Experimental study on the pathogenicity of avian influenza A/Ch/It/5093/99(H7N1) virus in chicken

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ABSTRACT

In this study, the pathogenicity of A/Ch/It/5093/1999 H7N1 which had been isolated from chicken during the outbreak in Italy was assessed in chicken by experimental infection virus. Ten SPF chickens of four week-old were inoculated with this virus, and five chickens were inoculated with uninfected allantoic fluid. For determination of virus shedding, oropharyngeal and cloacal swabs were taken from experiment and control groups, on days 1-5 post infection (p.i.) and used in a reverse transcription-polymerase chain reaction (RT- PCR) assay for detection of avian influenza virus (AIV). On day (2-5 d.p.i.) certain organs such as lung, brain, liver and kidneys collected from dead birds for virus titration, and histopathological investigations. In this study we obtained high titers in oropharyngeal swabs in 48 hours p.i. (h.p.i.). The first clinical signs observed were anorexia and depression in chickens. The results obtained with the virus isolation were confirmed by RT-PCR. All of the chickens inoculated with A/Ch/It/5093/99 died between 2d.p.i and 5 d.p.i. (2-5 d.p.i.). On day 1 to 3 d.p.i. relatively high titer of infectious virus could also be isolated from oropharyngeal swab $10^{2.3}$ to$10^{5.9}$ 50% egg infectious doses (EID_{50}/ml), whereas virus shedding from the cloaca ($10^{1.5}$ to $10^{3.2}$ EID_{50}/ml) was considerably less. The present study was conducted to determine the pathogenicity of A/Ch/It/5093 H7N1 in chickens. In our data show that infection of chicken with H7N1 virus leads to viral replication on the respiratory tract resulting in severe lung damage. The most consistent and severely affected organ by this virus were lung, kidney and brain, severity of the lesions in each organ was probably related to tissue tropism.
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Keywords: Avian Influenza, H7N1, Pathogenicity, Chicken

INTRODUCTION

Avian influenza (AI) is a viral respiratory disease of many species of domestic and wild bird. AI viruses have been circulating previously among domestic poultry over the past 100 year (Tumpy *et al* 2004). AI viruses may cause two different diseases on the basis of the severity of clinical signs they induce in susceptible species. Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry caused by some viruses of the H5 and H7 subtypes (Capua *et al* 2000). In Italy low pathogenic avian influenza (LPAI) has affected various avian species over the last 40 year, but it has particularly affected turkeys (Bread 1999 & Zanella *et al* 2002). In Italy, both HPAI and LPAI have been isolated through the years (1999-2000) (Puzelli *et al* 2005). Evidence of HPAI in 1935 to 1937 was

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reported and no other out breaks being reported until 1997, when a virulent H5N2 virus was isolated in north-eastern Italy (Capua *et al* 2000a). Since March of 1999, a serious H7N1 AI epidemic has been occurring in Northeastern Italy, in an area limited to the provinces of Verona, Bresica and Mantora, and this epidemic has caused heavy losses, particularly in turkeys, but also in breeder and layer (Capua *et al* 1999). From March to December 1999 approximately 65% of Italian industrial poultry population is raised with LPAI (H7N1) (Capua *et al* 2000b). On 17 December 1999 HPAI (H7N1) confirmed by OIE Laboratory. The HPAI epidemic resulted directly or indirectly in the death or culling of over 13 million birds, which inevitably resulted in the disruption of the marketing system and great economic losses to poultry industry and social community (Capua *et al* 2002). The HPAI virus emerged from mutation of a low pathogenic strain of the same subtype which had circuited in poultry for approximately nine months (Capua *et al* 2001). The circulation of the virus in a susceptible population for several months caused the emergence of a highly pathogenic virus with an IVPI of 3.0 and the presence of multiple basic amino acids in the deduced amino acid sequence for the cleavage site of the haemagglutinin molecule (Capua *et al* 2000). The pathogenicity of AIV varies considerably depending on the strain of virus and the host. Although the avian are sensitive to AIV but the pathogenicity studied caused by A/Ch/It/5093/1999 in chicken and other avian species are limited. In this paper we describe the gross, clinical, virological finding and viral shedding finding of chicken experimentally infected with field isolated of A/Ch/Italy/5093/1999 H7N1 virus.

