FEDERATION COLOMBOPHILE INTERNATIONALE
Présidence & Secrétariat
Rue de Livourne 39 – 1050 Bruxelles

FZ/ LJ/ie

COMMISSION VETERINAIRE & SCIENTIFIQUE
Procès-verbal de la réunion du 25.09.2006

PRESENTS :

Président de la Commission : **Mr Horst MENZEL** (Allemagne)

Membres : **Dr. Jurgen RADDEI** (Allemagne)
Ing. Jean-Pierre DUCHATEL (Belgique),
Dr. Christophe ARNOULT (France)
Dr L. VAN DER WAART (Pays-Bas)
Prof Piotr SZELESZOZUK (Pologne)
Dr Marc RYON (Portugal)
Dr Ulrich FREI-ZULAUF (Suisse)

Observateurs : **Mr Ton EBBEN** (Commission Europe - Pays-bas)
Mr Stef SWINNEN (Commission Europe - Belgique)

Secrétaire Administratif : **M. Luc JORIS** (Belgique)

Excusés : **Dr H.J.M. DE WEERD** (Pays-Bas)
Dr Jean-Pierre NELL (Suisse)

Absent : **Dr Gabor SCHINDLER** (Hongrie)

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Mr MENZEL, Président de la Commission Vétérinaire & Scientifique, souhaite la bienvenue aux participants. Il constate que des traductions en anglais et en français sont nécessaires.

1. Approbation du procès-verbal du 3.04.2006

Le procès-verbal est approuvé à l'unanimité.

Echange d'information :

- **Mr SWINNEN** distribue le tableau édité par la Commission EU quant aux mesures actuelles dans les pays de l'Europe pour combattre la grippe aviaire. Il insiste sur le fait que ce tableau est le fruit d'une collaboration entre la commission EU et la FCI.

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- **Mr SWINNEN** est déçu du fait que la Commission EU soit capable de présenter les mesures valable dans tous les 27 pays de l'Europe tandis que la F.C.I. n'avait reçu que 9 rapports.
  L'aviaire influenza est une grande menace pour le sport colombophile et il est incompréhensible que si peu des membres européens aient répondu à l'enquête.
  La liste présentée donne une vue générale des mesures en vigueur avant tout pour les concours et le vol libre. (**annexe 1**).

  Ce qu'il faudra rechercher maintenant sont les règles des différentes pays pour l'automne et l'hiver (expositions, marchés, olympiades,…)
  La Commission espère que les informations livrées les derniers 500 jours, seront suffisantes pour éviter des mesures restrictives pour la colombophilie.
  L'olympiade aura lieu fin janvier 2007. Les passages des oiseaux migrateurs sont terminés. On calcule que l'Olympiade à Ostende ne sera pas contrariée par la maladie (grippe aviaire).

  Pour l'automne et l'hiver, les pays représentés par la commission citent les restrictions suivantes :
  Pologne : pas de restriction actuelle. Expositions et marchés libres.
  Allemagne : pas de restriction, comme l'année passée.
  France : actuellement pas de restriction. L'AFSSA est d'avis que les restrictions ne sont pas utiles. Le gouvernement décidera en autonomie. Malheureusement, l'année passée, il avait fixé des sanctions malgré l'avis contraire de l'AFSSA.
  Pays-Bas : toujours pas de restrictions sauf le fait qu'il faut annoncer au moins 1 mois d'avance au département vétérinaire toute exposition de pigeons.
  Portugal : pas de restriction momentanément, mais le gouvernement portugais exige des définitions valables pour le terme 'concours internationaux" :
  a) concours lors des lâchers regroupant différents pays;
  b) lâcher des pigeons nationaux dans un autre pays (pays étranger);
  c) One Loft Races, rassemblements des pigeonneaux des différents pays.
  Pour la Commission européenne, un concours international est défini par le lâcher des pigeons nationaux hors frontières.
  Autriche : Mr Swinnen rapporte qu'en Autriche actuellement des expositions de pigeons sont autorisées mais sans pigeons vivants.

2. Problématique "Grippe aviaire"
   a. Projet "CERVA"
   b. Projet "OSTERHAUS"

La farde de la commission contient 2 rapports scientifiques : le résultat du **groupe Klopfleisch (annexe 2)** et le résultat présentés par **l'Ingénieur Duchâtel (annexe 3** - expérience Cerva-Coda T. Vandenberg).

Les 2 recherches montrent le même résultat : pas d'infection de poulets sentinelles par des pigeons infectés expérimentalement.

Ce résultat est très important pour le grand public, puisqu'il n'a pas été démontré que des pigeons voyageurs infectés expérimentalement étaient capables de contaminer des poulets sensible au virus. Ces résultats montrent que le pigeon est beaucoup moins réceptif au virus que les poules !

Les premiers résultats de l'expérience **Osterhaus** sont rapportés par **Mr EBBEN** (Pays-Bas) :
il était impossible d'infecter des pigeons vaccinés avec le virus. Pas le moindre signe de maladie.

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Certains pigeons non vaccinés montraient des signes de manque de santé : des pressions, apathies. Il n'y a pas des pigeons qui sont morts.

Dans la discussion des résultats, il est estimé pour une future expérimentation, qu'il faudra déplacer les pigeons infectés dans une autre cage avant d'y introduire les sentinelles. Ceci afin d' éviter que les sentinelles ne soient infectées par un reste de virus challenge.

La Commission discute à propos des buts de la recherche Osterhaus :

1. Le Prof. Osterhaus fait partie de la Commission E.U. Responsable pour les décisions concernant les pigeons voyageurs.
   Avec ces recherches, il augmente sa compétence concernant ce sujet et il peut mieux combattre les décisions défavorables au sport colombophile.
2. Si une pandémie de l'Influenza Aviaire devait se produire, toute volaille de production serait vaccinée immédiatement. Dans l'hystérie que se produira lors d'un tel événement, un pigeon non vacciné sera regardé comme un danger général pour toute la production de volaille et même pour la santé humaine. Deux vaccins pour la volaille ont déjà été approuvés par la E.U. qui en produit un stock permettant de combattre la maladie immédiatement si elle devait se manifester.
   Si actuellement nous commençons à nous intéresser à un vaccin de pigeons, on aurait un retard de 2 ou 3 ans sur l'actualité.
3. La recherche de vaccin pour volaille se concentre sur la production de vaccins marqués qui permettra de faire la distinction entre une immunité suite au vaccin et une immunité causée par la maladie. Une distinction favorable à notre sport.
   On est d'avis qu'il est nécessaire de faire un résumé sur ces recherches pour deux raisons :
   a. informations commentées aux autorités des décisions de l'Europe et des différentes nations.
   b. information de la base colombophile sur les résultats des recherches et les avantages de celles-ci pour assurer le financement des recherches encore à accomplir. Cette information sera à publier dans les journaux colombophiles. On évitera les grands médias. Un résumé, une argumentation pour le grand public sera également preparée, mais sera tenue en réserve pour le cas d'urgence (éviter la réaction hystérique).

