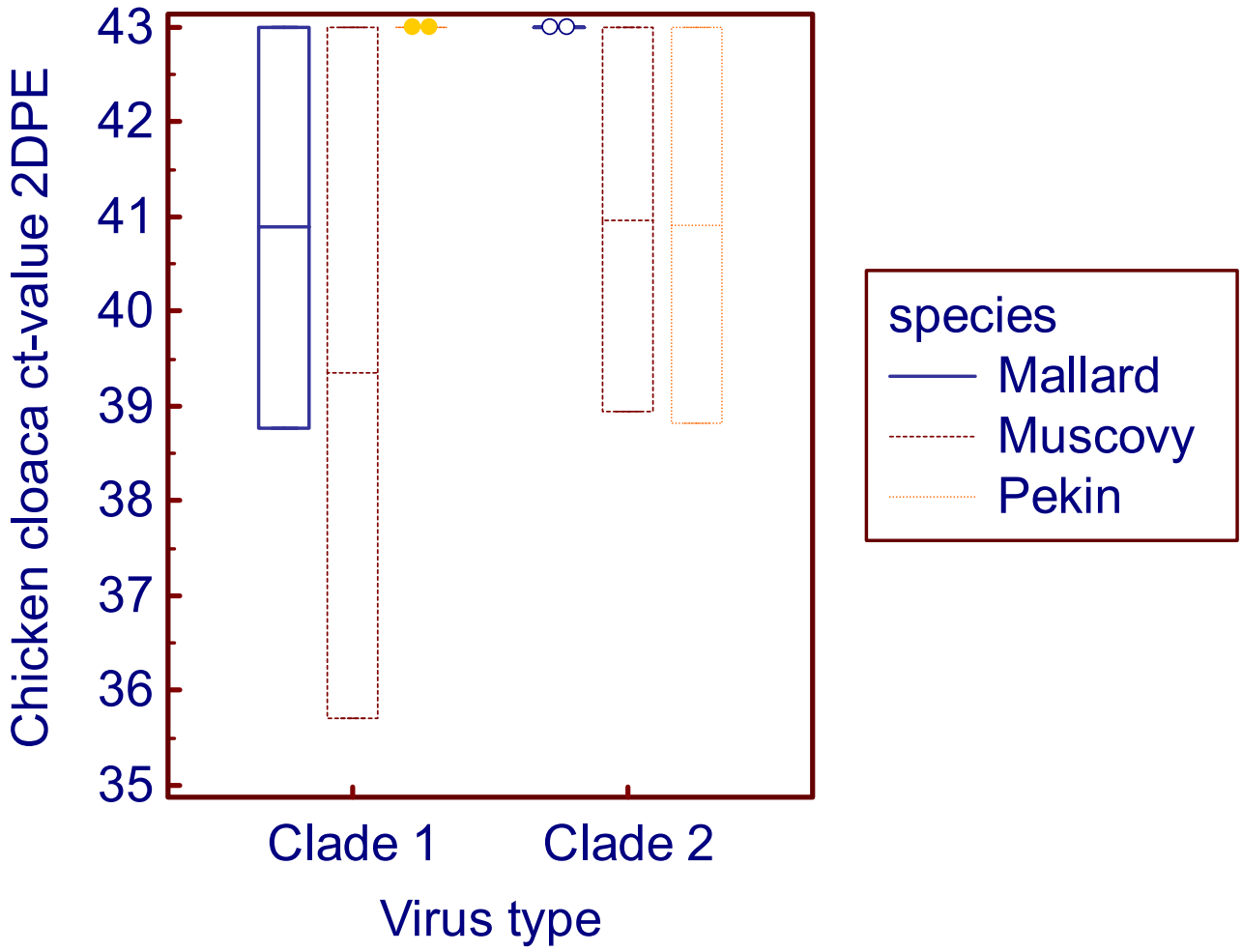

Figure 4.39. Clustered multiple variables graph: Cloacal Swab ct-values of chickens exposed to Clade 1 and 2.2 virus 2DPE

4.4.6.2 Tracheal and Cloacal Swab Analysis 3rd Day Post-Exposure (d.p.e) Chickens

When the mean tracheal and cloacal ct-values were compared between the chickens exposed to the duck species, i.e Muscovy, Mallard and Pekin in the two virus groups at 3 d.p.e, it was found that the mean tracheal swab ct-values for chickens in the Muscovy ducks group was the only highly positive mean ct-value (clade 1, μ=19.02; clade 2.2, 23.65) in the virus groups (Fig. 40). Also, the mean cloacal swab ct-values for chickens in the Muscovy ducks group was the only highly positive mean ct-value (clade 1, μ=20.55; clade 2.2, 23.43) in the virus groups (Fig 41). A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, showed that at 3 d.p.e, there was a significant difference (Bonferroni corrected P=0.001) in the mean tracheal ct-values of the chickens exposed to infected Muscovy ducks when compared with the chickens exposed to infected Mallard ducks.There was also a significant difference (Bonferroni corrected P=0.0035) in the mean tracheal ct-values of the chickens exposed to infected Muscovy ducks when compared with the chickens exposed to infected Pekin ducks. There was no significant difference (Bonferroni corrected P=0.94) in the mean tracheal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 irrespective of the duck species of exposure at 3 d.p.e. This result also applied to the mean cloacal ct-values. There was a significant difference (Bonferroni corrected P=0.004) in the mean cloacal ct-values of the chickens exposed to infected Muscovy ducks when compared with the chickens exposed to infected Mallard ducks. There was also a significant difference (Bonferroni corrected P=0.004) in the mean cloacal ct-values of the chickens exposed to infected Muscovy ducks when compared with the chickens exposed to infected Pekin ducks. There was no significant difference (Bonferroni corrected P=0.67) in the mean cloacal ct-values of chickens exposed to clade 2.2 when compared with chickens exposed to clade 1 irrespective of the duck species of exposure at 3 d.p.e.

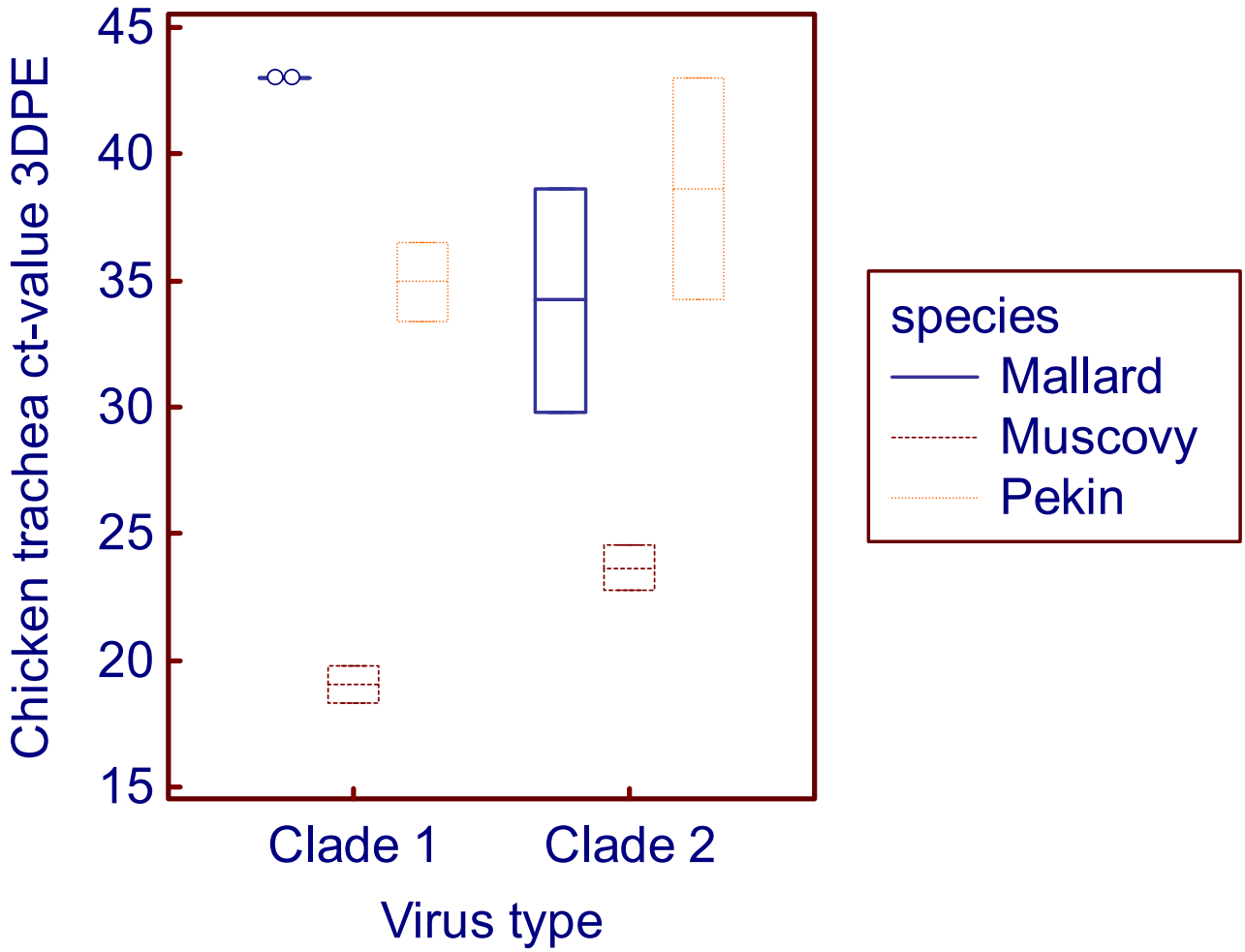

Figure 4.40 Clustered multiple variables graph: Trachea Swab ct-values of chickens exposed to Clade 1 and 2.2 virus 3DPE

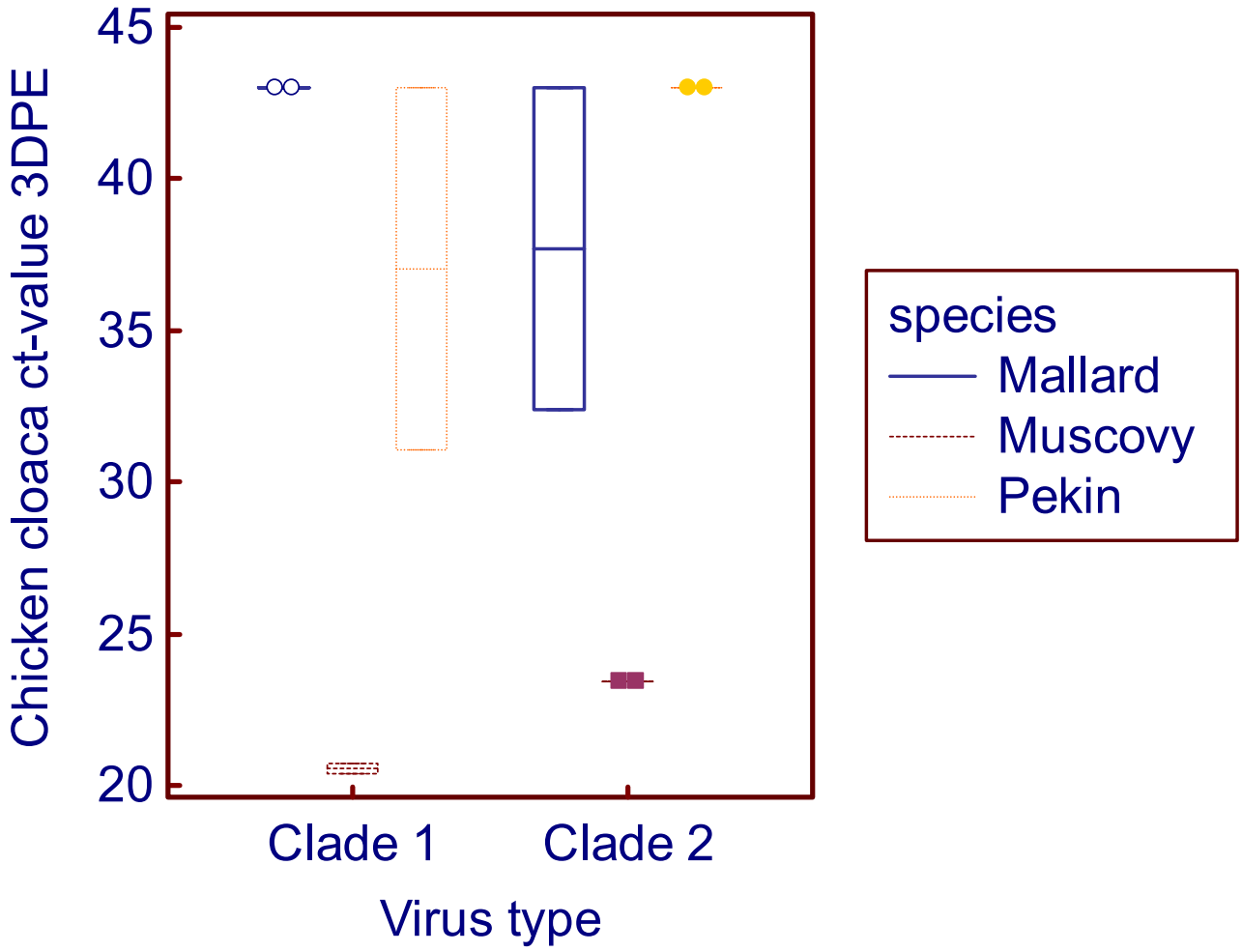

Figure 4.41 Clustered multiple variables graph: Cloaca Swab ct-values of chickens exposed to Clade 1 and 2.2 virus 3DPE