MATERIALS AND METHODS

Virus. The avian influenza virus A/Ch/Italy/5093/99 H7N1 was isolated from chicken during the outbreak of HPAI in Italy and was provided by Institute zooprofilatica sperimetale delle Venezie and national reference laboratory Italy. Virus stock was propagated in the allantoic cavity of 10-day-old embryonated chicken specific pathogen free eggs for 30hr at 37 °C. Allantoic fluid was harvested, clarified by centrifugation, aliquoted and stored at -70 °C. Fifty percent egg infectious dose (EID$_{50}$) titers were determined by serial titration of virus in SPF egg and endpoints were calculated by the method of Read and Munch (Read and Munch 1938). All experiments using live viruses were conducted in BSL3 containment facilities in national reference for laboratory zooprofilatica sperimentale delle Venezie, Italy.

Chicken. We obtained fifteen SPF chicken of four week-old from instituto Zooprofilatico sperinentale delle Venezie of Italy. Before inoculation, blood was collected from all birds to ensure that the birds were serologically naive to influenza viral antigens. All the serum samples collected tested negative for anti-influenza viral antibody with agar gel precipitin and RT-PCR test. All chicken were divided into a control group and a virus-inoculated group. The control group included 5 chickens and experiment group which contained ten chicken were hosed in stainless steel cabinets ventilated under negative pressure with HEPA filtered air and maintained under continues lighting. Feed and water were provided ad libitum. The birds in the sample group were inoculated via oculo-nasal route with 0.1ml of inoculums containing 10^4EID50 of A/Ch/It/5093/99 H7N1 virus. The birds in control group received the same volume of normal of uninfected allantoic fluid. After infection, chicken were monitored daily for disease signs. For determination of virus shedding, oropharyngeal and cloacal swabs were taken from control and virus-inoculated groups, on day's 1-5 p.i. Oropharyngeal and cloacal swabs from birds processed for virological assay and RT-PCR analysis. When chickens were dead (2-5 p.i.) lung, brain, liver and kidney of each chicken were collected aseptically and were used for the titration

of virus, RT-PCR and histopathological investigations. The initial clinical signs in virus-inoculated group were anorexia and reduced water consummation which progressed to depression.

Virus titration. On the sampling days (2-5), the organs collected from dead bird were homogenized to obtain a 10% W/V suspension in phosphate-bufferd sailin (0.05M to PH 7.0-7.4) with antibiotics (penicillin: 1000 IU/ml, streptomycin: 10mg/ml nystatin: 5000IU/ml, gentamycin sulfate: 25µg/ml) and clarified by centrifugation. The tissue homogenates from birds were inoculated into 10-day-old embryonated chicken eggs and incubated for 48hr at 37 °C. Individual oropharyngeal and cloacal swabs were placed in 1 ml of brain heart infusion medium containing antibiotics, clarified swabs samples tittered for virus infectivity in eggs. The undiluted and ten-fold serially diluted supernatants were used for the titration of virus infectivity in embryonated eggs according to EU guidelines (CEC 1992). The titers of virus were calculated by the method of Reed and Muench and expressed as the EID_{50} per ml for oropharyngeal and cloacal swab. All experiments were conducted under biosafety level 3 conditions.

RT-PCR. Samples were prepared using the commercial kit (High RNA of pure TM RNA extraction kit; Roche diagnostic GmbH, Mannheim, Germany) and cDNA was synthesized with "High capacity cDNA archive kit" according to manufactures instructions. A RT-PCR was performed on homogenized tissue, oropharyngeal and cloacal swabs samples with primers conserved regions in the matrix gene segment after analysis (Fouchier *et al* 2000). Primers that used were matrix gene-specific PCR primer set M+25 and M-124. (Spackman *et al* 2002). The following protocol for PCR was 20 minutes at 42°C and 5 minutes at 95°C followed by 40 cycles at 94 °C for 1 minute and 60°C for 1 minute and at 72 °C for 1 minute and following by 72 °C for 10 minutes.

Histopathology. Tissues (lung, kidney, liver and brain) from dead birds separated daily for histologic evolution were fixed by submersion in 10% phosphate neutral buffered formalin, they were dehydrate, embedded in paraffin, and cut into 7µm thick section. Sections were stained with hematoxylin and eosin using a standard method.