3. Préparation d'une séance d'information sur la grippe aviaire durant l'Olympiade 2007 en Belgique (27.01.2007 - juste avant le congrès)

Ce congrès étant ouvert aux grands spécialistes vétérinaires et aux petits amateurs colombophiles, la conception suivante est décidée :

a. Conférence de base sur le virus et ses conséquences pour les amateurs.
b. Résumé des travaux scientifiques actuels par le Prof. Oosterhof et le Prof. Van den Berg.
c. Résumé de la situation politique actuelle par Mr Pridman, responsable pour l'influenza aviaire dans la commission E.U.
d. Discussion générale sous la direction de Mr MENZEL.

4. Problématique DOPING.

Lors de la réunion des commissions, la sous-commission "doping" de la Commission Sport présentera ses propositions en octobre 2006.

Propositions seront à discuter au printemps 2007 pour les membres à disposition des pays membres pour la saison sportive 2007.

Sur demande, **Mr MENZEL** mettra les règlements anti-doping en vigueur en Allemagne, en Belgique et aux Pays-Bas à la disposition de la Pologne.

5. Etat actuel des dispositions FCI concernant le transport des pigeons voyageurs.

Mr MENZEL distribue la nouvelle version du texte proposé.

Il sera discuté au printemps 2007 pour mettre à la disposition des pays membres pour la saison sportive 2007 **(annexe 4).**

6. Publication des projets des recherches sur la CIRCOVIROSE.

La commission décide d'ajouter les résumés des recherches sur la circovirose **(annexe 5 et 6).**

7. DIVERS

La prochaine commission Vétérinaire et Scientifique est fixée au **26.03.2007 à 10h30.**

Mr MENZEL remercie les présents pour le travail constructif effectué.

Fin de la session : 14h20

Le Secrétaire Administratif,
Luc JORIS.

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Annexe I

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate D – Animal Health and Welfare
D1 - Animal Health and Standing Committees

SANCO/10555/2006

Information received from Member States on pigeon races

SCOFCAH 5-6 September 2006

RESTRICTIONS THAT MEMBER STATES INTEND TO APPLY TO PIGEON RACES

Member State	General Ban	Ban In High Risk Areas	Time period	Exercise Flights	Longer Exercise Flights	National Races	International Races	Other Remarks
Austria	-	-		+	+	+	+	No races from and through banned zones/areas
Belgium	-	-		+	+	+	+	No races from and through banned zones/areas
Cyprus	No pigeon races take place in Cyprus.							
Czech Rep.	-	-	In place since 11/05/2006 unchanged	+	+	+	+	No races starting/finishing in high risk areas or under restrictions
Denmark	+	-	15/09/2006-15/11/2006	+	+	-	-	No races in restricted zones due to an outbreak, permission for national and international races in areas of "medium risk"
Estonia	-	-		+	+	+	+	No races take place in Estonia, no permissions requested.
Finland	-	-		+	+	+	+	Finish racing pigeons do not participate in races outside FIN and no international races take place in FIN.
France	-			+	+	+	+	According to the epidemiological situation.
Germany	-	-		+	+	+	+	
Greece	-	-		+	-	+	-	
Hungary	-	-		+	+	+	+	In case of an outbreak of HPAI all races would be banned.
Ireland	-	-		+	+	+	+	From UK and Northern France under review

RESTRICTIONS THAT MEMBER STATES INTEND TO APPLY TO PIGEON RACES								
Member State	**General Ban**	**Ban In High Risk Areas**	**Time period**	**Exercise Flights**	**Longer Exercise Flights**	**National Races**	**International Races**	**Other Remarks**
Italy	-	-		-	-	-	+	
Latvia	-			+	+	+	+	
Lithuania	-	-		+	+	-	-	
Luxembourg	+	-	15/09/2006-15/12/2006	+	-	-	-	
Malta	-			+	+	+	+	
Netherlands	-	-		+	+	+	+	
Poland	-	-		+	+	+	+	On the condition that HPAI is not suspected or confirmed.
Portugal	-	+	Rules since 05/2006	+	+	+	+	No start in risk zones, inetrnat
Slovakia	-	+	1/11/2006-31/12/2006	+	+	+	+	
Slovenia	+		01/10/2006-31/12/2006	+				No permissions in case of HPAI outbreak, or in high risk zones
Spain	-	+	1/9/2006 - undefined	+	+	+	+	All permissions based on favourable outcome of Risk evaluation
Sweden	+	-	Ban in place since first appearance of AI	+	-	-	-	Possible derogations, races ceased in August.
United Kingdom	+		Permanent general ban	+	+	+	-	Permissions under general license
Norway	-			+	+	+	+	
Switzerland	-	+	1/10/2006 -					

RESTRICTIONS THAT MEMBER STATES INTEND TO APPLY TO PIGEON RACES								
Member State	**General Ban**	**Ban In High Risk Areas**	**Time period**	**Exercise Flights**	**Longer Exercise Flights**	**National Races**	**International Races**	**Other Remarks**
			undefined					
Bulgaria	+	-	1/09/2006 – 31/03/2007	-	-	-	-	
Romania	-	+	15/10/2006 - 15/12/2006					

annexe II

Vet Pathol 43:463–470 (2006)

Neurotropism of Highly Pathogenic Avian Influenza Virus A/Chicken/Indonesia/2003 (H5N1) in Experimentally Infected Pigeons (*Columbia livia f. domestica*)

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Abstract. This investigation assessed the susceptibility of experimentally infected pigeons to the highly pathogenic avian influenza virus (HPAIV) H5N1 that caused recent outbreaks of avian influenza in birds and humans in several countries of Asia. For this purpose 14 pigeons were infected ocularly and nasally with 10^8 EID_{50} and clinical signs were recorded and compared with five chickens infected simultaneously as positive controls. The chickens demonstrated anorexia, depression, and 100% mortality within 2 days postinoculation. Three of the pigeons died after a history of depression and severe neurological signs consisting of paresis to paralysis, mild enteric hemorrhage, resulting in a mortality of 21%. Gross lesions in these pigeons were mild and inconsistent. Occasionally subcutaneous hyperemia and hemorrhage and cerebral malacia were observed. Microscopic lesions and detection of viral antigen were confined to the central nervous system of these pigeons. In the cerebrum and to a minor extent in the brain stem a lymphohistiocytic meningoencephalitis with disseminated neuronal and glial cell necrosis, perivascular cuffing, glial nodules, and in one bird focally extensive liquefactive necrosis could be observed. The remaining nine pigeons showed neither clinical signs nor gross or histological lesions associated with avian influenza, although seroconversion against H5 indicated that they had been infected. These results confirm that pigeons are susceptible to HPAIV A/chicken/Indonesia/2003 (H5N1) and that the disease is associated with the neurotropism of this virus. Although sentinel chickens and most pigeons did not develop disease, further experiments have to elucidate whether or not *Columbiformes* are involved in transmission and spread of highly pathogenic avian influenza.

