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Isolation of influenza A virus, subtype H5N2, and avian paramyxovirus type 1 from a flock of ostriches in Europe

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A total of 146 of 506 ostriches (*Struthio camelus*) introduced into a quarantine in Denmark died within the first 23 days. The majority of deaths were in young birds up to 10 kg body weight. Avian influenza A viruses (AIVs) were isolated from 14 pools of organ tissues representing seven groups each of three or four ostriches, which died over the first 3 weeks. The AIVs were detected in respiratory tissues, kidneys and intestines. All were subtype H5N2. The intravenous pathogenicity index of each isolate for chickens was 0.0 and the four isolates examined each had the amino acid sequence -P-Q-R-E-T-R*G-L-F- at the cleavage site of the haemagglutinin protein, typical of non-pathogenic AIVs. In addition, an avirulent avian paramyxovirus type 1 virus was isolated from one pool of kidney tissues.

Bacteriological examination gave no significant results. The most characteristic pathological findings were impaction of the proventriculus and gizzard, enteritis with stasis and multi-focal necrotic hepatitis.

Introduction

Avian influenza virus (AIV) is a well known threat to poultry production worldwide, although the prevalence of infection varies considerably in terms of geographic region and of time. Besides losses due to morbidity and mortality, AIV infections give rise to restrictions on production and trade. Consequently, AIV infections are notifiable in many countries, e.g. the member states of the European Union. AIVs are divided into 15 H (H1 to H15) and 9 N (N1 to N9) subtypes according to their haemagglutinin and neuraminidase antigens, respectively.

The pathogenicity of AIV varies considerably depending on the strain of virus and the host. The pathogenic AIV strains for chickens have all been of either H5 or H7 subtypes. High pathogenicity in chickens of H5 and H7 AIV strains is associated with presence of multiple basic amino acids at the cleavage site of the haemagglutinin protein (Bashiruddin *et al.*, 1992; Wood *et al.*, 1993). AIVs of low virulence may cause severe disease in the presence of exacerbating conditions or infections with other organisms. Very little is known concerning the pathogenicity of AIV strains in ostriches compared to chickens and other avian species. AIV has been isolated from a substantial number of feral and domestic avian species. It is known that some species e.g. waterfowls are refractory to AIV infections with respect to development of disease (Alexander, 1995). At present there are very few reports on isolation of AIV from ratites. Allwright *et al.* (1993) isolated AIV subtype H7N1 from an outbreak of clinical disease in ostriches in the Republic of South Africa. The isolated strain was apathogenic to fowls while it caused disease in ostriches, especially young chicks. Panigrahy *et al.* (1995) reported isolation of AIV subtypes H5N2 and H7N1 from emus and rheas in the USA, some of which had a history of respiratory disease.

Probably all avian species are susceptible to avian paramyxovirus type 1 (APMV-1) and, similar to AIV, the pathogenicity of APMV-1 strains

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varies with the strain of virus and host species. Samberg *et al.* (1989) reported an outbreak of Newcastle disease in ostriches associated with 28% mortality in 5- to 9-month-old birds. Huchzermeyer & Gerdes (1993) isolated virus from three outbreaks of ND in ostriches with low mortality.

The present study describes the epidemiological and pathological findings associated with the isolation of AIV and APMV-1 from a flock of ostriches with high mortality during the initial 23 days after introduction in Denmark.

Materials and Methods

Population and records of disease and mortality

The investigation comprises the monitoring of a group of 506 ostriches (*Struthio camelus*) during the first 23 days of quarantine in Denmark, i.e. from March 26th to April 18th 1996. The birds were introduced into quarantine on 26th March 1996 after approximately 6 h transport on lorries within the European Union. They were mixed sex and it was recorded that 317 (63%) were 2 to 3 months of age, 121 (24%) approximately 6 months of age and 68 (13%) were adults. During the study period the flock was kept indoors and considered as one unit in terms of epidemiology. The flock was monitored and mortality recorded by a public veterinary officer. All dead birds were submitted for laboratory examination during the study period.

Post-mortem examination

Three submissions of dead ostriches from the flock were received at the laboratory. Post-mortem examination was performed on a representative number of birds from each submission, and samples were taken for bacteriology, virology and histopathology. Histopathology was performed in accordance with the macroscopic pathological lesions, e.g. extensive examination of liver specimens was carried out whereas intestinal specimens were examined to a lesser extent.