4.4.6.3 Tracheal and Cloacal Analysis 4th Day Post-Exposure (d.p.e)

When the mean tracheal and cloacal ct-values were compared between the chickens exposed to the remaining duck species at 4 d.p.e, i.e Mallard and Pekin in the two virus groups, it was found that the mean tracheal swab ct-values for chickens in the Pekin ducks group was highly positive (clade 1, μ=19.24; and clade 2.2, 22.23) in the two virus groups (Fig.4. 42). The mean cloacal swab ct-values was highly to moderately positive (clade 1, μ=18.92; and clade 2.2, 25.02) in the two virus groups (Fig 4.43). A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, of the mean tracheal and cloacal swab ct-value showed that at 4 d.p.e, there was a significant difference (Tracheal: Bonferroni corrected P=0.0001; Cloacal: Bonferroni corrected P=0.0025) in the mean tracheal and cloacal ct-values of the chickens exposed to infected Pekin ducks when compared with the chickens exposed to infected Mallard ducks. There was no significant difference (Tracheal: Bonferroni corrected P=0.53; Cloacal: Bonferroni corrected P=0.8) between the mean tracheal and cloacal ct-values of chickens exposed to clade 2.2 and those exposed to clade 1 at 4 d.p.e irrespective of wether they were exposed to Pekin or Mallard ducks.

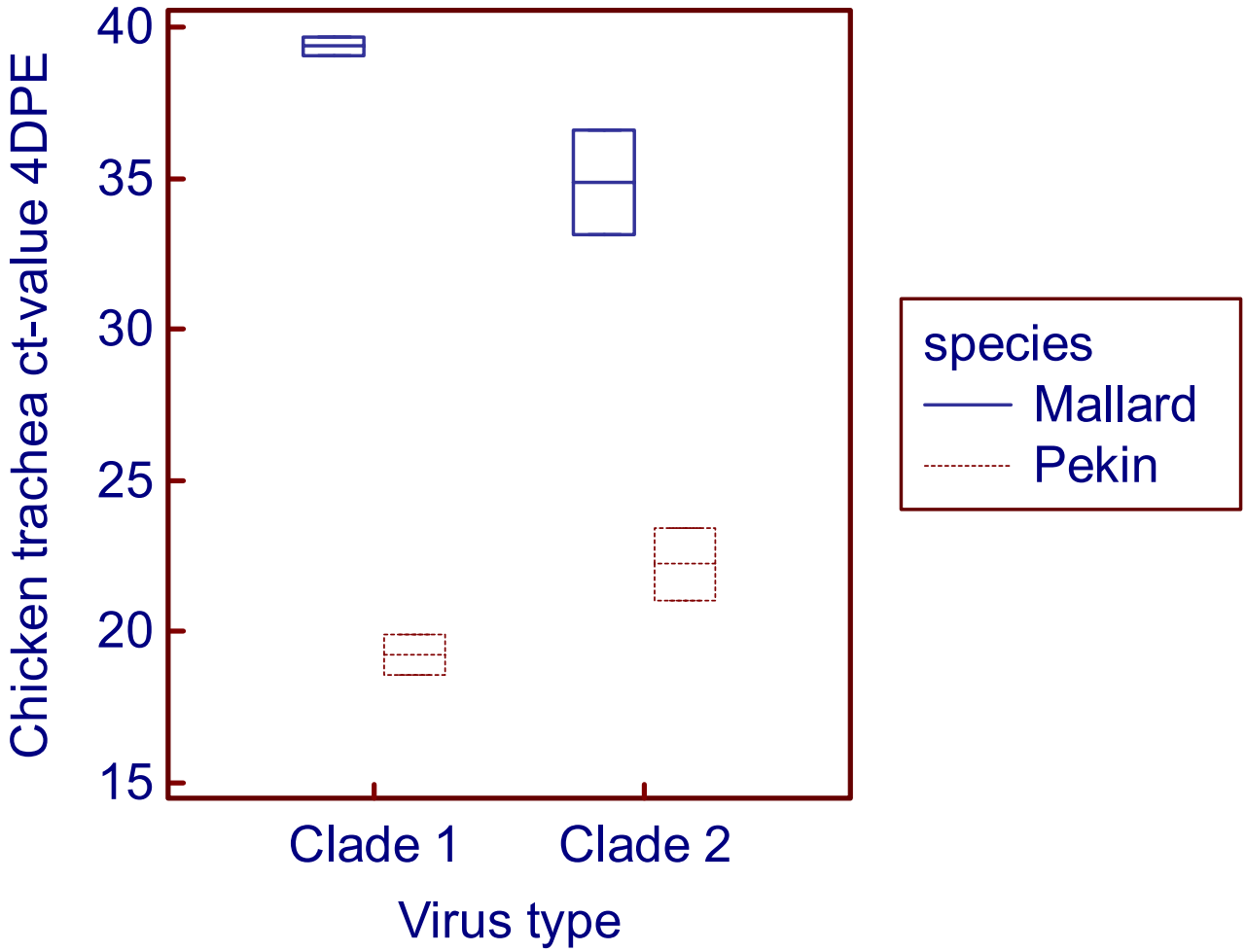

Figure 4.42. Clustered multiple variables graph: Trachea Swab ct-values of Clade 1 and 2.2 virus 4DPE

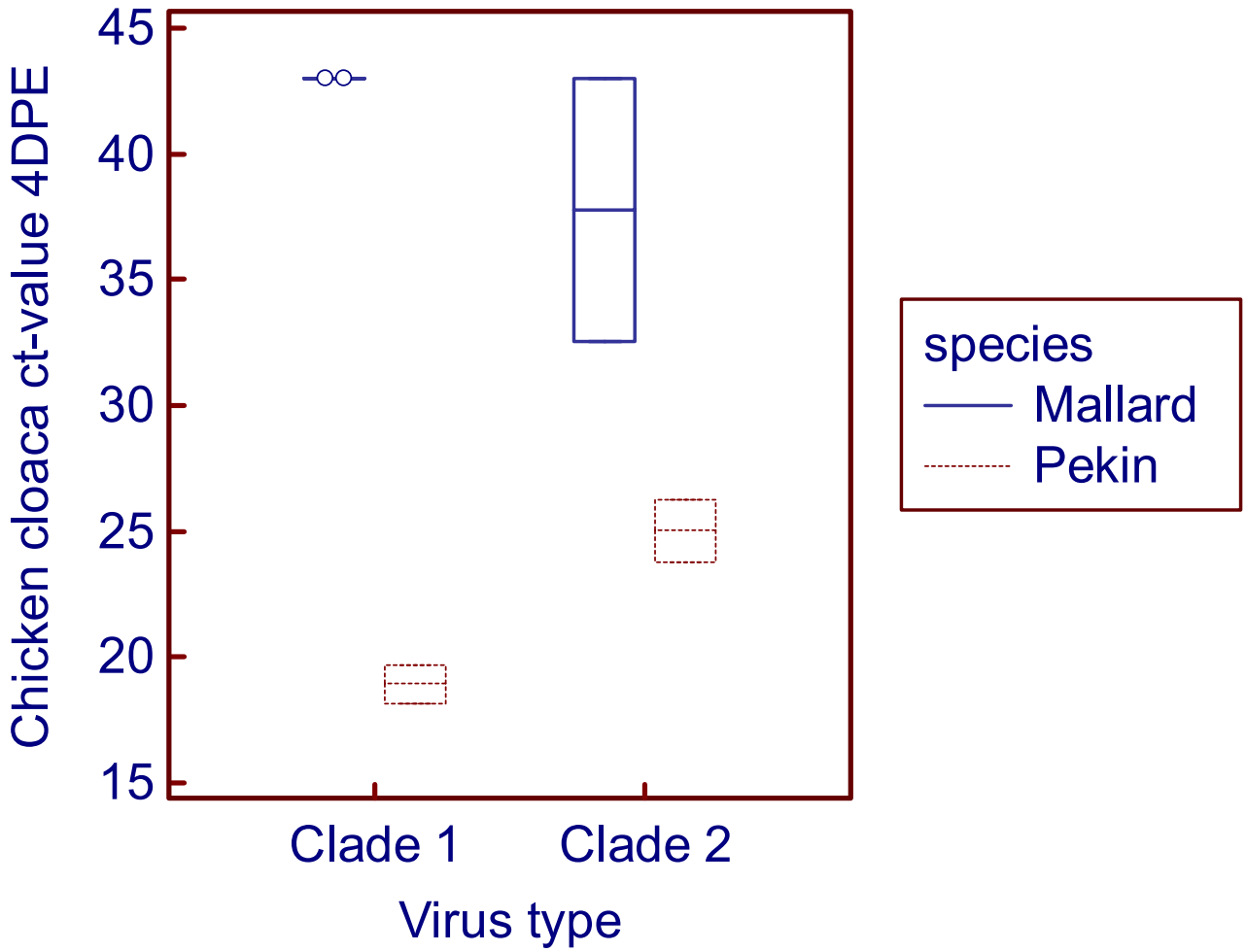

Figure 4.43. Clustered multiple variables graph: Cloacal Swab ct-values of Clade 1 and 2.2 virus 4DPE

4.4.6.4 Tracheal and Cloacal Analysis 5th Day Post-Exposure (d.p.e)

When the mean tracheal and cloacal ct-values were compared between the chickens exposed to Mallard and Pekin in the two virus groups 5 d.p.e, it was found that the mean tracheal swab ct-values for chickens in the Pekin ducks group was highly positive in clade 2.2 (μ= 20.34) but negative in clade 1(Fig 4.44). The mean cloacal swab ct-values for chickens in the Mallard and Pekin ducks group were moderately positive (Mallard, μ= 25.48; Pekin, μ= 25.52) only in clade 2.2 virus group (Fig 4.45). A pairwise comparisons of a two-way analysis of variance using Medcalc software version 11.5.0, 2011, of the mean tracheal swab ct-value showed that at 5 d.p.e, there was a significant difference (Tracheal: Bonferroni corrected P=0.01) in the mean tracheal swab ct-values of chickens exposed to clade 2.2 in contrast to those exposed to clade 1 at 5 d.p.e irrespective of whether they were exposed to Pekin or Mallard ducks. This was not so with the cloacal swab mean ct-value, as there was no significant difference (Bonferroni corrected P=0.06) in the mean cloacal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 irrespective of the duck species of exposure at 5 d.p.e. There was no significant difference (Bonferroni corrected P=0.08) in the mean tracheal ct-values of the chickens exposed to infected Mallard ducks when compared with the chickens exposed to infected Pekin ducks. There was no significant difference (Bonferroni corrected P=0.39) in the mean cloacal ct-values of the chickens exposed to infected Mallard ducks when compared with those exposed to infected Pekin ducks. Total trachea and cloaca ct-values are shown in figures 4.46 and 4.47.