Key words: Avian influenza; chicken; meningoencephalitis; neuronal necrosis; pigeon.

Introduction

In the past, a large number of avian influenza viruses (AIV), belonging to 16 hemagglutinin (H1-H16) and nine neuraminidase (N1-N9) subtypes, have been recovered from a variety of domestic and wild avian species worldwide. All subtypes have been found in wild birds from at least nine different orders, but the main reservoir hosts seem to be birds of the orders *Anseriformes* (geese, ducks, swans) or *Chadriiformes* (gulls, terns, surfbirds, sandpipers).[25] The wide distribution in these asymptomatically infected wild birds may result in spread to domestic poultry.[2] Highly pathogenic avian influenza (HPAI) is an acute, generalized, fatal disease etiologically restricted only to those AIV strains of the subtypes H5 and H7, which code for a furin-sensitive cleavage site in their hemagglutinin protein and, therefore, are capable of inducing systemic infections. HPAI occurs characteristically in chickens and turkeys.[1] However, other gallinaceous birds such as quails or guinea fowls, as well as ducks, geese, ratites, passerine birds, flamingos, herons, and raptors are also susceptible for the disease.[18,19] Since 2003, an epidemic emergence of H5N1 in Vietnam, Indonesia, and Thailand has resulted in the culling of millions of birds and led to 173 confirmed cases of human H5N1-influenza, with a total of 93 fatalities reported to the World Health Organization (WHO) until 27 February 2006 (http://www.who.int/csr/disease/avian_influenza). Controversial reports exist about the susceptibility of domestic birds, especially racing pigeons and wild pigeons, and their role as a link between wild and domestic birds and transmission and spread of HPAIV during epizootics over long distances.[10] Whereas several transmission experiments suggest a resistance, one report describes a minimal susceptibility of *Columbiformes* to infection with HPAIV of the H5 subtype.[16,18,20] Here, we focus on the clinical and pathological findings and distribution of viral antigen after experimental inoculation of pigeons with the HPAIV A/chicken/Indonesia/2003 (H5N1).

Materials and Methods

Viruses

The HPAIV A/chicken/Indonesia/2003 (H5N1) was isolated in the national reference laboratory for avian influenza at the Friedrich-Loeffler-Institut, Isle of Riems, Germany, from chicken organs received from Indonesia. The isolate was once propagated in the allantoic cavities of 10-day-old embryonated specific pathogen-free (SPF) chicken eggs. The allantoic fluid was harvested and stored at −70°C.[25]

Animals and experimental design

Fourteen 4-month-old, male and female racing pigeons *(Columbia livia f. domestica)*, serologically negative for H5-specific hemagglutination-inhibiting antibodies, were inoculated ocularly and intranasally in each case with 0.5 ml allantoic fluid containing 1×10^8 EID_{50} (50% embryo infective dose) of the Indonesia/2003 HPAIV. For detection of virus excretion five 12-week-old serologically AIV-negative SPF White Leghorn chickens (VALO SPF, Lohmann, Cuxhaven, Germany) were added to the aviary with the pigeons 48 hours postinoculation (pi). Four pigeons were not inoculated with HPAIV and served as a negative control group. Furthermore five SPF chickens of the same age and breed were simultaneously and identically infected as positive controls and kept separately. Pigeons and chickens were housed in aviaries with free access to food and water. The animals were observed for 19 days pi (dpi), and clinical signs were recorded. All animals used for these investigations were kept under isolated conditions in the biosafety level 3 containment facilities on Isle of Riems. The experimental studies were permitted by the German Animal Welfare Committee, and general care was provided as required.

Sampling

All pigeons and chickens that died or were killed were necropsied for determination of gross lesions and histopathology. Animals were killed when they developed severe clinical signs consisting of severe depression, reluctance to move, neurological signs such as torticollis, paralysis, and loss of consciousness, or after 19 dpi. Tissue samples of all animals were fixed in 4% phosphate-buffered neutral formaldehyde and processed for paraffin-embedding.

Serology

Sera of pigeons and chickens were collected before infection and at the time of euthanasia or at 19 dpi. For detection of H5-specific antibodies the hemagglutination-inhibition test (HI-test) with inactivated H5 antigens was used.[25]

Generation of anti-nucleoprotein serum

The influenza virus strain Influenza A/FPV/Rostock/34 was used for the amplification of the viral sequence encoding the nucleoprotein (NP) gene by reverse transcription-polymerase chain reaction (RT-PCR) following standard procedures. The obtained RT-PCR fragment encompassing the complete coding region of the NP gene was cloned into the baculo transfer vector pAcGHLT-A (Pharmingen, Heidelberg, Germany), which resulted in a plasmid pAcGHLTA-NP-Ros encoding a fusion protein of the vector-derived glutathione-S-transferase (GST) and the open reading frame of the NP protein. After generation of a recombinant baculovirus using Baculo-Gold DNA (Pharmingen, Heidelberg, Germany), a recombinant NP expressing baculovirus was generated. The recombinant protein (GST-NP) was purified according to the manufacturer's instructions using GST-binding resin (Novagen, Bad Soden, Germany). Rabbits (SPF, Harlan, and Winkelman; Borchen, Germany) were inoculated intramuscularly four times with 100 µg of the purified GST-NP. The specificity of the anti-NP serum was monitored by Western Blot analysis using lysates of infected and noninfected cell cultures.

Histopathology and immunohistochemistry

Paraffin-wax sections (3 µm) were dewaxed and stained with hematoxylin and eosin (HE). To analyze distribution of HPAIV antigens, sections were mounted on positively charged SuperFrost Plus microscope slides (Menzel, Braunschweig, Germany), dewaxed, and rehydrated. The sections were incubated with the rabbit anti-NP serum. This antibody was applied at a dilution of 1:500 in Tris-buffered-saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6). As linker-antibody for the avidin-biotin-complex (ABC) method, a biotinylated goat anti-rabbit (IgG1) (Vector, Burlingame, CA; diluted 1:200 in TBS) was used. As negative control the preimmunization serum of the same rabbit was applied. By means of the ABC method and an immunoperoxidase kit (Vectastain Elite ABC Kit, Vector), a bright red signal was produced from the substrate, 3-amino-9-ethylcarbazole (DAKO AEC substrate-chromogen system; Dako, Carpinteria, CA). The sections were counterstained with Mayer's hematoxylin and sealed with aqueous medium (Aquatex; Merck, Darmstadt, Germany).