Virology

Virological examination was carried out according to guidelines in E.U. Council Directives 92/40/EEC and 92/66/EEC (CEC, 1992a,b). Briefly, tissue samples were homogenized in sterile antibiotic medium and made to 10 to 20% w/v suspensions. The clarified supernatant was inoculated into the allantoic cavity of each of six 9-day-old SPF chick embryos. Allantoic fluid harvested from eggs with dead embryos and from eggs incubated for 6 days was examined for haemagglutination activity. Up to two serial blind passages were made. Tissue samples from groups of three or four birds were pooled at homogenization. The procedure was performed on tissues from the trachea and lungs, kidneys and intestinal tract including intestinal contents. Inoculations in cell cultures were not carried out.

Identification of haemagglutinating viruses

Sterile harvest of allantoic fluid was tested according to the procedures described for haemagglutination (HA), haemagglutination inhibition (HI) and immunodiffusion (ID) test in the above mentioned directives (CEC, 1992a,b). HA and HI tests were performed using SPF chicken red blood cells.

AIV subtyping was done using chicken polyclonal antisera to reference strains of influenza A viruses as described (Alexander & Spackman, 1981).

The APMV-1 virus was typed using reference chicken antisera and further characterized by assessing its ability to cause infected Vero cells to react with a panel of 26 mouse monoclonal antibodies in immunoperoxidase monolayer assay (IPMA) tests as described (Alexander *et al.* 1997).

Pathogenicity tests

APMV-1 and AIV isolates were tested for pathogenicity by means of intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) tests, respectively, according to the E.U. guidelines (CEC, 1992a,b).

Sequence analysis of the haemagglutinin cleavage site

Four representative isolates covering each of the submissions of ostriches were subjected to nucleotide sequencing in the region of the genome coding for the cleavage site of the haemagglutinin molecule as described (Wood *et al.*, 1994, 1997).

Results

Epidemiology

During transport and the following 3 days, 45 ostriches died. Two were above 100 kg, five were 10 to 100 kg and 38 were below 10 kg body weight. From the fourth to the fifteenth day, 77 birds died. Of these, four were 10 to 100 kg body weight while the rest were below 10 kg. During the rest of the study, 24 birds died. Two were 10 to 100 kg body weight and the rest were smaller. Thus, a total of 146 (29%) ostriches died during transport and initial 23 days after arrival. Approximately 91% of the dead birds were below 10 kg body weight. From the hatching records of the flock it was evident that ostriches below 10 kg body weight (March/April) were 2 to 3 months of age. At least 50 more ostriches died within 2 months of the end of the study period. These were not included in the study due to lack of exact records and laboratory investigations.

Pathology and bacteriology

The gross pathological lesions are summarized in Table 1. The most consistent findings were impaction of the proventriculus and gizzard, and pathological changes related to the intestine and multi-focal necrotic hepatitis.

Intestinal and liver specimens from three birds from the initial submission (96-72170) with gross pathological lesions of haemorrhagic enteritis/stasis had histopathological lesions of enteric stasis, haemorrhage and a dense mononuclear cellular infiltrate in the lamina propria, and necrotic hepatitis, respectively.

The most characteristic histopathological lesions in the two remaining submissions (96-72420 and 96-72588) were multi-focal necrotic hepatitis with characteristic irregular contours of the lesions.

Aerobic bacterial culture was performed on livers from all birds examined, as well as on samples from various organs when indicated by the pathological findings, with negative or non-specific results.

Table 1. *Gross pathological findings at* post-mortem *examination of three submissions of dead ostriches from a quarantine in Denmark*

Identification of submission	No. of birds received	No. of birds examined	Impaction of proventriculus and gizzard	Haemorrhagic enteritis/stasis	Enteritis	Typhlitis	Multi-focal necrotic hepatitis	Airsac-culitis	Focal pneumonia	Petechia		Subcutaneous bleeding
										Epicardium	Trachea	
96-72170	45	45	25[1]	19	18					3	1	3
96-72420	77	20	8		10		7	2	3			
96-72588	24	12	4			2	3	1	1			

[1]The number of birds with the different types of pathological findings.

Table 2. *Results of virological examination of three submissions of dead ostriches from a quarantine in Denmark*

Identification of submission	Date received	No. of birds received	No. of birds examined	No. of groups examined	Virus isolations[a]		
					Lung and trachea	Kidneys	Intestines and intestinal contents
96-72170	29 Mar 96	45	45	15	2/15[b] AIV	1/15 AIV 1/15 PMV1	1/15 AIV
96-72420	10 Apr 96	77	20	5	2/5 AIV	3/5 AIV	4/5 AIV
96-72588	18 Apr 96	24	12	4	0/4	0/4	1/4 AIV

[a]The number of organ pools from which virus was isolated together with the identity of the virus isolates. AIV = Avian influenza virus (all subtype H5N2). PMV1 = paramyxovirus serotype 1.
[b]The number of organ pools from which virus was isolated over the number of pools examined.