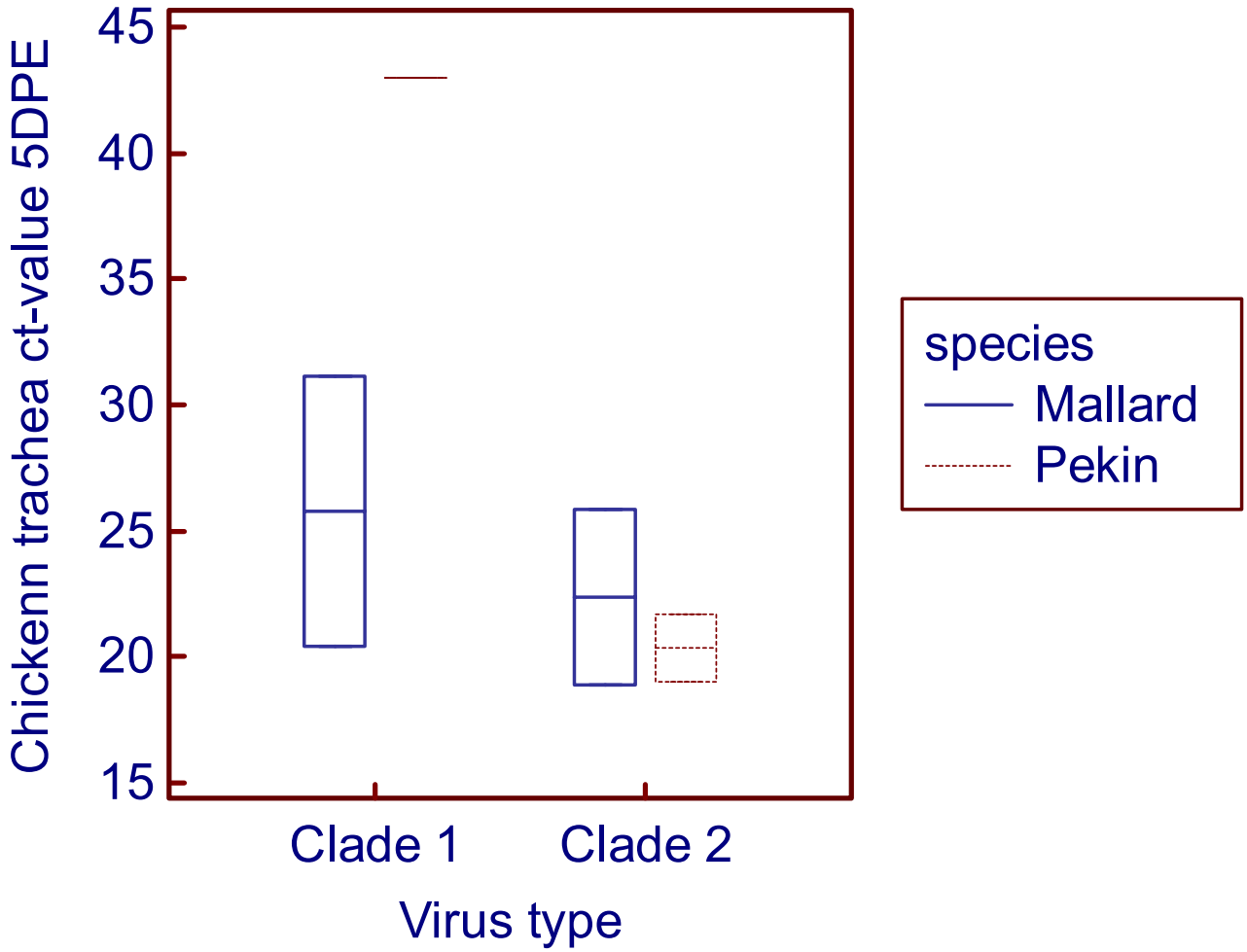

Figure 4.44: Clustered multiple variables graph: Tracheal Swab ct-values of Clade 1 and 2.2 virus 5DPE

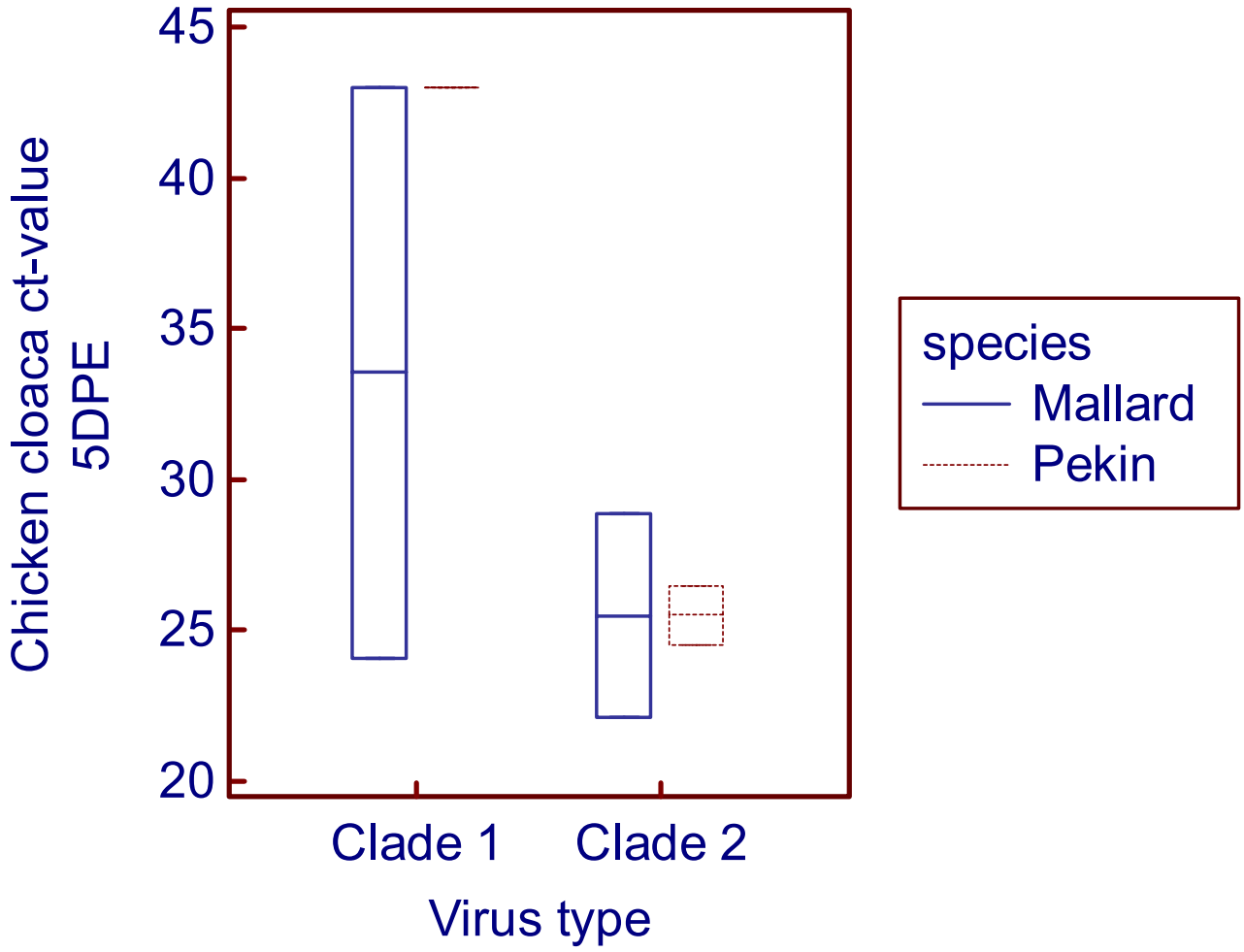

Figure 4.45: Clustered multiple variables graph: Cloaca Swab ct-values of Clade 1 and 2.2 virus 5DPE

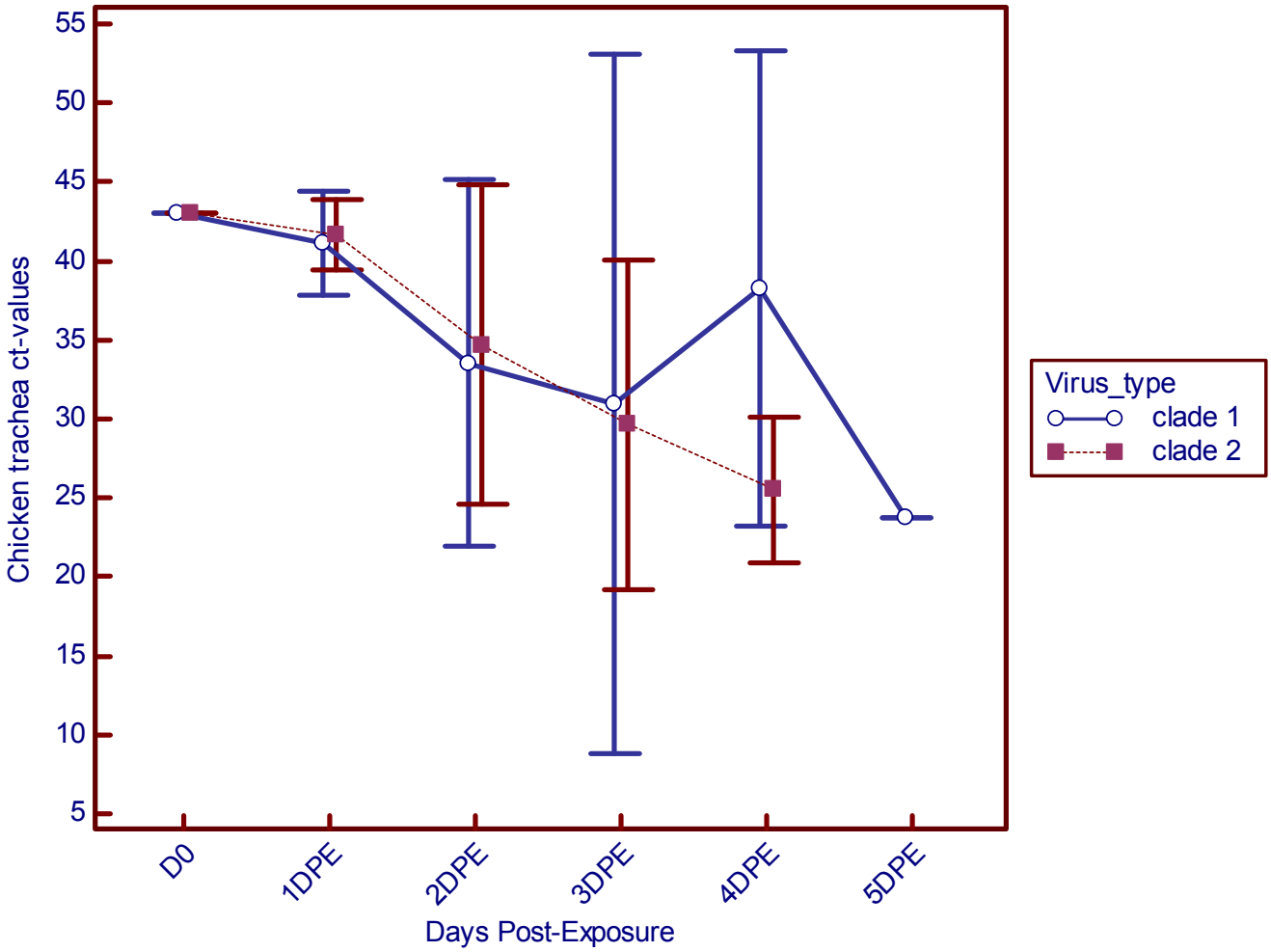

Figure 4.46: Clustered multiple variables graph: tracheal Swab ct-values of Clade 1 and 2.2 virus