RESULTS

The first clinical signs observed were anorexia and depression in chickens. Feed consumption dropped and the birds appeared sleepy and depressed such as paralysis of wings, incoordination and tremors of the head and neck, there was no mortality in the control group after 7 days. The results obtained with the virus isolation were confirmed by RT-PCR, all samples that were positive by virus isolation were also found to be positive by RT-PCR (Figure 1). In addition, brain tissue sample in 2 d.p.i was negative by virus isolation and positive by RT-PCR (Table2).
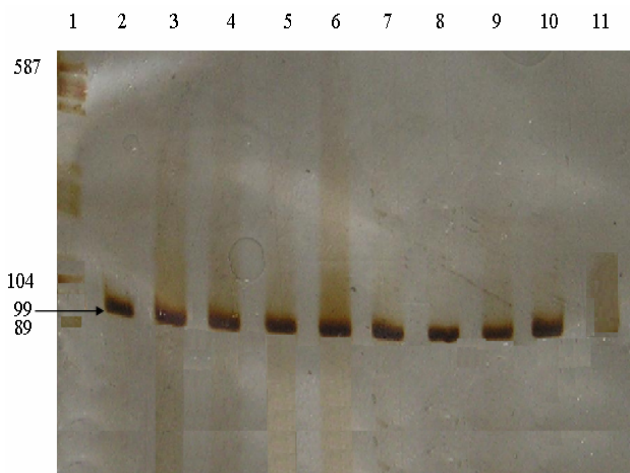
Figure 1. Gel electrophoretic analysis of PCR product. lane 1: marker , lane 2: positive control, lane 3: oropharyngeal swab(2 d.p.i), lane 4: cloacal swab(2 d.p.i), lane 5: oropharyngeal swab(3 d.p.i), lane 6: cloacal swab(3 d.p.i), lane 7: oropharyngeal swab(4 d.p.i), lane 8: cloacal swab(4 d.p.i), lane 9: oropharyngeal swab(5 d.p.i), lane 10: cloacal swab(5 d.p.i), lane 11: negative control

All of the chickens inoculated with A/Ch/It/5093/99 died between 2-5 d.p.i and virus was recovered from each of the tissues tested (lungs, kidneys, liver and brain). On day 1 to 3 d.p.i. relatively high titer of infectious virus could also be isolated from oropharyngeal swab 2.5 to 5.9 $\log_{10}EID_{50}$/ml (Table 1) .Whereas virus shedding from the cloaca (1.5 to 3.2 $\log_{10}EID50$/ml) was considerably less.

**Table 1.** Comparison shedding of oropharyngeal and cloacal swab (A/Ch/It/5093/1999) H7N1 virus in chicken

	Oropharyngeal swabs					Cloacal swab				
Days	1	2	3	4	5	1	2	3	4	5
titer	2.5	3.5	5.9	5.8	4.9	1.5	2	3.2	3.5	3.1

Mean virus titer expressed as Log_{10} EID_{50}/ml

Table2. Detection of AIV in tissue sample obtained from chicken infected with influenza virus A/Ch/It/5093/1999 by different methods

Chi. No	Day p.i	Dead/total	Lung		Liver		kidney		Brain	
			VI	RT-PCR	VI	PCR	VI	RT-PCR	VI	RT-PCR
1	1	0/10	-	-	-	-	-	-	-	-
2	2	2/10	+	+	-	-	+	+	-	+
3	3	5/10	+	+	-	-	+	+	+	+
4	4	8/10	+	+	-	-	+	+	+	+
5	5	10/10	+	+	-	-	+	+	+	+

These results indicate that the severity of disease caused by the A/Ch/It/5093/99 virus was associated with broad tissue tropism and with high virus titers in multiple organs particularly the lung and trachea. The kidneys were appeared enlarged and friable and contained urate deposits. Mononuclear cells

infiltrations in interstial tissue were observed, degenerative changes were found in the kidneys (Figure 3).
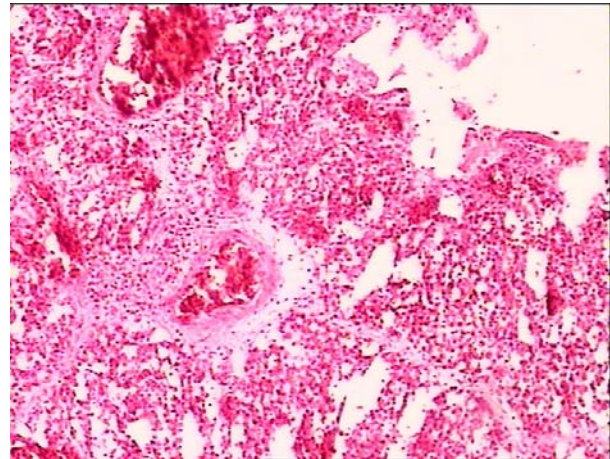
Figure 2. Lung, inflammatory cells infiltration in the interstial area and prevascular cuffing of chicken infected with H7N1 AI (H&E×40).
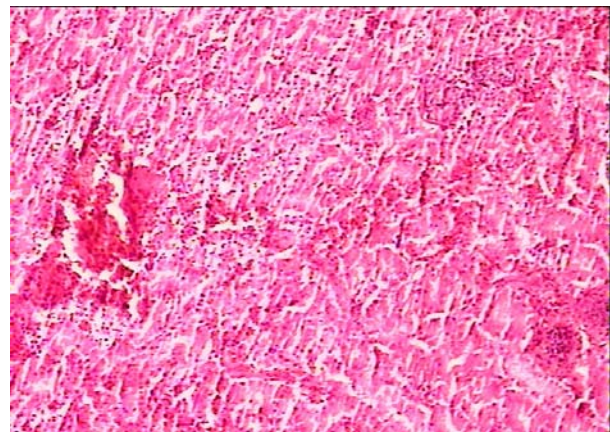
Figure 3. Kidney, Congestion with infiltration of mononuclear inflammatory cells (H&E×10).