Virology

The number and identity of virus isolates from all three submissions of dead ostriches collected over a 23 day period are shown in Table 2. AIV was present in all three submissions and a total of 14 AIV isolates was made. Non-agglutinating viruses were not isolated.

Forty-five ostriches from the first submission were divided into 15 groups which were examined virologically. AIV was isolated from two of the 15 groups, from respiratory tissues of one group and from all types of tissues of the other group. Due to the large number of birds in the second submission 20 birds forming five groups each of four birds were selected for examination. AIV was isolated from intestines of one group, from intestines and kidneys of one group and from all three types of tissues of the two groups. One group yielded no virus. Finally, AIV was isolated from intestines from one group of birds of the last submission. All AIV isolates were made from dead birds weighing less than 7 kg and the majority were less than 4 kg. Out of five groups examined from the second submission, AIV was isolated from four, all of which represented birds weighing less than 6 kg. No virus was isolated from the remaining pool, which originated from birds weighing 24 to 54 kg.

The vast majority of the viruses were isolated in the first passages. Only two AIV isolates needed a blind passage before isolation. The HA titres of the allantoic fluid harvested were in the range 1:32 to 1:1024.

All AIV isolates were identified as influenza type A viruses in ID test, and as subtype H5N2 by HI and neuraminidase inhibition tests.

Nucleotide sequencing showed each of the four viruses tested to have the deduced amino acid sequence, -P-Q-R-E-T-R*G-L-F-, at the cleavage site of the haemagglutinin molecule. The virulence of each of the AIV isolates was determined in IVPI tests in 6-week-old chickens. None induced any clinical signs and an index of 0.0 was recorded for each virus.

A single isolate of APMV-1 was obtained from a pool of kidney tissues from a group of small ostrich chicks of the initial submission. The identity of this virus was confirmed by HI test and when tested by immunoperoxidase test it gave patterns identical to those obtained when testing Ulster2C-like viruses (group G/Q) (Alexander *et al.*, 1997). The ICPI of the isolate was 0.0.

Discussion

The stress connected with transport and introduction to an in-door environment may have influenced the mortality of the ostriches. Allwright *et al.* (1993) reported that an outbreak of AIV subtype H7N1 associated disease in ostriches was also associated with influence of stress. In this study, 63% of the birds weighed less than 10 kg at the arrival. Nevertheless, 91% of the birds that died weighed less than 10 kg; It is therefore concluded that the mortality was highest in youngest birds. Allwright *et al.* (1993) reported the highest mortality in young ostriches with mortality rates of 15 to 60% in ostriches up to 8 months of age and even higher in chicks under 1 month of age.

In the present study clinical symptoms were not recorded systematically. The macroscopic pathological findings were confirmed by the histopathological examination. Allwright *et al.* (1993) reported similar gross pathological and histological lesions in the liver and intestine of dead AIV-infected ostriches. Panigrahy *et al.* (1995) found that AIV infections in emus and rheas were associated with respiratory disease and pathological lesions of respiratory organs. In general, pathological lesions associated with AIV infections show considerable variation, but have been most intensively studied in fowls and turkeys. A variety of congestive, haemorrhagic transsudative, and necrobiotic changes

have been associated with highly pathogenic AIV strains. In chickens experimentally infected with fowl plague virus necrotic foci have frequently been observed in liver, spleen and lungs (Easterday & Hinshaw, 1991).

In contrast to the present study, Allwright *et al.* (1933) reported invariable presence of several bacterial and fungal concurrent infections in AIV-infected ostriches and Panigrahy *et al.* (1995) found *Escherichia coli* in affected organs of AIV-infected emus and rheas.

AIV was most efficiently isolated from kidneys and intestines. The finding of AIV as well as APMV-1 in the kidneys indicates viraemic phase, of both viral infections in the ostriches. Serology could not be performed since blood samples from the flock were not available. The subtype of AIV isolated from the Danish flock of ostriches was H5N2. The vast majority of AIV strains pathogenic to fowls have been either H5 or H7 subtypes but also many apathogenic strains of these subtypes have been isolated (Easterday & Hinshaw, 1991). Panigrahy *et al.* (1995) found H5N2 and H7N1 subtypes AIV in emus and rheas with respiratory disease and Allwright *et al.* (1993) isolated H7N1 AIV subtype from outbreaks in ostriches. The AIV strains isolated in both these studies proved to be apathogenic for chickens, similar to the H5N2 isolate of the present investigation.