Results

Pigeons

Mortality and clinical signs. Ocular and intranasal administration of HPAIV Indonesia/2003 resulted in death of 5 of the 14 pigeons, during a time period of 19 dpi. Survival times varied from 5 dpi (pigeon No. 1) to 7 dpi (pigeons Nos. 2 and 3) to 19 dpi (pigeons Nos. 4 and 5, euthanized both with neurologic symptoms). Clinical signs were observed in all of these five animals (Table 1). Disease

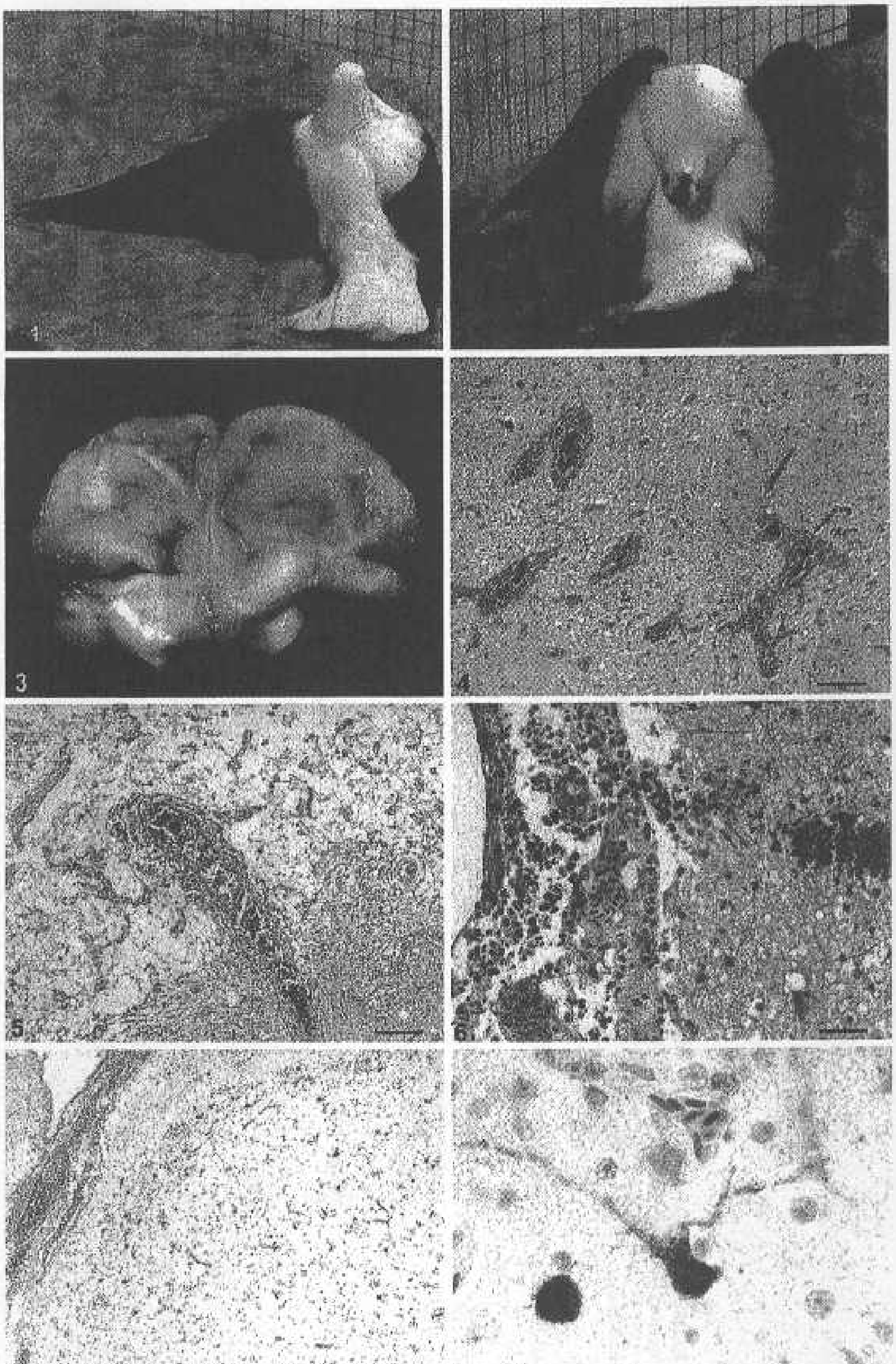
Table 1. Clinical signs, time to death, gross and histopathology, and results of immunohistochemistry of pigeons and chickens intranasally and ocularly inoculated with HPAIV A/chicken/Indonesia/2003 (H5N1)

Animal Nos.	Clinical Signs	Time to Death	Gross Lesions	Histopathology†	Immunohistochemistry‡
Pigeon 1	Neurologic signs	5 dpi	Subcutaneous hemorrhage	Cerebrum: meningoencephalitis (++)	Cerebrum (***)
Pigeon 2	Depression, neurologic signs	7 dpi	Subcutaneous hemorrhage, hydropericardium	Cerebrum, brain stem: meningoencephalitis (++)	Cerebrum (***)
Pigeon 3	Depression, neurologic signs	7 dpi	(/)	Cerebrum: meningoencephalitis (+++)	Cerebrum (*)
Pigeon 4	Neurologic signs	19 dpi*	(/)	Cerebrum: encephalitis (+)	Cerebrum (*)
Pigeon 5	Neurologic signs	19 dpi*	Cerebral malacia	Cerebrum, brain stem: meningoencephalitis, malacia (+++)	Cerebrum (**), brain stem (*)
Pigeons 6–14	(/)	19 dpi*	(/)	(/)	(/)
Chickens 1–5	Depression	2 dpi	Subcutaneous hemorrhage, hydropericardium	Disseminated epithelial and lymphoid necrosis, necrotizing vasculitis, interstitial pneumonia, necrotizing pancreatitis	Lung, intestine, adrenal, heart, liver, kidney, spleen (*) pancreas, thymus (**)

* Killed humanely.

† Histopathological grading of the lesions: (/) = none, (+) = mild, (++) = moderate, (+++) = severe.