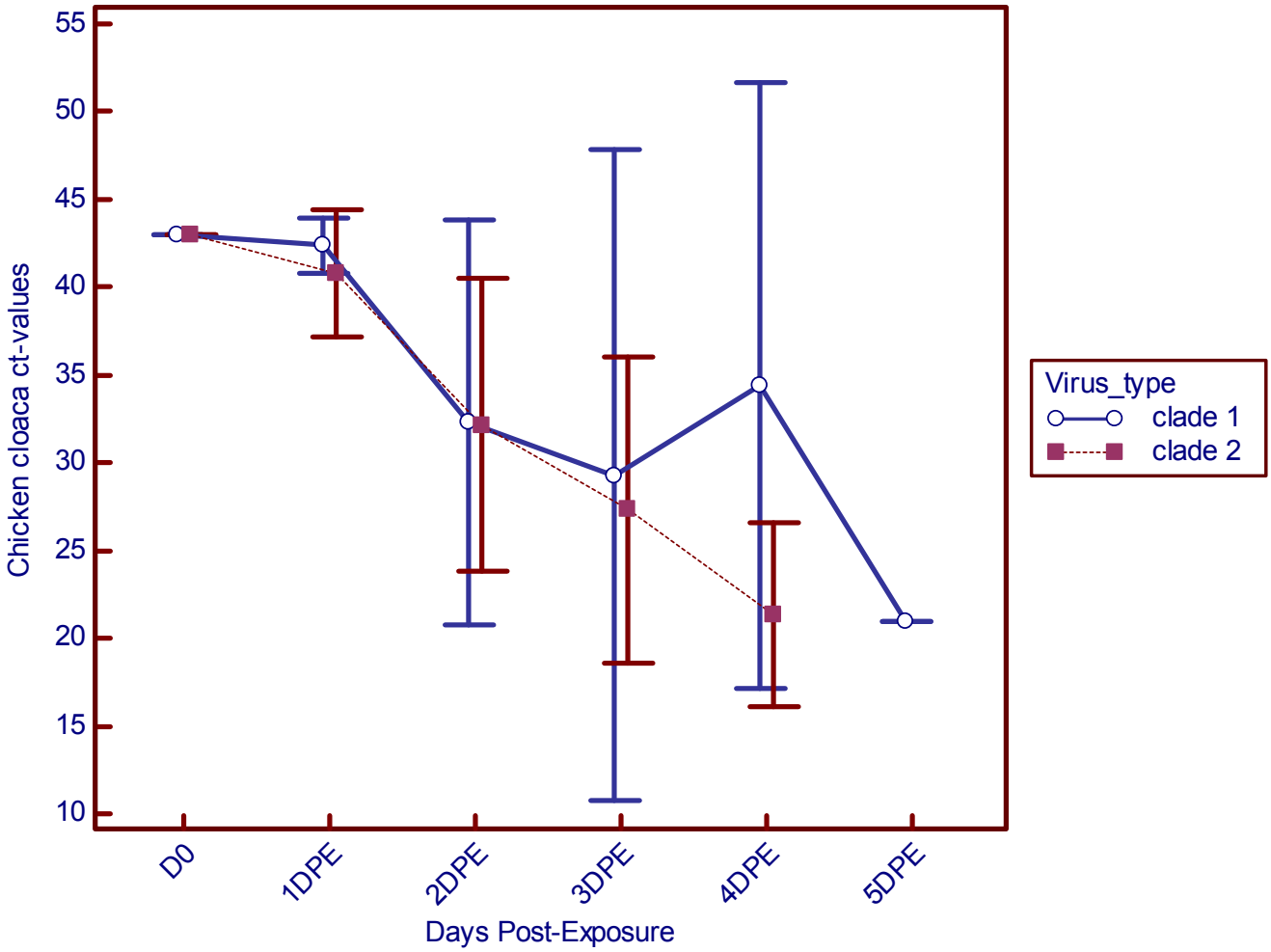

Figure 4.47 Clustered multiple variables graph: Cloacal Swab ct-values of Clade 1 and 2.2 virus

4.4.7 Comparative analysis of *Influenza A/Cygnuscygnus/Germany/R65/2006* of clade 2.2 subtypes H5N1 virus exposure in Pekin ducks(Anas platyrhynchos)

4.4.7.1 Clinical signs and Mortality

At 3dpe, The Pekin duck with the Muscovy infected group in Clade 2.2 virus group, died without clinical sign of disease. While the Pekin duck with the Mallard group at 5 days p.e, showed somnolence, and squatted on the floor, and was sacrificed on the 6 days p.e. The Pekin duck with the Pekin group showed no sign of sickness and was sacrificed at 6days p.e.

4.4.7.2 Gross, Histopathology and immunohistochemical detection

Pekin duck in the Muscovy group (Sacrificed 3DPE): Grossly this duck showed splenomegaly, pulmonary congestion and pancreatic necrosis. Within the cerebellum were multifocal glia nodule formations and the conjuctival epithelium was desquamated. The feather follicular keratinocyte of the eyelid showed multifocal necrosis, and the trigeminal nerve branch of the nares had glia nodule formation and neuronal degeneration. There was adrenal cortical and medulla necrosis. There was mild lymphoid depletion within the bursa of Fabricius follicles. Microscopically, there was mild lymphoid depletion with numerous hemosiderin laden macrophages in the spleen. The airsac had foci of extending epithelia necrosis while the trachea epithelium was infiltrated by moderate lymphocyte, plasma cell and heterophils. Microscopically, numerous hemosiderin laden macrophages were seen within parabronchi and the pulmonaryatria epithelium which were desquamated and showed BALT necrosis. The jejunum intramural ganglia showed neuronal degeneration and the liver had fatty infiltration. The gross pancreatic multifocal necrosis was evident as foci of acinar necrosis with scattered lymphocyte infiltration microscopically. Immunohistochemical detection of H5 antigen was found in the following cell types: strongly (++) positive within the eyelid and feather follicle. It was mildly (+) positive in the nares and within the respiratory epithelium. The mononuclear cells of the adrenal gland were strongly (++) positive. The cardiomyocytes were mildly (+) positive. Single cells in the air sac epithelium were mildly (+) positive. The jejunum intramural ganglion was mildly (+) positive and single acinar cell of the pancreas was mildly (+) positive.

Pekin duck in the Pekin group (Sacrificed 6DPE): Grossly, the spleen was enlarged and the pancreas showed multifocal necrosis. In the cerebrum and cerebellum were multifocal perivascular lymphocyte cuffs with glia proliferation and foci of glia degeneration. The eyelid had focal dermal edema with multifocal lymphocyte infiltration and the nasal turbinate showed multifocal epithelia degeneration with subepidermal edem,a lypmhocytes and macrophage infiltration. There was multifocal cardiomyofibrillar degeneration and lymphocytes infiltration. The trachea was infiltrated by moderate heterophils and macrophage. The alveolar epithelia and subepithelia have diffuse moderate heterophilic infiltration that extended into the parabronchi and the interstitium contain edema fluid admix with mild heterophil. Microscopically, mild depletion with numerous hemosiderin laden macrophages. The (GALT) within the esophageal submucosal was prominent and had desquamated epithelium. Microscopically, single acinar cell epithelia degeneration with focal lymphocyte infiltration. Within the myofibril of thigh skeletal muscle were multifocal lymphocyte and plasma cells infiltration. Immunohistochemical detection of H5 Antigen was found in the following cell types: Neurons of the cerebrum showed mild (+) positivity. Within the comb, the keratinized epithelium showed moderate (++) positivity and in the bursa of Fabricius, follicular mononuclear cells showed mild (+) positivity.

Pekin duck in the Mallard group (Sacrificed 6DPE): The only gross lesion shown by this duck was splenomegally.There was cerebral and cerebellar multifocal glia proliferation with submeningeal and perivascular lyphoplasmacytic infiltration. The spinal cord showed moderate glia proliferation and the nasal palatine epithelium showed degeneration. There was multifocal mild lymphocytic adrenalitis with ganglioneuritis. Splenic periateriolar hyperplasia and lymphoid depletion waqs seen microscopically. Pulmonary consolidation and edema were seen microscopically with diffuse moderate heterophilic infiltration into the alveolar. The liver showed fatty change. Immunohistochemical detection of H5 antigen was in the following cell types: The neuron and glia cell of the cerebrum and cerebellum were strongly (++) and mildly (+) positive respectively. The nasal septum was strongly (++) positive.

4.4.7.3 Real-Time Reverse Transcriptase Polymerase Chain Reaction (real-time RT-PCR) of Tracheal and Cloacal Swabs

All tracheal swabs were negative at day zero. Two of the ducks (one exposed to Mallard and the other to Muscovy) were positive for the clade 2.2 HPAIV on day 1 p.e, but the duck with Pekin was not positive until 2nd p.e before been killed on the 3rd day p.e. The remaining ducks were killed on the 6th dpe (Fig 4. 48). The cloacal swabs rRT-PCR were irregular and undulation in the Pekin duck in the Mallard group (Fig 4.49), while the duck in the Pekin group had a ct-value of less than 25 (very positive) at 2 days p.e.

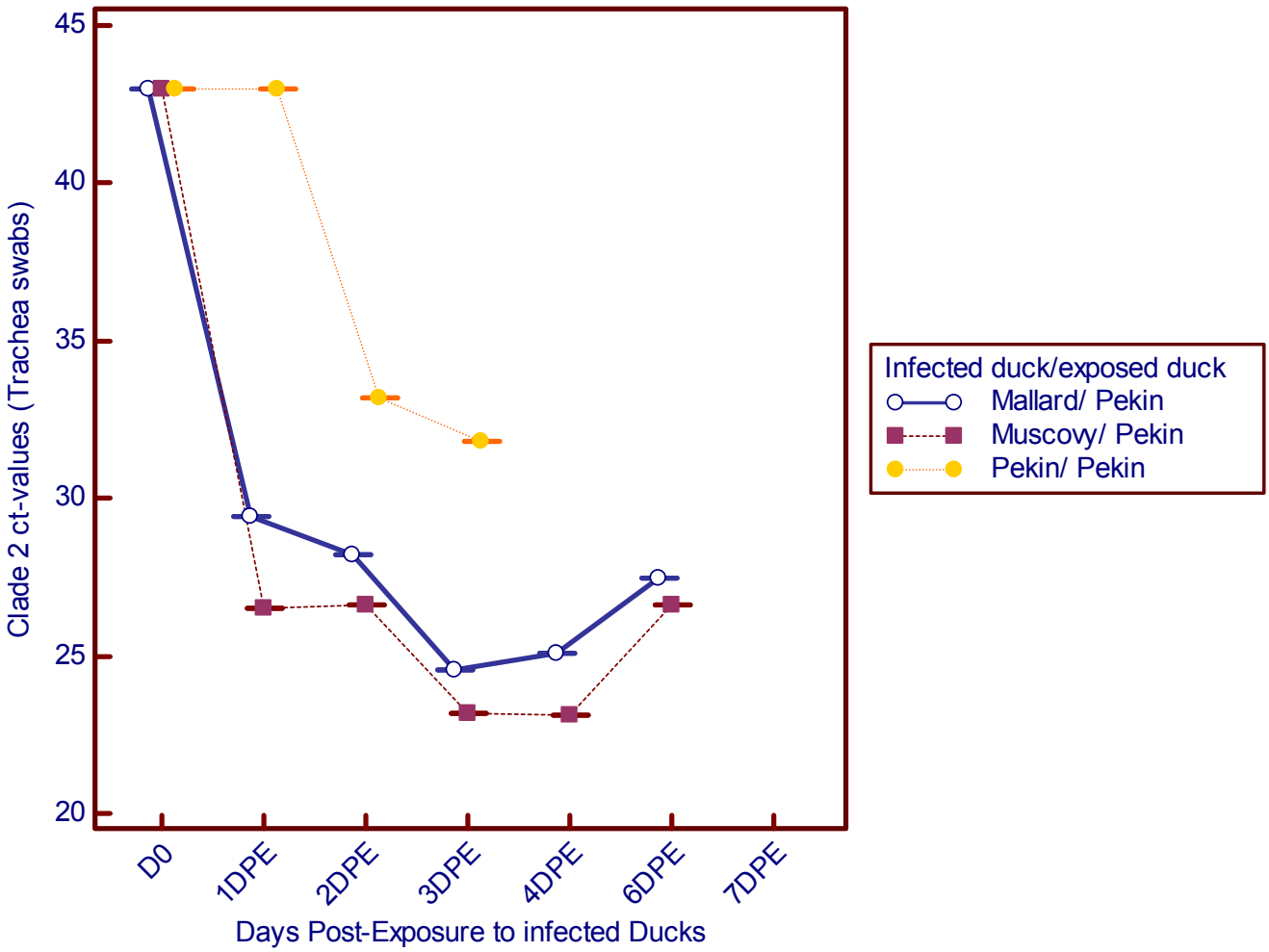

Figure 4.48. Clustered multiple variables graph: Trachea Swab ct-values of Clade 2 virus