Very few results of systematic investigations on pathogenicity of AIV in ostriches have been published until now. If the disease and the mortality in the flock of ostriches in the present study was significantly influenced by the AIV infections, pathogenicity tests carried out in fowls may not indicate the true pathogenicity of AIV strains for ostriches. Collaborative studies on experimental AIV infections in ostriches are in progress.

APMV-1 was isolated from kidney tissue of one group of 2 to 3 months old ostrich chicks. The virus isolate was apathogenic to fowls and antigenically related to Ulster 2C-like APMV-1 strains, which are frequently isolated from feral waterfowl (Alexander *et al.*, 1997). As with AIV, only few investigations on the pathogenicity of APMV-1 in ostriches have been published. Most recently Samberg *et al.* (1989), and Huchzermeyer & Gerdes (1993) reported isolation of APMV-1 viruses from diseased ostriches. Samberg *et al.* (1989) observed morbidity and mortality as highest in young chicks while older ostriches were unaffected. Due to concurrent AIV infection and the conditions of the investigation it was not possible to evaluate the role of the APMV-1 isolate for the development of disease and mortality in the present study. The origin of the AIV and APMV-1 in the flock of ostriches is unclear. Nevertheless, both viruses were isolated from birds that died during transport and a few days after arrival. Until now AIV has not been isolated in Denmark and according to certificates, the flock had received no ND vaccine prior to the entrance in Denmark. Vaccination against ND is not practised in Denmark. Influenza A viruses prevalent in Danish swine are of H1 and H3 subtypes. Consequently, the possibility of the flock having contracted the infections prior to the shipment and the APMV-1-isolate being vaccine derived cannot be ignored.

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References

Alexander, D.J. (1995). The epidemiology and control of avian influenza and Newcastle disease. *Journal of Comparative Pathology, 112*, 105–126.

Alexander, D.J. & Spackman, D. (1981). Characterization of Influenza A viruses isolated from turkeys in England during March–May 1979. *Avian Pathology, 10*, 281–293.

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

Allwright, D.M., Burger, E.P., Geyer, A. & Terblanche, A.W. (1993). Isolation of influenza A virus from ostriches (*Struthio camelus*). *Avian Pathology, 22*, 59–65.

Bashirruddin, J.B., Gould, A.R. & Westbury, H.A. (1992). Molecular pathotyping of two avian influenza viruses isolated during the Victoria 1976 outbreak. *Australian Veterinary Journal, 69*, 140–142.

CEC (1992a). Council Directive 92/40/EEC of 19 May 1992 introducing Community measures for the control of avian influenza. *Official Journal of the European Commission, L167*, 1–15.

CEC (1992b). Council Directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease. *Official Journal of the European Commission, L260*, 1–20.

Easterday, B.C. & Hinshaw, V.S. (1991). Influenza. In: B.W. Calnek, H.J. Brenes, C.W. Beard, W.M. Reid & H.W. Yoder (Eds), *Diseases of Poultry*, 9th edn (pp. 532–551). Ames: Iowa State University Press.

Huchzermeyer, F.W. & Gerdes, G.H. (1993). Newcastle disease virus isolated from ostriches in South Africa. *Journal of the South African Veterinary Association, 64*, 140.

Panigrahy, B., Senne, D.A. & Pearson, J.E. (1995). Presence of avian influenza virus (AIV) subtypes H5N2 and H7N1 in emus. (*Dromaius novaehollandiae*) and rheas (*Rhea americana*): virus isolation and serological findings. *Avian Diseases, 39*, 64–67.

Samberg, V., Hadash, D.V., Perelman, B. & Meloz, M. (1989). Newcastle disease in ostriches (*Struthio camelus*): field case and experimental infection. *Avian Pathology, 18*, 221–226.

Wood, G.W., McCauley, J.W., Bashirruddin, J.B. & Alexander, D.J. (1993). Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. *Archives of Virology, 130*, 209–217.

Wood, G.W., Banks, J., McCauley, J.W. & Alexander, D.J. (1994). Deduced amino acids sequences of the haemagglutinin of H5N1 avian influenza virus isolates from an outbreak in turkeys in Norfolk, England. *Archives of Virology, 134*, 185–194.