‡ Numbers of IHC-anti-H5N9 positive cells: (/) = none, (*) = few, (**) = moderate numbers, (***) = numerous.



progressed over a period of 1 to 3 days from depression to enteric hemorrhage and severe neurological signs consisting of torticollis, nystagmus, and wing paresis to paralysis (Figs. 1 and 2). No clinical signs were observed in the nine pigeons (pigeon Nos. 6–14) euthanized 19 dpi (Table 1).

Serology. In all of the nonsymptomatic pigeons Nos. 6–14, significant H5-specific titers were detected on day 19 pi (ranging from 1 : 32 to 1 : 64).

Gross pathology. At necropsy, only mild and inconsistent lesions were present (Table 1). Whereas pigeons Nos. 1 and 2 showed a focal, well-circumscribed, 2-cm-diameter area of hyperemia and hemorrhage in the subcutaneous venous plexus of the neck, a hydropericardium was found in pigeon No. 2 (Table 1). A focally extensive area of unilateral cerebral malacia was observed in pigeon No. 5. In this case the complete anterior part of the left hemisphere showed a focal, well-delineated indentation with softening and yellow-brown discoloration of the neuroparenchyma as well as hyperemia of adjacent meningeal vessels (Fig. 3). Gross lesions could not be observed in pigeons Nos. 3 and 4 and the animals were killed at 21 dpi.

Histopathology. Histologic lesions were confined to the central nervous system (CNS) of the pigeons that developed clinical signs of HPAI (Table 1). Diffusely, lesions were present mainly in the cerebral gray matter but also in the brain stem. In the cerebrum, multifocal, moderate to severe necrosis of neurons and glial cells and hemorrhages were found in pigeons Nos. 1–5. Corresponding to these lesions, prominent multifocal glial nodules and occasional neuronophagia could be observed in the cerebral gray and white matter. Vessels showed multifocal perivascular cuffing with one to four layers of numerous lymphocytes, fewer histiocytes, and small numbers of heterophils (Fig. 4). In pigeon No. 5, additional extensive, multifocal to coalescent, irregularly shaped, moderately demarcated areas of liquefactive necrosis were found in the cerebrum, characterized by central complete loss of neuropil, leaving empty spaces that contained preexisting arterioles, small amounts of pale eosinophilic material, and few finely vacuolated macrophages (gitter cells; Fig. 5). Milder lesions were found in the brain stem but not in the cerebellum of pigeons Nos. 1 and 5. Rarely, swelling, degeneration, and necrosis of endothelial cells were observed. Four of the five affected pigeons showed moderate diffuse meningitis with small numbers of mainly perivascular mononuclear cells and mild edema (Fig. 6). Histologic examination of the heart, aorta, larynx, lung, spleen, pancreas, kidney, proventriculus, gizzard, and duodenum revealed no lesions. No lesions were observed in the pigeons that did not develop clinical signs.

Immunohistochemistry. Influenza virus antigen strongly colocalized with the histologic lesions in the brain. Intranuclear and intracytoplasmic staining for NP antigen was seen in numerous viable as well as degenerating and necrotic neurons of the cerebrum and brain stem as well as in a few glial cells and capillary and arteriolar endothelial cells of pigeons Nos. 1–3 (Figs. 7 and 8). In contrast, in pigeons Nos. 4 and 5 only a few NP antigen positive neurons and glial cells were detected in the cerebrum and the brain stem but no staining of endothelial cells was observed. In pigeon No. 5 few positive neurons surrounded closely the area of liquefactive necrosis. In pigeons Nos. 1–5 no viral antigen as detected in heart, larynx, lung, spleen, pancreas, kidney, or intestine. Furthermore, viral

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Fig. 1. Pigeon No. 4, 19 dpi. Animal with hemiparalysis of the wing.

Fig. 2. Pigeon No. 5, 19 dpi. Animal with severe torticollis.

Fig. 3. Transversal section of formalin-fixed brain: Pigeon No. 5, 19 dpi. Left hemisphere of the cerebrum with moderate reduction in size and irregularly shaped, fairly demarcated area of liquefactive necrosis of white and gray matter.

Fig. 4. Cerebrum: Pigeon No. 4, 19 dpi. Severe perivascular cuffing of cerebral vessels and mild diffuse gliosis. HE. Bar = 250 µm.

Fig. 5. Cerebrum: Pigeon No. 5, 19 dpi. Area of liquefactive necrosis with complete loss of neuropil, remaining blood vessels and few finely vacuolated macrophages (gitter cells). HE. Bar = 250 µm.

Fig. 6. Cerebrum: Pigeon No. 4, 19 dpi. Moderate meningitis with small numbers of mononuclear cells and mild edema. HE. Bar = 175 µm.

Fig. 7. Cerebrum: Pigeon No. 5, 19 dpi. Immunohistochemical staining for AIV nucleoprotein in a moderate number of cerebral neurons and glial cells. ABC method with hematoxylin counterstain. Bar = 500 µm.

Fig. 8. Cerebrum: Pigeon No. 2, 7 dpi. Intranuclear and intracytoplasmic staining for AIV nucleoprotein in a cerebral neuron. ABC method with hematoxylin counterstain. Bar = 25 µm.

antigen could not be detected in pigeons Nos. 6–14 (Table 1).

Chickens (positive control group)

Inoculation with HPAIV A/chicken/Indonesia/2003 (H5N1) resulted in 100% mortality in chickens. Clinical signs were mild depression, anorexia, and minimal dyspnea. After occurrence of first clinical signs, the disease progressed rapidly to death within 8–12 hours at 2 dpi. At necropsy, lesions in the chickens consisted of ruptured ovarian follicles with diffuse serofibrinous "egg yolk" peritonitis and subserosal hemorrhages, scattered mucosal hemorrhages in the proventriculus, petechiation in adipose tissue as well as moderate to marked congestion of the lungs, liver, and spleen.

Histologic lesions were observed in a wide variety of organs and consisted of disseminated fibrinoid and necrotizing vasculitis with necrosis of endothelial cells, mild to moderate perivascular, mainly lymphohistiocytic, infiltrates and lymphoid depletion with necrosis in spleen, Peyer's patches, bursa, and perilaryngeal lymph follicles. The lungs showed marked congestion and mild infiltration with few lymphocytes and heterophils within the mildly edematous interstitial connective tissue. Furthermore, discrete foci of single cells or group necrosis of the pancreatic acinar epithelium, proventricular mucosa, and renal tubular epithelium as well as hepatocytes could be observed. Inflammatory response corresponding to these lesions was absent or minimal and consisted of few infiltrating heterophils. Immunohistochemically, intralesional, intranuclear, and intracytoplasmic staining for viral antigen was found within leukocytes of the Peyer's patches, bursa, perilaryngeal lymph follicles as well as cortical and medullary epithelium of the adrenal glands, renal tubules, pancreatic acini, and in few pneumocytes. Endothelial cells of small vessels and capillaries were invariably and diffusely positive for HPAIV antigen.