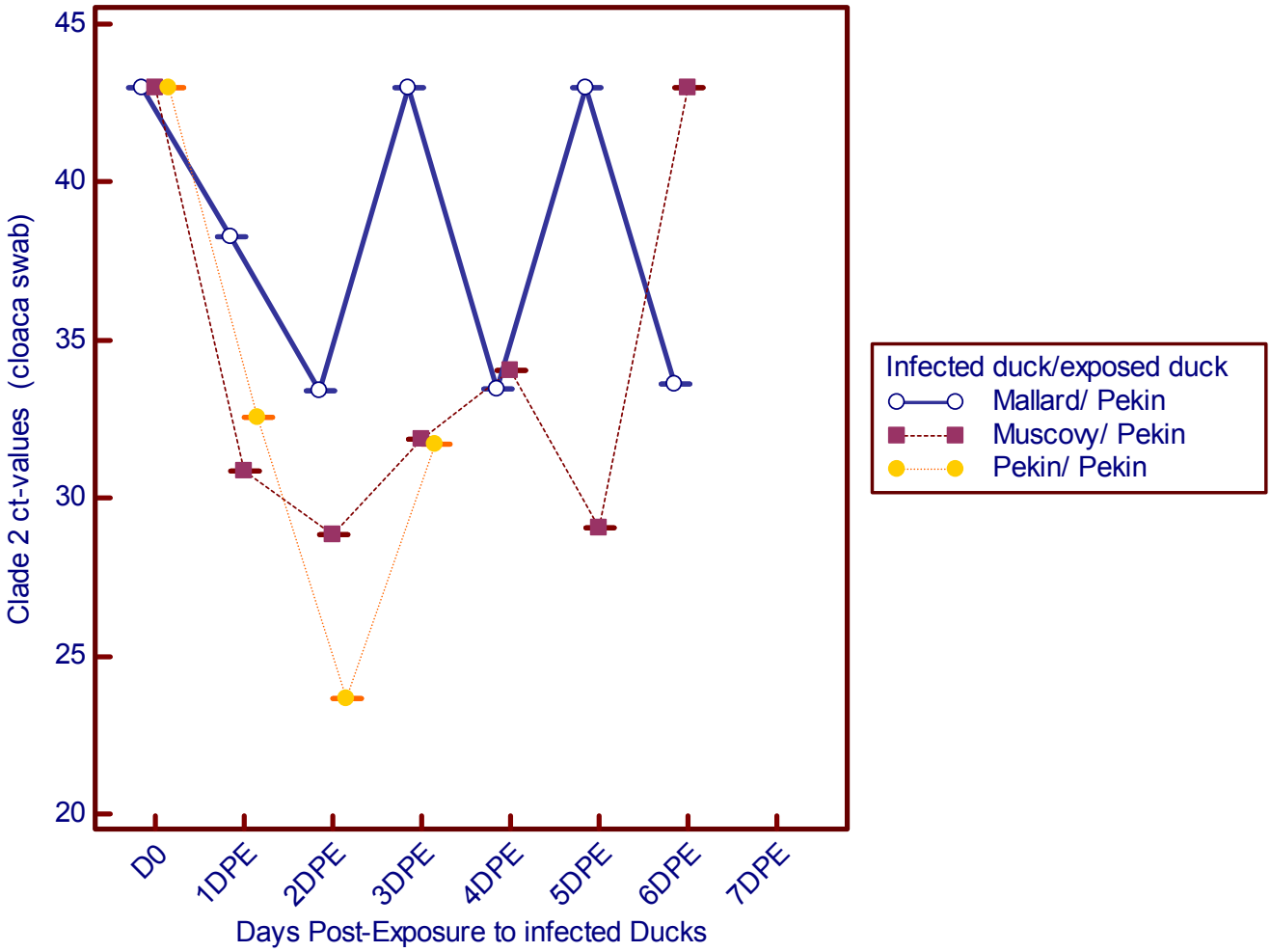

Figure 4.49. Clustered multiple variables graph: Cloaca Swab ct-values of Clade 2 virus.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Study 1: The Epidemiology and Pathology of Highly Pathogenic Avian Influenza (HPAI) H5N1 outbreaks in Commercial and Backyard poultry between 2006 and 2008 in Nigeria.

Documented case histories obtained directly from farmers or AI desk officers who reported to the Central Diagnostic Laboratory (CDL) of NVRI in Vom, used in this study, showed that one million, one hundred and six thousand, five hundred and seventy birds (1,106,570) including chicken (broiler, cockerel, pullet and layer), duck, turkey, geese and guinea fowl were lost between 2006 and 2007 as against over 1.2million birds earlier reported (Maina, 2008). There were discrepancies in the figures given as total bird losses (as a result of death or stamping out of infected flock), during the course of HPAI infection in the country. It is also possible that many more bird were lost as a result of the cases that were not reported due to farmers' ignorance and fear of depopulation/stamping out policy which was the control measure adopted(Fusaro *et al.,* 2010). The bird losses in commercial poultry were highest in the North-western and South-western parts, and this could be attributed to the high density poultry populations in these areas. The bird loss figures in the commercial poultry sector was higher than the backyard poultry because the intensive poultry units have an ideal viral breeding ground due to the artificial density of individual host which increases spread to other individuals; amongst other reasons (Caron *et al*., 2009). The results showed a more devastation of the poultry economy in the North-western and South-western parts and is contrary to the initial reports of earlier investigators (Fasina *et al*., 2008a; Saidu *et al*., 2008), who reported that the disease devastated the poultry sector of the economy, in the north-central part of Nigeria. Although more outbreaks reporting were recorded in the north-central part of the country, more birds were infected/stamped out in both North-western and South-western parts of the country. For backyard poultry, the bird loss figure is highest in the North-west region; this may partly be because of the proximity of this region to the Central Diagnostic Laboratory of NVRI in Vom. It may also be because some of the reporting states were areas which habour a large backyard poultry population and the highest concentration of domestic ducks, reared under free-range conditions, providing opportunities for contact between wild birds and backyard poultry, as previously reported

for this region (Cecchi *et al*., 2008). The finding of high mortality in poultry in the North-western States is consistent with earlier reports by Adene *et al.*, 2006 and Saidu *et al.*, 2008. Under the sector 1, 2 and 3 of the Poultry production systems practiced in Nigeria (Adene and Oguntade, 2006), the commercial layer-type chicken was the most hit by the highly pathogenic avian influenza outbreaks. With a total flock size of 939,620 lost in 127 farms and holdings having confirmed outbreaks, the commercial layer-type chicken suffered a major economic loss and devastation. This is attributed to the large number of laying birds in individual flocks as compared to other chicken type under these sectors. This significantly affected the economy not only because a whooping sum of N631 million (US$5.43 million) was paid as compensations to farmers (Maina, 2008, Durosinlorun *et al.,* 2010) but also because laying hens contribute huge resources to the national poultry flock and this emphasizes the importance of commercial layer flocks for Nigeria's economy (Fasina *et al.,* 2008). It was observed, that as at the time of reporting by livestock owners and farmers, the presence of disease as evident by high death rate was between 2-3 days post onset of disease. This study showed also, that mortality rate was least in commercial layers (11.11%) and highest in broilers (73.92%). It was higher in cockerels (males = 45.51%) than in pullets (females = 26.84%), for no obvious reasons.This may suggest that young males, in this case cockerels were more susceptible to the virus than females (pullets) during these outbreaks in Nigeria. This mortality pattern explains the initial observation of variable clinical signs from the index case of HPAI in Nigeria (Kumbish *et al*., 2006). The signs varied with age of bird, sex, concurrent diseases and the environment. In an attempt to relate outbreaks clusters with geo-ecological features especially wetlands, because of their importance in the ecology of AI, one of the outbreak clustered around the Hadeija-Nguru wetland in Jigawa State which is significant for the wild bird wintering activities (Fusaro *et al.,* 2009). Some isolated HPAI detection points in 2008 after a nine month period of lack of outbreaks especially in Gombe and Kebbi also had presence of wetlands. This may suggest that there is an association between HPAI occurrence and presence of wetland but not necessarily transmission of HPAI virus from wild birds. Detailed epidemiological investigation, wild bird monitoring and phylogenetic analysis of isolates in wild birds and backyard poultry in areas of wetlands will possibly elucidate this dichotomy as it was shown that offal from infected ducks was responsible for an infection in backyard poultry in Germany in 2007 (Harder *et al*., 2009). It is known that the Clade 2.2 HPAI virus originated in April 2005 during a large outbreak of a phylogenetically distinct H5N1 virus among wild bird populations at Qinghai Lake in western China (Chen *et al*., 2004, Liu *et al*., 2005)

and rapidly spread west through central Asia and Europe, eventually reaching Africa in 2006 (Salsberg *et al*., 2007). The southwestern outbreaks cluster, though clustered around some wetlands, does not have significant wild bird activity. The necropsy finding in a total of four hundred and sixty-eight (468) carcasses from two hundred and thirty-three (233) farms/holdings in 25 states and FCT showed that, the clinical signs and lesions were more severe in younger birds and layers with older flocks showing more overt symptoms and lesion. During the HPAI outbreaks in Nigeria in affected backyard flocks, acute mortalities were experienced but with little or no premonitory signs and with or without gross lesions of petechiation of coronary fat as we have earlier observed (Akanbi *et al.,* 2007). It was also observed that there was high mortality and minimal clinical evidence in free range chickens. This has been attributed to be one of the potential clinical outcomes of AI infection (Swayne, 2007) in some part of the world (Perkins and Swayne, 2002a) but not in free-range chickens in Nigeria. Since the first reported outbreak in backyard poultry flock in chickens and duck in Potiskum and Jakusko towns in Yobe State on the 16th of February, 2006 (NVRI records, 2006), earlier post mortem findings of high mortality and minimal clinical evidence in free-ranging chickens from Taraba State (NVRI records, 2006) made us to suggest the probable acuteness of the infection in this chicken type (NVRI records, 2006, Kumbish *et al*., 2006), but the overall post mortem findings of the free-ranging chickens during the HPAI outbreaks in Nigeria as shown by the results of this study showed similar trend of little or no clinical signs with high mortality. It has been observed that pathobiological changes are abnormal physiological and anatomic changes that occur as a result of virus replication within the cell, tissue, organ, or a combination (Swayne, 2007). In general, as virus replication titers increase, so does the severity of pathobiological changes such as gross and microscopic lesions with the most pathogenic virus strains causing major cell damages and death if it is sufficiently severe to affect critical organs (Swayne, 2007). This study therefore submits that free-ranging chickens otherwise called local chickens are less resistant to infection by HPAI H5N1 viruses in Nigeria, hence birds died per-acutely without exhibiting clinical signs. The lesions in other species examined, especially in ducks and turkeys are characteristics of findings already reported in the literature e.g nervous signs and neuronal necrosis, although this was only seen in 4 (13.7%) of ducks and 1 (2.7%) of turkeys. Other lesions were multi-systemic affecting the cardiovascular, respiratory and GI systems. More than half (7(58.3%)) of the geese showed nervous signs and neuronal necrosis and more than half (8(57.1%)) of the guinea fowls died suddenly. Adene *et al*. 2006 reported that the infection was rather fulminating in turkeys with 100% mortality while

mortality in geese was 50% in the maiden case. In commercial layers, similar signs and lesions as seen in this study were earlier reported by investigators of the index case and earlier outbreaks of HPAI in Nigeria (Adene *et al.,* 2006; Joannis *et al.,* 2006*;* Kumbish *et al*., 2006). In addition to what has been reported, this study found lesions of airsacculitis, pneumonia, pancreatic necrosis and pancreatitis in the commercial birds that were necropsied. Only 20% of the 248 dead birds examined had histories of neurologic signs and lesions contrary to what was observed in the index case where only young stocks had nervous system signs (Adene *et al.,* 2006). Also, other investigators reported presence of nervous signs and lesions with no particular reference to age (Joannis *et al.,* 2006) and with reference to adult birds (De Benedictis *et al.,* 2007). The development of neurological signs or lesions vary from bird to bird, as also shown by the pathobiology of a prototype H5N1 HPAI virus, A/chicken/Hong Kong/220/1997 with only a few chickens having neurological dysfunction (Perkins and Swayne, 2002a). Neurological signs in these birds show that the virus has strong tropism for the central nervous system (Perkins and Swayne, 2002a). This may also relate to the finding that the factors influencing the clinical course relate to species, age of animals, and the virus strain (Pantin-Jackwood and Swayne*,* 2007*;* Pantin-Jackwood *et al.,* 2007).

5.2 Study 2: The Influence of mixed species poultry farming on the mortalities of highly pathogenic avian influenza (HPAI) H5N1 outbreaks in Backyard poultry between 2006 and 2008 in Nigeria.