Wood, G.W., Banks, J., Brown, I.H., Strong, I. & Alexander, D.J. (1997) The nucleotide sequence of the HA1 of the haemagglutinin of an H1 avian influenza isolate from turkeys in Germany provides additional evidence suggesting recent transmission from pigs. *Avian Pathology, 26*, 347–355.

RÉSUMÉ

Isolement d'un virus Influenza de sous type H_2N_2 et d'un paramyxovirus de type 1 à partir d'un troupeau d'autruches en Europe

Cent quarante six autruches (*Struthio camelus*) sur un total de 506, mises en quarantaine au Danemark, sont mortes dans les 23 premiers jours. La majorité des morts étaient des jeunes oiseaux d'un poids maximum de 10 kg. Des virus Influenza de type A (AIVs) ont été isolés à partir de 14 pools d'organes correspondant à 7 groupes de 3 ou 4 autruches qui sont mortes dans les 3 premières semaines. Les AIVs ont été mis en évidence à partir des tissus respiratoires, des reins et des intestins. Tous les virus appartenaient au sous type H_5N_2. L'indice de pathogénicité intraveineux de chaque isolat pour le poulet a été de 0,0 et les 4 isolats étudiés avaient la séquence en acides aminés: P-Q-R-E-T-R*G-L-F au site de clivage de la protéine hemagglutinante, typique des AIVs non pathogènes. De plus, un paramyxovirus aviaire de type 1 non-virulent a été isolé à partir d'un des pools de reins. Les examens de types bactériologiques n'ont pas donné de résultats significatifs. Les résultats pathologiques les plus caractéristiques ont été observés au niveau du proventricule et du gésier, une entérite avec stase et une hépatite avec de multiples foyers de nécrose.

ZUSAMMENFASSUNG

Isolierung von Influenza A-Virus des Subtyps H5N2 und aviärem Paramyxovirus 1 aus einer Straußenherde in Europa

Insgesamt 146 von 506 Straußen (*Struthio camelus*), die in eine Quarantäne in Dänemark gebracht wurden, starben innerhalb der ersten 23 Tage. Die Mehrzahl der Todesfälle ereignete sich bei jungen, bis zu 10 kg schweren Vögeln. Aviäre Influenza A-Viren (AIVs) wurden aus 14 Organgewebe-Pools isoliert, die 7 Gruppen von je 3-4 Straußen repräsentierten, welche im Verlauf der ersten 3 Wochen starben. Die AIVs wurden in Geweben der Atmungsorgane, Nieren und Därmen nachgewiesen. Alle waren vom Subtyp H5N2. Der intravenöse Pathogenitäts-Index für Küken war bei allen Isolaten 0,0. Die 4 untersuchten Isolate hatten alle die Aminosäuresequenz -P-Q-R-E-T-R*G-L-F- an der Spaltungsstelle des Hämagglutinin-Proteins, was für nicht-pathogene AIVs typisch ist. Außerdem wurde ein avirulentes aviäres Paramyxovirus vom Typ 1 aus einem Nierengewebe-Pool isoliert.

Die bakteriologische Untersuchung lieferte keine wesentlichen Ergebnisse. Die charakteristischsten pathologischen Befunde waren Drüsen- und Muskelmagenverstopfung Enteritis mit Stase und multifokale nekrotische Hepatitis.

RESUMEN

Aislamiento del virus influenza, subtipo H5N2, y el paramixovirus aviar tipo 1 procedentes de un grupo de avestruces en Europa

Un total de 146 de 506 avestruces (*Struthio camelus*) en cuarentena en Dinamarca murieron durante los primeros 23 días. La mayoría de las aves muertas eran jóvenes hasta un peso de 10 kg. Se aislaron virus de influenza aviar (AIVs) a partir de macerados de tejidos que reresentaban 7 grupos de 3–4 avestruces cada uno que habían muerto durante las tres primeras semanas. Se detectaron AIVs del tipo H5N2 en tejidos respiratorios, riñones e intestinos. El índice de patogenicidad intravenosa de cada aislamiento para las gallinas fue 0.0 y los 4 aislamientos examinados tenían una secuencia aminoacidica -P-Q-R-E-T-R*G-L-F- en el lugar de excisión de la proteina hemoaglutinina, típica de AIVs no patógenas. Se aisló además un paramixovirus aviar tipo 1 de un macerado de tejido renal.

El examen bacteriológico no produjo resultados significativos. Los hallazgos patológicos más significativos fueron la imapctación de la molleja, enteritis con estasis y hepatitis multifocal necrotizante.