Sentinel chickens and negative control pigeons

Sentinel chickens and non-infected pigeons were euthanized at day 19 pi. None of the animals showed any clinical signs. Gross pathology and histopathology revealed no lesions in the brain, heart, vessels, larynx, lung, spleen, pancreas, kidney and intestine. Immunohistochemical staining detected no viral antigen in these tissues. Sera of the sentinel chickens revealed no significant H5-specific antibodies on day 19 pi.

Discussion

Ocular and nasal administration of HPAIV A/chicken/Indonesia/2003 (H5N1) resulted in moderate morbidity and mortality in pigeons within 5 to 19 dpi. Clinically, the affected birds showed depression and neurological dysfunction. Histopathology and immunohistochemistry demonstrated a pronounced neurotropism of HPAIV A/chicken/Indonesia/2003 (H5N1) for the cerebrum and brainstem in these pigeons. This is the first complete investigation integrating increased mortality with histopathology and distribution of viral antigen after infection of pigeons with HPAIV of the subtype H5.

Several transmission experiments addressed the susceptibility of pigeons to characterized and quantified HPAIV of the subtypes H5 and H7.[5,10,15,16,18,20] However, so far only one report demonstrated death of 1/19 pigeons after intranasal administration of HPAIV A/turkey/Ontario/7732/66 (H5N9).[24] In contrast to these results, our experiments demonstrate that pigeons are susceptible to HPAIV A/chicken/Indonesia/2003 and that this influenza virus exhibits a strong neurotropism in this species. The seroconversion of the nine pigeons that did not develop signs of avian influenza shows that these animals, although primarily infected, were obviously able to clear the virus. Furthermore the lack of clinical symptoms and of seroconversion in the sentinel chickens shows that bird-to-bird transmission of the virus did not occur.

Infections of the CNS with HPAIV of the subtype H5 have been observed in a wide variety of avian and mammalian species. Particularly in mice and rats but also in humans and ferrets, this virus displays a marked neurotropism.[3,9,21,29] Among avian species, the brain was consistently affected in emus, geese, house finches, and budgerigars, but microscopic lesions and viral antigen were also detected in other organs.[17,19] In chickens, zebra finches, Japanese and Bobwhite quails, turkeys, guinea fowl, pheasants, and partridges, infection was evenly distributed in the body including the brain.[17,19] Consequently, the pronounced neurotropism in pigeons observed in our experiments seems to be unprecedented at least in avian species. However, the virus may be more widely distributed in pigeons at earlier time points after inoculation.

Multiple basic amino acid residues defining a furin-sensitive cleavage site of the hemagglutinin protein and cellular proteases determine the virulence of avian influenza viruses by rendering them processable in virtually all receptor-positive cells throughout the body.[11,22] In addition, an influence of the structure of the polymerase PB2 for virulence of Hong Kong H5N1 influenza A virus in mice could be shown.[7] The molecular basis of the neurotropism of certain influenza virus strains is not known but obviously can be influenced by the host and the virus strain.[25] Furthermore, viral adaptation to the host might be important, since enhanced neuropathogenicity of an influenza virus strain (H5N3) could be produced by repeated passages through air sacs and brains of chickens.[23]

It has been shown that highly pathogenic influenza virus replicates in endothelial cells throughout the body during early stages of infection in chickens, and virus spread in the body occurs following viremia.[4,12,26] After penetrating the blood-brain barrier, infection is propagated to glial cells and neurons.[23] In mice, CNS infections may occur by ascending transsynaptic transmission of H5N1 via vagal and trigeminal nerve fibers.[13,25] Hence, viral antigen was found mainly in vagal and trigeminal ganglia, and vagal and trigeminal nuclei of the brainstem but not in the cerebral cortex.[14] The exact mechanism of intraneural transport of avian influenza A is unknown but a dependence on integrity of intermediate filaments such as neurofilament has been shown.[14] In contrast to the situation in mice, the more diffuse infection of cortical and midbrain neurons and the sparing of brain stem nuclei of cranial nerves raises the question of whether infection of the CNS in the pigeons follows rather a hematogenic than a neurogenic route. Nevertheless, the exact pathogenesis remains to be further elucidated and is currently under investigation in our laboratory. Whether the observed malacia in pigeon No. 5 was directly caused by viral infection of neuronal structures with necrosis or apoptosis in this area or by ischemia caused by endothelial damage after infection and consequent thrombotic infarction remains unclear.[8] Although adjacent neurons and glial cells stained for AIV NP-antigen and no thrombi could be detected, the focally extensive pattern and the limitation to one animal favors a vascular pathogenesis.

While reported isolations of HPAIV H5N1 were limited to one dead wild pigeon during the outbreak of avian influenza in Hong Kong 2002, the results obtained from this experiment support the view that pigeons are not resistant to infection and might be at least hypothetically involved in transmission of avian influenza.[6] Further experimental studies are ongoing to investigate the shedding of avian influenza virus by infected pigeons.

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annexe III

Study

Challenge of Pigeons

Question of Transmission to Sentinel Birds

Experimental Setup

Animals:

- 1 group (10 birds) of pigeons,
  No Vaccination
- 3 sentinel birds (SPF chicken) (9 weeks of age)
  No vaccination
- Animals were kept in BSL3 isolators according to standing biosecurity and bioethical procedures at the VAR. They were provided with water and food ad libitum.
  All animals were identified individually
  Chickens and pigeons received different type of foods from different reservoirs. In addition no litter was present in the isolators, which means feces dropped below the grid.

na 14 dagen onderzocht

na 8 d. 2 duif kalmer doch direct geëuthanaseerd

1	1429
2	1002
3	9981
4	0321
5	1044
6	1061
7	0795
8	9962
9	0771
10	1051

Challenge:

19/05/2006 (9 weeks of age):

The pigeons were inoculated oculonasally with 100 µl inoculum containing 10^6 EID_{50} A/crested_eagle/Belgium/01/2004 (H5N1) (50 µl eye, 50 µl nose).

Three days after infection of the pigeons the 3 sentinels were added to the isolator to check transmission. Leghorn SPF chickens are highly susceptible to HPAI, so we have an extern control of the experiment.

Clinical follow-up pigeons and sentinel contact birds

Methods :

We used a standardized cross-out table to register the clinical symptoms (end of document) observed in the pigeons and sentinel SPF-chickens.