Under the sector 4 of the (backyard poultry) production systems practiced in Nigeria (Adene and Oguntade, 2006; FAO, 2008), the backyard domestic chicken was most hit by the highly pathogenic avian influenza outbreaks experienced by the Nigerian village poultry household. With a total flock size of 14512 lost in 80 backyard flock having confirmed outbreaks, the backyard domestic chicken suffered a major economic loss and devastation. This is because the predominant species in the rural poultry sector of Africa is the scavenging indigenous domestic fowl (*Gallus domesticus*) (Kitalyi*,* 1998). Majority of the cases included in this study were reported from the northern part (north central, north east and northwest) of Nigeria as earlier noted above. It cannot be ascertained whether this result is a reflection of the finding of Cecchi *et al.* (2008), that this area sustains a large backyard poultry population and the highest concentration of domestic ducks, reared under free-range conditions, providing opportunities for contact between wild birds and backyard poultry. Or it was just that HPAI cases reporting were more from this part of the country. This study result revealed that amongst single species backyard poultry flocks, percent mortality was highest in ducks (90.16%) and turkeys (89.39%) and followed by chicken (76.38%) leaving mortality lowest in guinea fowls (10.4%). Compiteli *et al.* (2004) reported that the mortality due to AI may be low in the ducks and geese, and field observations indicated that highest mortalities have been recorded mostly in chickens and turkeys (Aly *et al.,* 2006). But in Nigeria, Saidu *et al.* (2008) reported that the mortality rates were high for both geese and ducks and even higher than the mortality rates for turkeys, chickens, pigeons and guinea fowls. The result presented in this study is a national analysis as compared to that done for the North-western region by Saidu *et al*. (2008). The number of backyard poultry that died most from natural infection with HPAI in Nigeria was from the North-western region. The finding of high mortality in the North western states is consistent with earlier reports by Adene *et al*. (2006) and Saidu *et al*. (2008). The result here also indicates that mixed species backyard poultry especially chicken, duck and turkey combination recorded the highest percent mortality (90.72%) amongst all kinds and species combination of backyard poultry. This is also confirmed by the odds ratio analysis which was 3.02 higher in chicken, duck and turkey only flocks than in chicken only flocks and represent the highest odd ration in this study. This is

consistent with the finding of Aly *et al.* (2006), but differs in the species combination. Having found out that the percent proportion of number dead in the duck only flocks seems to be a major factor in single species backyard poultry flocks and it also influences the percent proportion of number dead in mixed species backyard poultry flocks and multiple mixed species backyard poultry flocks.

5.3 Study 3: Determination of the Usefulness of Archival Formalin-fixed Paraffin-embedded (FFPE) Tissues from Chickens naturally infected with HPAI H5N1 Retrospective Diagnosis of AI in Nigeria

All FFPE tissue samples from the ten outbreaks of HPAI-H5N1 in commercial chickens in Nigeria taken from the NVRI archive in 2006 and 2007 included in this study proved useful for immunohistochemical evaluation. Samples of different tissues demonstrated lesions consistent with acute infections seen in natural and experimental cases of HPAIvirus H5N1. At least three tissues from all, except outbreak D/06, (only the comb and wattle vascular endothelium) and G/07, (cardiomyocytes and proventricular endothelium) were immunoreactive. In these tissues, Immunohistochemistry (IHC) reactivity varied from strongly positive comb and wattle to mildly positive lung and spleen, proving tissue localization of the virus. In this study, seven out of ten RNA samples tested positive by rRT-PCR assays targeting the genes for the viral matrix protein and four samples for the haemagglutinin gene (HA2 part), respectively. The real-time PCR assay with the probe specific for the HA cleavage site of clade 2.2 viruses revealed weakly positive signals only for two samples (A/07 and D/06) with threshold cycle (CT-values) in the HA2-PCR of about 30. The remaining 5 positive samples (B/07, D/06, F/06, H/06, and J/06) were too weak for successful detection using the cleavage site rRT-PCR which is known to have a reduced sensitivity compared to the other both rRT-PCR assays (Goelz *et al*., 1985). When the rRT-PCR result was compared with the IHC, the 3 rRT-PCR negative RNA samples from outbreaks C/06, E/06, G/07 had at least 2 tissue immunoreactivity by IHC, this was attributed to the absence of strongly immunoreactive lung, spleen, comb and wattle in the pooled tissues. In contrast to the good results of the M and HA2 rRT-PCR assays, all experiments to amplify the RNA samples by conventional RT-PCR were not successful. This might be due to the higher product sizes aimed in these assays and/or reaction conditions which were not adapted to this special material (Godfrey *et al*., 2000). The specificity of the rRT-PCR results was further validated by sequencing of four of the HA2 rRT-PCR positive samples (A/07, B/07, D/06 and I/06). Although only 154 nucleotides were included in the analysis, differences could be shown which might result from the co-circulation of different strains in Nigeria (Ducatez *et al*., 2007). Beside the nucleotide and amino acid comparison of the four sequences, basic local alignment search tool (BLAST) search might suggest that the viruses belong to different sub-clades although this should be verified by longer sequences. Previously, Ducatez and co-workers

(Ducatez *et al.,* 2006) identified three HPAI H5N1 virus strains circulating in Nigeria, which had close genetic relationships with viruses from Kurgan, Astrakhan and Egypt. The findings of this work might be a different co-circulation of viruses, other than the result of Ducatez et al. Formalin-fixed paraffin-embedded tissue samples have been used on molecular level especially for gene expression analyses in neoplasms (Abramovitz *et al*., 2008; Bibikova *et al*., 2004). Few studies were published about virus RNA detection from FFPE material (Krafft *et al*., 1997; Wakamatsu *et al*., 2007). Due to the degradation of RNA during the formalin fixation process which results in RNA species with an average size of ~200 nucleotides, the efficiency of RT-PCR assays is often clearly reduced compared to assays with RNA prepared from fresh tissues (Masuda *et al*., 1999). However, real-time RT-PCR assays with their increased sensitivity and aiming at products of small size can compensate this disadvantage (McKinney *et al*., 2009). In summary, this study has shown the suitability of 2-3 years old archival FFPE tissues from HPAIV infected chicken in the detection of H5N1 viral antigen by IHC, extraction of RNA, and generation of first short sequence information from PCR products. It was also partially possible to classify the viruses into sub-clade 2.2.via HA cleavage site- specific RT-PCR. This study also provided the opportunity to correlate retrieval of proteins for IHC and extraction of nucleic acids from archival HPAIV H5N1 paraffin embedded tissue sections. It will support studies on older archival materials in the near future, which would be useful in tracing the epidemiology of earlier H5N1 virus introductions into Nigeria, and this will serve as a control measure for prevention of future introductions.

5.4 Study 4: Susceptibility, Carrier States, Transmission, Pathogenesis and Pathology in Three Duck Species and their In-contact Chickens and ducks infected with clade 1 H5N1 and clade 2.2 H5N1 Viruses

Results from this experiment showed the susceptibility of the three ducks- Muscovy, Mallard and Pekin- to HPAIV infection. This is consistent with the findings of Chen *et al. (*2004); Cooley *et al*. (1989) and Shortridge *et al*. (1998) who reported that domestic ducks are also susceptible to AI virus infection. Clade 1 and 2.2 H5N1 viruses used in this experiment caused severe neurological signs and death as early as 3 days p.i in Muscovy ducks. No clinical signs (Pekin) or death was recorded in Pekin and Mallard ducks at the termination of the experiment 8 days p.i in clade 2.2 infections. Whereas in clade 1 virus infection at 5 and 8 days p.i, 30% of Pekins and 25% of Mallard respectively, died before the remaining in both species were sacrificed for animal welfare reasons after exhibiting neurological signs. This showed differences in the virulence of the viruses and susceptibility of the ducks to infection. Clade 1 H5N1 virus was more virulent than clade 2.2 H5N1 virus. Muscovy ducks were highly susceptible to both clade of viruses while Pekin and Mallard were moderately susceptible to clade 1 virus and not susceptible to clade 2.2 virus. Lack of clinical signs have however been observed in infected ducks in a fattening farm in Germany with no overt excess daily deaths (Harder *et al*., 2009). Earlier report showed that field and experimental data suggest that an introduction and subsequent spread of HPAIV (H5N1) in duck flocks is likely clinically silent (Smith *et al.,* 2006) and lack any clinical signs suggestive of an HPAIV infection, especially neurologic manifestations. But this experiment was able to show that neurological clinical signs were present in all the ducks (Muscovy, Pekin and Mallard) infected with clade 1 H5N1 virus and only in Muscovy ducks infected with clade 2.2 H5N1 virus. Therefore, it is possible that clade 1 H5N1 virus is more pathogenic than clade 2.2 H5N1 virus and that the presence of clinical signs in ducks infected with HPAIV virus is dependent on the virus strain used and the duck species. This finding also explains why Mallard ducks naturally infected with HPAIV remained apparently healthy in both live bird markets (Fusaro *et al*., 2009; Meseko *et al*., 2010); and backyard flock (Wakawa *et al*., 2008) in Nigeria. But the Muscovy ducks and geese in the same backyard flock showed severe clinical signs. This result has also shown Muscovy ducks has one of the duck species which show high virus replication and severe clinical signs contrary to earlier findings in ducks (Alexander *et al.,* 1978, 1986*;* Perkins and Swayne*,* 2002b). Intranasal and oral

administration of HPAIV A/ Duck/Vietnam/12/05 (H5N1) to ducks, in an earlier study by Harder *et al*. 2009, showed that clinical signs were observed to have started at 2 days p.i, with conjunctivitis and slight depression, progressing over a period of 1–3 days to severe neurologic signs consisting of torticollis, incoordination, tremors, and seizures. This is similar to the findings of this experiment, in particular with the clade 1 HPAIV H5N1 duck isolate. Also from Vietnam in the same year (*Starick, Per. Comm.,* 2009), in which the clinical sign of weakness was seen in the Muscovy ducks at 2days p.i. By 3dpi, the Muscovy ducks in both the clade 1 HPAIV H5N1 duck isolate and clade 2.2 HPAIV H5N1 swan isolate started to show severe neurologic signs consisting of torticollis, incoordination, tremors, seizures and death. While Mallard and Pekin only started to show clinical signs of weakness 4dpi in clade 1, and only Muscovy and Pekin showed clinical sign of weakness in clade 2.2 H5N1 virus infection at 4 dpi. Grossly, in clade 1 H5N1 infection, only one Muscovy had multifocal pancreatic necrosis with duodenal vascular congestion, all other Muscovy that died or were sacrificed at 3dpi showed no visible lesion as compared to six Muscovy ducks in clade 2.2 with multifocal pancreatic necrosis. Suggesting that the pancreatic cells are one of the tissues that are infected and die early in HPAIV infection in Muscovy ducks. The histopathological CNS lesion, as evident by multifocal perivascular lymphocyte cuff in the cerebrum and meningoencephalitis corroborate the neurological signs exhibited by the Muscovy ducks in clade 1 infection before death at 3days p.i. However in both clade infections, Muscovy ducks examined at 3days p.i showed no gross lesion in the cardiovascular and GIT systems. Histopathological lesion was also in intestinal intramural ganglia (degeneration and necrosis) in clade 2.2 infection, while immunoreactivity was only present in the intramural ganglia of the GIT tissues, testis and ovary in clade 2.2 infections. In addition, the microscopic findings in the central nervous system (cerebrum) and in the peripheral nervous system (intramural ganglia) suggest the strong tropism of the virus to these tissues during early HPAIV infection in Muscovy ducks. Gross lesion was rare in clade 2.2 virus infected Mallard ducks that were sacrificed on 4days p. i and was only seen in one Mallard, showing severe diffuse pancreatic necrosis. Histopathological lesions included severe diffuse meningo-encephalitis with multifocal lymphocytic perivascular cuffs. In clade 2.2 infection at 4 days p.i, bursa of Fabricius in Pekin duck shows follicular necrosis and repopulation by mononuclear (repopulation lymphocytes) cells and the heart had diffuse necrotizing myocarditis. With the occurrence of pancreatic (acinar) cells necrosis and neuronal necrosis in cerebrum and intramural ganglia, it can be said that these tissues are sensitive to the replication of HPAI virus in all ducks- Muscovy, Mallard and Pekin-