The trachea and lungs appeared to be congested and hemorrhagic lesion were present in the parabronchi mild to severe infiltration of lymphocytes, mononuclear cells inflammation were observed in the interstitial area in the lungs with prevascular cuffing (Figure 2). Histopathological features detected in the brain included the presence of hyperemia,

prevascular infiltration of lymphocytes and polymorphonuclear cells in the parenchyema.

DISCUSSION

Although some isolates of H7 AIVs are considered to be low pathogenicity, the high mutation rate of AI virus is in thought to allow these subtypes to change to a highly pathogenic AI virus with alarm frequency (Kawaoka *et al* 1987 & Govorkova *et al* 2005). In contrast of most other HPAI outbreaks, the Italian HPAI H7N1 virus affected a wide variety of avian species including chicks, turkeys, quails, geese and ostriches. Infection was never detected in any wild or domestic mammals and humans (puzelli *et al*, 2005). The present study was conducted to determine the pathogenicity of A/Ch/It/5093/99 H7N1 in chickens. In our data show that infection of chicken with H7N1 virus leads to viral replication on the respiratory tract resulting in severe lung damage. In poultry the nasal cavity is the predominant initial site of AI virus replication with release of virions and infection of other cells in the respiratory tract and intestinal tract (Swanye 2008). Illness or deaths is most often from respiratory damage so I obtained shedding of virus in oropharyngeal and cloacal swabs. In recent studies with three H7N1 HPAI viruses isolated from Italy, A/Ch/It/5093/99 isolate was the least pathogenic in mice infected, they have reported that the pathogenicity of the ostrich isolate (Os/984/00) for mice appears to be intermediate level compared to other isolates, all the three H7N1 viruses isolates were pathogenic for mice, although varying degree of pathogenicity were observed (Rigoni *et al* 2007) but in our study A/Ch/It/5093/99 is highly pathogen in chicken. For the whole animal, HPAI viruses vary in the pathophysiological mechanism responsible for the illness and death. HPAI viruses like this virus in poultry have initial replication in nasal epithelium and after 24hr virus replicates within endothelial cells and spread via the vascular or lymphatic system to infect and replicate visceral organ in cell types within, brain and skin (Swayne 2008). This initial visceral replication may be seen as early as 24hr after intranasal inoculation, and by 48hr, the virus titer are high (Swanye 2007). In this study we obtained high titers in oropharyngeal swabs in 48 h.p.i. (table1). Infection of chickens with H7N1 virus resulted in higher titers of infections virus recovered from the oropharynx in comparison with cloaca. HPAI viruses grow to high titers in the respiratory and intestinal tracts of chickens and turkeys and are shed in the respiratory secretions and feces. LPAI viruses can be shed in asymptomatically infected or minimally affected flocks but HPAI viruses are unlikely to cause significant human diseases (Spickler *et al* 2008). Systemic spreads of virus was also observed after inoculation with A/Ch/It/5093 (table 2) and after inoculation with this isolate were detectable in the internal organ. These results indicate that the severity of disease caused by the A/Ch/It/5093/99. Virus was associated with broad tissue tropism and with high titers in respiratory tract. Collectively, our data show that infection of chicken with influenza virus A/Ch/It/5093/99/H7N1 leads to viral replication in the respiratory tract resulting in severe lung damage. This virus affected organs particularly kidneys, lung and brain not limited to respiratory tract. The necrotic and inflammatory changes in histopathologic description for experimental studies vary with individual viruses as results of varitiations inoculums dose, species and strain of birds, rout of inoculation and passage history of the virus. Gross lesions are variable depending on the virus strain, length of the time from infect to death, the host species and the presence of secondary pathogenesis. The

most consistent and severely affected organ by this virus were lung, kidney and brain, severity of the lesions in each organ was probably related to tissue tropism (Mehrabanpour *et al*, 2007)

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