Results:

Challenge was carried out 19/05/2006 (= 0 dpi). On the 3d day post-inoculation, 2 pigeons showed depression (ring numbers 6011061 and 6010795) but recovered on day 5. At 26/05/2006 (8 dpi) two pigeons were quieter than the others (but they recuperated soon). Consequently it is hard to say if this quietness was a consequence of the virus or not.

The sentinel white leghorn SPF chickens showed no clinical symptoms throughout the experiment.

Conclusion:

No clear clinical consequences of the challenge with the HPAI A/crested_eagle/Belgium/01/2004 (H5N1) became visible throughout the experiment neither for the pigeons, neither the contact sentinel SPF-chickens.

Detection of virus in pigeons after necropsy

Ring number	Sample	CT LU+TR	CT BRAIN	CT BOW	B-actin
*6011061	*1	38,15	29,15	-	+
*6009981	*2	-	-	-	+
*6011051	*3	-	-	-	+
*6010446	*4	-	37,47	-	+
*6011002	*5	-	-	-	+
*6010771	*6	-	-	-	+
*6010795	*7	-	35,34	-	+
*6009962	*8	-	-	-	+
*6011429	*9	-	-	-	+
*6010312	*10	-	-	-	+
Standaard		+	+	+	+

Three pigeons were considered as positive 15 days after challenge (CT value below 40). Two of them were those which presented some depression 2 days after infection. The highest viral load was found in the brain of one pigeon. This pigeon was also positive in the lungs

Virus excretion after challenge

Methods:

Sampling:

Cloacal and oropharyngeal swabs were taken from each animal at 3, 5 and 11 days post infection (dpi). Cotton swabs were immersed in 1,5 ml of Phosphate buffered saline (PBS) buffer containing antibiotics according to SOP/VIR/ANA/01 and stored at –70°C until further analysis.

DPI	Date
3	22/05/2006
5	24/05/2006
11	30/06/2006

Real time RT-PCR:

RNA was extracted according to an internal GLP procedure based on Roche's High Pure Viral Nucleic Acid Purification kit. The number of viral genome copies was quantified in a Taqman real time RT-PCR targeting the influenza A Matrix gene (1). An absolute quantification was done relative to a standard curve, based on tenfold dilutions of an in vitro transcribed RNA template. The virus quantification in this real time RT-PCR assay is strongly correlated with quantification using titration on CEF for virus amplified in eggs (cf ANNEX 1). Pigeon beta-actin mRNA was amplified from each sample as an internal endogenous control for sample quality and RNA extraction efficiency.

In the past, we obtained results of swab material on CEF and real-time that did not correlate well. This appeared to be a known inhibition-problem with swabs in real-time. Unpublished research in our lab (2) show a 100-fold PCR inhibition from cloacal swabs and a 10-fold inhibition from oropharyngeal swabs. Improvements have been made to our real-time protocol for swabs (2). Where the matrix RRT-PCR applied to PBS-diluted H5N1 virus has a sensitivity of 1 EID_{50}, this means that the sensitivity for cloacal and oropharyngeal swabs is about 100 and 10 EID_{50}, respectively.

Titration on CEF:

Virus titers were determined on primary chicken embryo fibroblast cultures according to SOP/VAC/ANA/15.

Results

Virus quantification using real time RT-PCR:

Several pigeons excrete the virus during the experiment. Pigeons excreting by oropharyngeal route do not necesseraly excrete cloacally and vice versa (Figure 2). Excretion peaks on average at 5 dpi (Figure 1 and 2). Most animals stopped excreting before 11 dpi.

Sentinel SPF-chicken did not excrete at any given time-point during the experiment. This means the virus changed its virulence towards chicken (eg by mutation), or the excreted concentration is to low for transmission or there is not enough contact between chicken en pigeons or the virus excreted is not alive (further tests will help to determine which option is most likely).

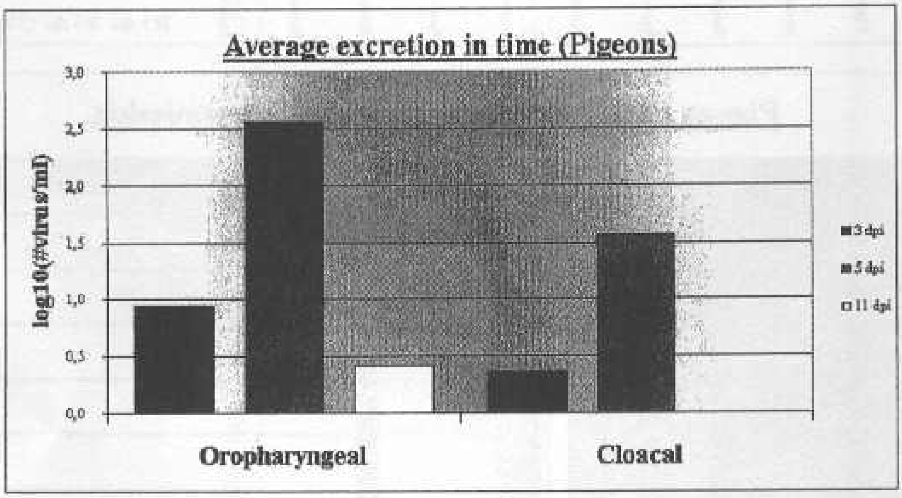
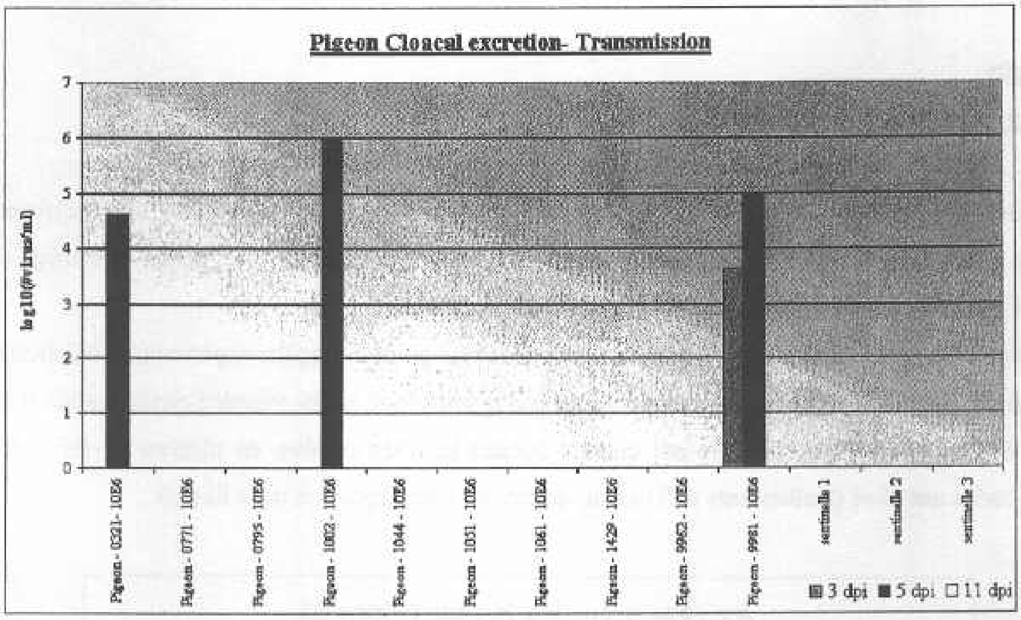


Figure 1: Bars represent the average number of virus genomes detected, based on excreting and non-excreting animals at 3,5 and 11 days post infection.