especially in early HPAIV infection. Also, Mallard ducks showed neuronal necrosis with diffuse lymphocytes and plasma cell infiltrates and the optic nerve show neuritis (optic neuritis). More gross lesions were recorded in the Pekin duck in clade infection at 6 dpi, including myocardial necrosis and myocarditis, hydrothorax, pulmonary congestion and edema, hepatic and pancreatic necrosis and pancreatitis. This signifies the occurrence of more gross and histopathological lesions with the progression of HPAIV infection up till 4 dpi. In Pekin and Mallard at 4 dpi, histopathological lesions included meningoencephalitis with lymphocytic perivascular cuffs, adrenal necrosis with lympho-plasma-histiocytic ganglioneuritis; and the duodenal intramural lympho-plasma-histiocytic ganglioneuritis which was evident in the 4^{th} and 5^{th} d.p.i Pekin ducks. Gross lesions of pancreatic necrosis and ecchymotic myocardial hemorrhages, in both Pekin groups are consistent with the gross changes observed in the HPAIV H5N1 clade 2.2 isolate infection in its swan host (Teifke *et al*., 2007). In addition, myocardial necrosis, myocarditis, pulmonary congestion and edema seen in Pekin ducks were also observed in the swans (Teifke *et al*., 2007). The histopathological lesions produced by the HPAIV H5N1 Swan isolate in experimentally infected ducks are similar to the lesions produced in the Swan from which it was isolated. This included mainly nervous, hepatic, adrenal and lymphoid histopathological lesions (Teifke *et al*., 2007). In addition, lymphoplasmacytic ganglioneuritis was common histopathological lesions in all duck species with immunohistochemical staining of the meyenteric intramural ganglia. Other histopathological finding seen in the lymphoid organs was lymphocytic depletion and necrosis. The cardiac lesion observed in Pekin and the lymphoid lesions in Mallard ducks infected with clade 1 and 2.2 viruses at 4 days p.i were not seen in 3 dpi Muscovy ducks, suggesting that cardiac and lymphoid lesion occurs as a result of the progression of infection by H5N1 in ducks. Also, Mallard ducks in clade 2.2 showed no gross lesion at 4 days p.i, an occurrence that was only seen in adult Swans as reported by Teifke et al. (2007), This may suggest some level of mild susceptibility to the infection. Histopathological lesion seen in clade 1 infection included jejunal intramural lymphocytic ganglioneuritis. This shows the sensitivity of Mallard nervous tissues to the replication of the two clade of HPAI virus. Although only one Mallard examined at 4 days p.i in the clade 1 had gross lesion of severe diffuse pancreatic necrosis, the major histopathological lesions in the Mallard included meningoencephalitis with lymphocytic perivascular cuffs. In clade 1 virus infection, two Mallards examined at 8 days p.i showed no gross lesion and although histopathological lesions were fewer, the disease was accompanied by lymphoplasmacytic meningoencephalitis and polioencephalitis with lymphocytic perivascular cuffs,

which was also seen in the eyelid and multifocal lymphocytic myocarditis. Also in clade 2.2 virus infection, Mallard ducks showed no visible gross lesion at 8 days p.i, In general, at 8 days p.i, no gross lesion was seen in Mallard ducks infected with either of these viruses, and nervous histopathologic lesions seen more in clade 1 than in clade 2.2 may be the major lesion (Akanbi *et al.,* 2010). Of note is the finding that no Mallard ducks exhibited any visible gross lesion in both clade of the H5N1 virus throughout the experiment, except one mallard at 4 days p.i that showed pancreatic necrosis. This suggests that gross lesion may not be reliable in the morphologic diagnosis of HPAIV in Mallard and perhaps in Muscovy ducks because in clade 2.2, one Muscovy each at 4 days p.i and 8 days p.i respectively did not show any visible gross lesion. But histopathological findings were consistent with those of 3 dpi Muscovy. This leaves Pekin ducks as the only duck specie in the two clade infections with generalized gross lesions irrespective of virus strain and days p.e. Hence evidence of gross lesion may be reliable in the morphologic diagnosis of HPAIV in the Pekin duck. Generally, in clade 1 virus infection, immunoreactivity was seen in the endothelial cells of some GIT tissues in Muscovy ducks examined at 3 days p.i, but none was seen in the entire GIT tissues of Pekin and Mallard ducks examined at 4 days p.i. Athough the serosal lining cells of these tissues were IHC positive in Mallard at 5 days p.i, they remained negative in the Pekins still and in both at 6 days p.i. In clade 2.2 Muscovy ducks, immunoreactivity was only in the intramural ganglia of the duodenum, jejunum, ileum, caeca and rectum. While immunoreactivity was rare in Mallard ducks at 4 days p. i. This may suggest that immunohistochemical detection may be possible or consistent in the GIT, as early as 3rd day post infection and that detection of viral antigen by immunohistochemistry in Muscovy ducks in the virus groups is not widespread. Hence immunohistochemical detection of HPAIV in ducks is dependent on days p.i. At 8 days p.i in clade 1, immunoreativity was restricted to the cerebral neurons and glia cells and around the vessels only in one Mallard and not detected in the second Mallard, whereas in clade 2.2, only one mallard duck showed immunohistochemical detection of H5 antigen in the ganglion and inner nuclear layer of the retina. This suggests that immunohistochemical detection may not be possible or consistent, as the days p.i increases and that detection of viral antigen by immunohistochemistry in mallard ducks in the virus groups is low. This study shows that Mallard ducks are most resistant to infection by the clade 1 and clade 2.2 viruses. Invariably, Muscovy ducks are highly and most susceptible to the clade 2.2 HPAI H5N1 virus with severe nervous system lesions and highest viral replication in all tissues while Mallard are least susceptible to the clade 2.2 virus with no visible gross lesion at 4 and

8days p.i. Pekin ducks are moderately susceptible to both viruses and showed immunohistochemical detection at 4days p.i. In the Muscovy ducks examined at 3 days p.i, the cerebrum, nasal cavity, lung and ovary/testis in both clade 1 and clade 2.2 virus groups, airsac (clade 1) and trachea, heart, spleen and muscle of clade 2.2 virus groups had high H5N1 viral genome load as detected by RT-PCR (mean ct- value ≤ 21). This means that both viruses replicated most highly in these tissues and they should be the target tissues of sampling when investigating these clade of viruses at 3 days p.i. However at 8 days p.i, in the only surviving Muscovy duck infected with clade 2.2 virus, H5N1 viral genome load detection by RT-PCR (mean ct- value < 27) was only in the cerebrum, emphasizing the importance of this organ for diagnosis. In the Pekin ducks examined at 4 days p.i, H5N1 viral genome load detection by RT-PCR (mean ct- value ≤ 23) was in the cerebrum, nasal cavity, airsac, heart, pancreas and ovary/testis in those infected with clade 1 virus and the RT-PCR mean ct- value ≤ 27 was in the cerebrum, nasal cavity, airsac and ovary/testis of those infected with clade 2.2 virus. This means that both viruses replicated most highly (clade 1) and moderately (clade 2.2) in these tissues and should be the target tissues of sampling when investigating these clades of viruses at 4 days p.i. In Mallard ducks examined at 4 days p.i, H5N1 viral genome load detection by RT-PCR was (mean ct- value ≤ 23) in the cerebrum, nasal cavity, airsac, heart and ovary/testis of those infected with clade 1 virus and (mean ct- value ≤ 27) in the cerebrum, nasal cavity, airsac, lung and ovary/testis of those infected with clade 2.2 virus. This means that both viruses replicated most highly (clade 1) and moderately (clade 2.2) in these tissues and they should be the target tissues of sampling when investigating these clades of viruses at 4 days p.i. However at 8 days p.i, in the surviving 2 Mallard ducks in the clade 1 virus group, H5N1 viral genome load detection by RT-PCR was high (mean ct- value ≤ 25) only in the cerebrum and ovary/testis and was very low (mean ct-value ≤ 35) in the cerebrum, nasal cavity, airsac, spleen and ovary/testis of clade 2.2. This implies that, when investigating clade 1 virus in the Mallard duck on 8 days p.i, the cerebrum and ovary/testis are the tissues of choice for high detection of the virus. While the cerebrum, nasal cavity, airsac, spleen and ovary/testis though gave weak detection, are still the tissues of choice for detecting the clade 2.2 virus. Although, virus replication titre were low in most organs except for cerebrum, nasal cavity, airsac, heart and ovary/testis of those infected with clade 1 virus with mean ct- value of ≤ 23 by RT-PCR and in the cerebrum, nasal cavity, airsac, lung and ovary/testis of those infected with clade 2.2 virus with mean ct- value of ≤ 27 by RT-PCR. This was responsible for the lack of clinical signs and low severity of lesions.As the virus replication titers increase, so do the

severity of pathobiological changes such as gross and microscopic lesions with the most pathogenic virus strains causing major cell damage and death if it is sufficiently severe to affect critical organs (Swayne, 2007). That Mallard duck is an example of some duck species which show no or limited virus replication and few clinical signs has earlier been observed by Alexander *et al.* (1978, 1986) and Perkins and Swayne (2002). Therefore, since tissues virus load and lesion varied from species to specie and virus type, the following tissues; cerebrum, nasal cavity, ovary, testis, air sac heart, pancreas and spleen are useful as sampling tissues for histopathology, immunohistochemistry and tissue RRT-PCR in Muscovy, Pekin and Mallard. Lung and eye should only be included for 3dpi sampling in Mallard ducks. This also confirms that Muscovy ducks are most susceptible to infection by the clade 1 and clade 2.2 viruses. Also, virus re- isolation in the brain of Muscovy ducks yielded higher titre in clade 1(log $10^{6.4}$) and clade 2.2 (log $10^{6.3}$) than the infectivity dose of EID_{50} log 10^{6} compared to other tissues of all duck species sampled in clade 1 and 2.2 virus group at 3 dpi, signifying higher virus replication rate in the brain of Muscovy ducks. In the natural HPAI virus infection of Muscovy ducks introduced to a backyard flock in Zaria, Kaduna state in Nigeria, it was found that the Muscovy ducks were highly susceptible to the infection showing severe neurologic clinical signs of torticollis, ataxia, excitation, cyclic movement, opisthotonus, tremor, paddling and prostration; and pathologic changes resulting in death as compared to Mallard ducks in the same flock which were apparently healthy (Wakawa *et al*., 2008). This may be as a result of high virus replication rate in the brain of Muscovy ducks shown by this experiment. It has been observed since 2002–2003, that new strains were identified that caused mortality in experimentally inoculated domestic ducks (Chen *et al*., 2004, Ellis *et al*., 2004, Sturm-Ramirez *et al*., 2004, Lee *et al*., 2005). The mortality was the result of systemic infections and was associated with increasing replication titers within respiratory tract and brain compared with nonlethal infections (Chen *et al*., 2004, Ellis *et al*., 2004, Sturm-Ramirez *et al*., 2004, Lee *et al*., 2005). All tissues of Muscovy ducks were affected by the effect of the two viruses. It was also evident from this study that Pekin ducks are moderately susceptible to infection by clade 1 virus and to infection by clade 2.2 virus. In all Muscovy ducks examined at 3 dpi, mean tracheal (μ=20.7) and cloacal (μ=27.8) swabs real-time RT-PCR ct-values were most positive, although tracheal swabs were more positive with high viral genome load. Also tracheal swabs were more positive for Mallard and Pekin ducks at 3dpi than cloacal swabs, and this result is similar to the finding in Muscovy ducks. Other investigators have found both tracheal (Meseko *et al*., 2010) and cloacal (Wakawa et al., 2008) swabs useful in

detecting HPAI virus in natural infections in Nigeria. Although no RT-PCR ct-values were given in both reports, the results of this current experiment have shown that tracheal swabs are more positive by real time RT-PCR and should be the swab of choice for H5N1 virus detection in ducks. All ducks tracheal and cloacal swabs were negative (ct-value ≥ 40) on the day of infection with clade 1 H5N1 virus by real time RT-PCR. The finding that tracheal swabs were more positive with high viral genome load than cloacal swabs suggests that viral replication was more in the respiratory epithelium than the intestinal epithelium. The tracheal swabs in the Muscovy ducks were the most positive, emphasizing the highest replication ability of the two clade H5N1virus in the Muscovy ducks respiratory epithelium throughout days 1, 2 and 3 PI when compared to Pekin and Mallard. Cloacal swabs of the Muscovy ducks were also most positive on the 2nd and 3rd days PI when compared to Pekin and Mallard in both clade of H5N1 virus. Very few Pekin and Mallard ducks had positive cloacal swabs, and this was only on the 3rd day PI, meaning the virus replicated well on 3 dpi in both virus infection. Surges in viral genome detection by real time RT-PCR, were observed in the tracheal swabs mean ct-values of Pekin ducks 6 days p.i in clade 1 infection. While surges in viral genome detection by real time RT-PCR, were observed in the tracheal swabs mean ct-values of Mallard ducks and in the cloacal swabs mean ct-values of Mallard ducks 6 days p.i. These surges signify higher virus shedding than the previous day shedding, which may be due to higher virus replication and may be useful for swab taking in Pekin and Mallard ducks 6 days p.i with clade 1 H5N1virus. Also in clade 1 infection, two peaks were observed in the Pekin ducks mean tracheal swabs real-time RT-PCR ct-values at 3 dpi. (μ=26.1), been lowest and at 6 dpi (μ=28.80). These were higher than the mean cloacal swab ct-value for the 3dpi (μ=31.59) and no surge was observed. In general, it can be said that the exposure of Mallard ducks to the two clades of H5N1 viruses resulted in infection of this duck, but with little host adaptation i.e the result of progressive genetic changes in a virus resulting in increasing efficiencies of binding, replication and release of the virus from a specific host species (Swayne, 2007). It is evident from this study that Muscovy ducks are highly susceptible to infection with H5N1 HPAI viruses with by widespread clinical, macro- and microscopic lesions, immunohistochemical detection, prolonged and highly concentrated viral shedding, viral re-isolation and RRT-PCR detection. Recent isolates of H5N1 viruses used in this experiment caused a high proportion of illness and death in the ducks and this is consistent with previous studies on ducks (Sturm-Ramirez *et.al*. 2004). The significant differences observed in the tracheal and cloacal shedding of the virus in Muscovy ducks compared with Mallard and Pekin in

the clade 1 and clade 2.2 at 1 and 2 days p.i is evidence that attest that the virus replication and shedding in the Muscovy duck is highest. Although, there was no difference between the tracheal and cloacal swabs of the ducks in the two clade of virus at 3 days p.i, there exists a significant difference in the cloacal swabs of Muscovy ducks compared with Mallard and compared with Pekin between clade 1 and clade 2.2 at 3 days p.i. It is worth noting that from 6^{th} and 8^{th}days p.i, in clade 2 virus group, cloaca swabs were negative in Mallard and Pekin ducks and also on 8^{th} dpi cloacal swabs were negative in the remaining Mallard and Pekin ducks in the clade 1 and clade 2.2 infected groups. This shows that during surveillance in HPAI virus infection, days post infection will affects the RT-PCR result of cloacal swabs and this should be taking into consideration.