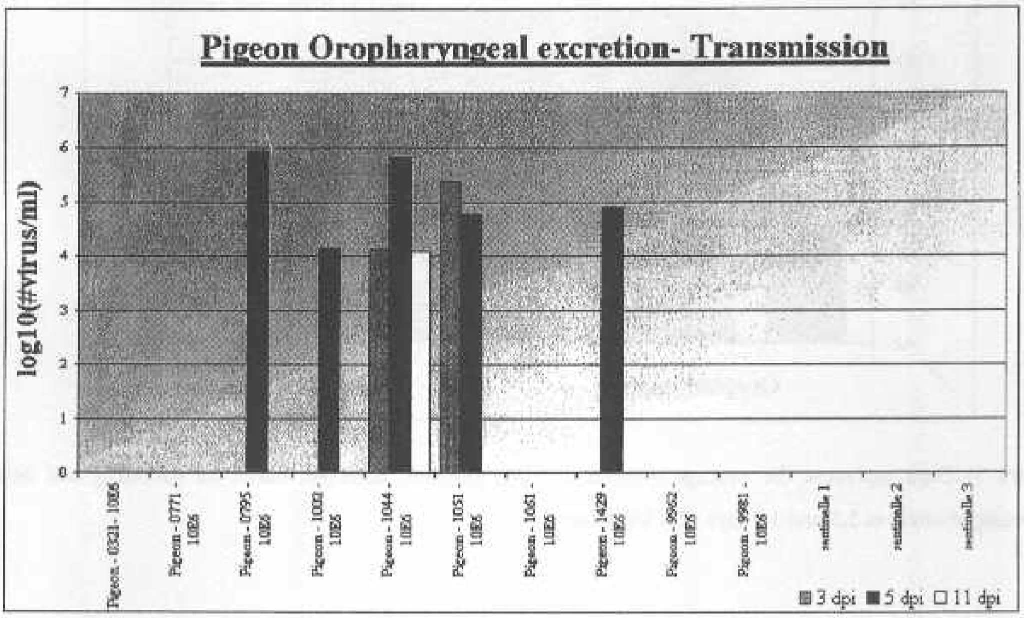


Figure 2: Cloacal (top) and oropharyngeal (bottem) virus excretion as determined by real time RT-PCR after infection with A/crested_eagle/Belgium/01/2004 (H5N1).

Titration on CEF:

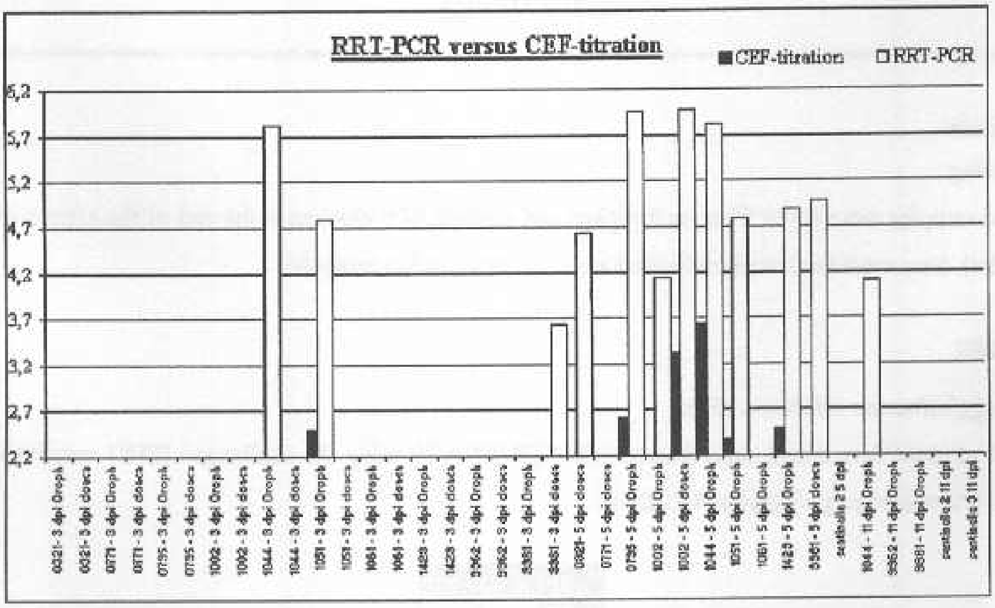


Figure 3: CEF-titration and RRT-PCR results compared for a selection of samples.

Titration of a selection of samples (all positives in real-time included) on primary chicken embryo fibroblast cell culture (Figure 3) shows that not all virus that was excreted had died. So the absence of clinical signs in the sentinels was not due to a lack of excretion of life virus. Even if no virus could be detected by CEF-titration does not mean no live virus was excreted because low concentrations of virus do not preserve well in swab material.

Serology

Methods:

Sampling:

Blood samples were taken from each pigeon and sentinel SPF chicken at the end of the experiment (14 dpi). Sera were harvested and stored at –20°C until further analysis.

Results:

Hemagglutination Inhibition (HI):

HI titres were determined according to SOP/SER/ANA/29 using the challenged **H5N1** inactivated strain (A/crested eagle/Belgium/01/2004).

	Log2 H5N1
121	6
[illegible]	7
1882	7
[illegible]	3
[illegible]	8
[illegible]	4
[illegible]	4
[illegible]	7
[illegible]	-
1429	-
senti 1	-
sent. 2	6
senti 3	6
mean	4,461538462
stdev	2,904461643

Most pigeons seroconverted (8/10), but not all at the same degree. Besides, no clear link could be made between viral excretion and seroconversion.

The seroconversion of two out of three sentinel birds can be explained. First, there is a limited contact between chicken and pigeons (feces drops below grid and they eat from different reservoirs). Secondly, a very low virus titer with which the chicken came in contact (due to little contact with excretions and due to low (live) virus titers in the excretions) the sentinel chicken did not become ill but seroconverted due to minor contact with the virus.

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