In exposed chickens, morbidity and mortality in the two virus groups was up to 100% at 4 (clade 2.2) and 5 (clade 1) days p.e. Clinical signs and death started at 2 d.p.e in both virus groups. With this development, it is evident that transmissibility by contact, which is the natural host-to-host spread (Swayne, 2007) is successful in these chickens, and chickens being the most frequently infected species with HPAI viruses (Swayne, 2007) came down with the virus infection producing high morbidity and mortality in them. The exposure in the in-contact chickens and ducks was successful as shown by the establishment of infection and disease. This is consistent with the finding of Songserm et al. (2006), which stated that through silently but productively infected ducks, an endemic status of HPAIV (H5N1) infection can be established and perpetuated. The HPAIV H5N1 viruses used in this experiment were highly pathogenic and induced clinical signs in the exposed chickens and ducks contrary to the report of Harder et al. (2009), who stated that strains isolated from such endemic infections induced no clinical signs in ducks but retain high pathogenicity for chickens and turkeys. Gross pathological manifestations of the disease are associated with the cardiovascular and gastrointestinal systems (clade 1) and cardiovascular, respiratory and in the pancrease (clade 2.2). Histopathologic lesions were consistent in all exposed chickens in clade 1 virus groups but was observed only in chickens that died 4 days p.e in clade 2.2 virus group. Although gross lesion was not seen in the respiratory system in clade 1 virus exposed group, exposure to both viruses however showed tracheal and atria epithelia necrosis. Villi and crypt necrosis in duodenum, jejunum, ileum, caeca and rectum was common to both virus groups; clade 2.2 group had additional proventricular and ventricular epithelia necrosis and vasculitis. Infected Muscovy appeared to have transmited the H5N1virus faster to the chickens in clade 1 and clade 2.2, as the viruses were detected earlier in the Muscovy group than in the other 2 at 2 days p.e first and

only in the trachea and cloacal swab of the chickens with the Muscovy ducks in clade 1 and clade 2.2. The difference in mean tracheal ct-value in the chickens exposed to Muscovy ducks compared to those exposed to Mallard and Pekin and between exposing chickens to clade 2.2 as opposed to exposing them to clade 1 at 2 dpe irrespective of the duck species of exposure is significant by a two-way Anova. Transmission has been observed to occur primarily by faecal–oral pathways through direct contact or indirect contact with contaminated surface water (Webster *et al.,* 1992). The high transmission capability of Muscovy ducks was also evident when the chickens in their groups tested by real time RT-PCR, were found to be highly positive for the influenza viral genome load in tracheal and cloacal swabs much more than chickens in other groups at 3 dpe. This high transmission capability of Muscovy ducks is statistically significant for both tracheal and cloacal swabs, although this is not the case with the virus type in tracheal and cloacal swabs, as there is no significant difference in the mean tracheal and cloacal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 irrespective of the duck species of exposure at 3 days p.e. At 4 dpe, findings from the remaining chickens in the Mallard and Pekin groups of the two virus groups showed that infected Pekin ducks transmitted the virus to their in-contact chickens significantly faster than infected Mallard ducks, but no significant difference in the mean tracheal and cloacal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 at 4 days p.e irrespective of either they were exposed to Pekin or Mallard ducks. At 5 days p.e, there is a significant difference in the mean tracheal swab ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 irrespective of whether they were exposed to Pekin or Mallard ducks. This was not so with the cloacal swab mean ct-value, as there is no significant difference in the mean cloacal ct-values of exposing chickens to clade 2.2 as opposed to exposing them to clade 1 irrespective of the duck species of exposure at 5 days p.e. This also apply to the duck specie of exposure as there is no significant difference in the mean tracheal and cloacal ct-values of the chickens exposed to infected Mallard ducks when compared with the chickens exposed to infected Pekin ducks and vice versa. It is worth noting that the order with which the three ducks shed the clade 1 and 2.2 HPAI H5N1 virus, i.e Muscovy(M)>Pekin(P)>Mallard(Md) is the same way the chickens exposed to them also shed the virus i.e M-chicken>P-chicken>Md-chicken.

In exposed Pekin ducks at 4 days p.e, HPAIV replication in the brain was associated with severe neurological alterations and histopathological lesion of meningoencephalitis and the correlating high ct-values in the real-time RT-PCR in all ducks, though there was no immunohistochemical demonstration of antigen in the 4 days p.e duck. Oro-pharygeal excretion of the virus was highest 4^{th} and 5^{th} day post exposure and this also corroborated the high ct-value (ct< 25) of the RT-PCR of the trachea, lung and air sac of the duck sacrificed on the 4^{th} dpe. In fact immunohistochemical detection of H5 antigen was only possible in the air sac of this duck. Though mortality in wild aquatic birds due to infection with highly pathogenic avian influenza viruses (HPAIV) is known to be a rare event (Teifke *et al.,* 2007), mortality due to H5N1 HPAIV was observed among mute and whooper swans from which the inoculated virus was isolated (Teifke *et al.,* 2007) as well as in the Pekin duck infected in this experiment but not in the Pekin duck experimentally exposed. Cloacal virus shedding was negative (ct>40) in all ducks on 1^{st} dpe, and inconsistent and undulating in all ducks throughout the experiment, while peaking at high ct-value (ct< 25) on the 3^{rd} dpe in one duck. No histopathological lesion was observed (except in esophagus in one duck) and immunohistochemistry were negative in the GIT of all ducks. When the three methods used in this experiment were compared, RT-PCR detected the H5N1 virus most efficiently. Apart from the eyelid, nares adrenal, airsac, pancreas, heart and jejunum virus predilection detected by IHC was not seen in other tissue at 3^{rd} dpe. Also presence of histopathological lesion did not always corroborate IHC detection but IHC detection corroborates most of the time with RRT-PCR detection. While in 6^{th} days p.e ducks, only the virus in the nares, cerebrum and cerebellum was detected by IHC. Even when lesion was present and RT-PCR was positive, IHC detection was not always positive.

CHAPTER SIX

6.0 CONCLUSION

The highly pathogenic avian influenza (HPAI) H5N1 virus outbreaks experienced in both commercial and local poultry populations in Nigeria during 2006-2008 was devastating in the 25 states, including the Federal Capital TerritoryAbuja. The re-emergence of the virus in domestic ducks at a live bird market in Gombe, Gombe State after 9-months of absence of outbreak was an insight to the transmission and persistence of the virus in Nigeria and an indication of the fact that through silently but productively infected ducks, an endemic status of HPAIV (H5N1) infection can be established and perpetuated as earlier reported by *Songserm et al.* (2006).This work has shown that commercial chickens and backyard poultry in the northwest of Nigeria were mostly affected by the outbreaks of HPAI in Nigeria during 2006-2008. The values presented in this work are based on reported, submitted and confirmed HPAI cases. This did not include unreported cases. The clustering of outbreaks points with presence of wetlands especially the Hadeija-Nguru wetland in Jigawa State (North-western region), and some isolated outbreak points detected in 2008 after a nine month period of lack of outbreak especially in Yobe, Gombe and Kebbi which also have presence of wetlands may suggest that there may be association between HPAI occurrence and presence of wetland. Mortality was highest in broilers and males (cockerels) had more death than females (pullets). The reason for this cannot be ascertained within the scope of this study. It is hoped that in the future, factors responsible for this finding will be looked into. Although HPAI virus caused high mortality with minimal clinical and postmortem evidence of disease in free-ranged chickens, clinical and postmortem evidence were more overt in younger bird and older flocks. It was important to discover that there is a higher risk of infection and mortalities due to HPAIV infection in multiple, mixed species backyard poultry (chicken, duck and turkey) than in single species backyard poultry. Archival FFPE tissues from HPAI virus infected chicken tissues are very useful in the retrospective diagnosis of AI using immunohistochemistry and RRT-PCR on extracted RNA. BLAST search might suggest that the viruses belong to different sub-clades although this should be verified by longer sequences, and it was partially possible to classify the viruses into sub-clade 2.2 via HA cleavage

site- specific RT-PCR, hence the usefulness in trace backs. HPAI viruses circulating in Nigeria showed very high homology (>96%) with those from south-east Asia and Europe. There are mutations and hence, genetic drift among some of the viruses investigated. The pathogenesis and pathology of HPAI infection in ducks are similar but with varied mortality to that of chickens. The role played by domestic ducks in the transmission and persistence of the virus was made known in both natural outbreak evaluation and by animal experiments. Both clades of HPAI viruses replicated faster and most in Muscovy than Pekin and Mallard ducks, hence serve as higher risk carriers to in-contact chickens and ducks. These challenge experiments also suggest that days post onset of HPAI disease has influence on the diagnosis and that there is need to review the swabs sampling and expand the list of organs and tissues harvested for AI surveillance and diagnosis to include wattle, comb (chickens), eyelids, spinal cord, cerebellum, cerebrum, nasal cavity and thymus, spleen and ovary/testis. Swabs sampling should always include tracheal swabs and tissue samples should always include cerebrum, nasal cavity, ovary, testis, airsac, pancreas and bursa of Fabricius; in addition to the traditional trachea, lung, heart, liver, spleen and intestine. It is hoped that the findings of this work would be put to use at the national and international level in the event of an HPAI H5N1 virus outbreak and during surveillance for the virus. Also the unanswered observations generated from this work would be exployed during post doctoral research.

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APPENDIX

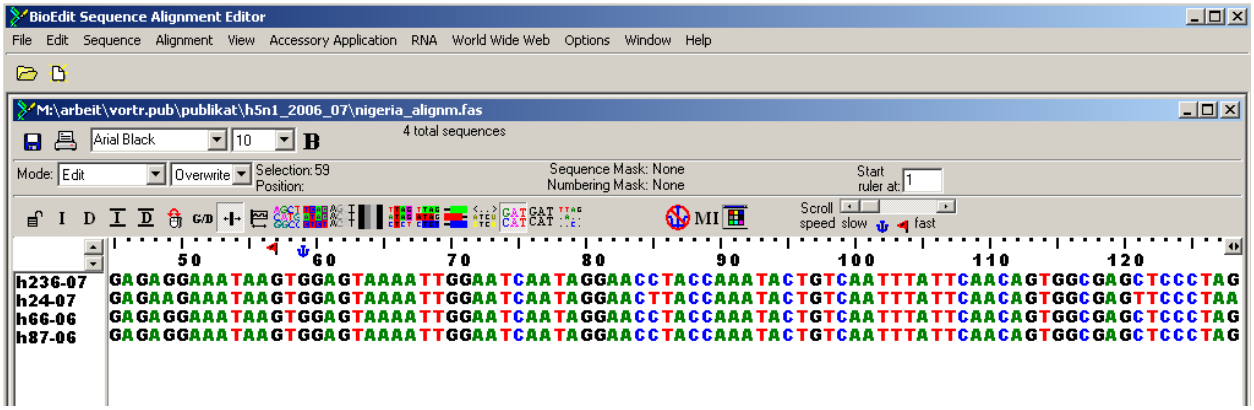

Figure 1. Full sequence of the four (4) Nigerian isolates from FFPE tissues