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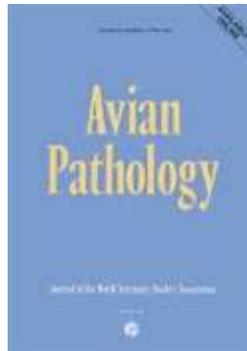
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Running title: High and low pathogenic Avian Influenza in turkey meat

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Conventional inactivated bivalent H5/H7 vaccine prevents viral localisation in muscles of turkeys infected experimentally with LPAI and HPAI H7N1 isolates

Anna Toffan¹, Maria Serena Beato¹, Roberta De Nardi¹, Elena Bertoli¹, Salviato Annalisa¹, Giovanni Cattoli¹, Calogero Terregino¹ and Iliaria Capua^{1*}

Abstract

Highly pathogenic avian influenza (HPAI) viruses cause viraemia and systemic infections, with virus replication in internal organs and muscles. In contrast low pathogenicity avian influenza (LPAI) viruses produce mild infections with low mortality rates and local virus replication. There is little available information on the ability of LPAI viruses to cause viraemia or on the presence of AI viruses in general in the muscles of infected turkeys. The aim of this study was to determine the ability of LPAI and HPAI H7N1 viruses to reach muscle tissues following experimental infection, and the efficacy of vaccination in preventing viraemia and meat localisation. The potential of infective muscle tissue to act as a source of infection for susceptible turkeys by mimicking the practice of swill-feeding was also investigated. The HPAI virus was isolated from blood and muscle tissues of all unvaccinated turkeys. LPAI virus could be isolated only from blood of one bird, and could be detected only by RT-PCR in muscles. In contrast, in vaccinated/challenged turkeys, no viable virus or viral RNA could be detected in muscles indicating that in vaccinated birds viral localisation in muscle tissue is prevented.

Introduction

Avian Influenza (AI) represents one of the major concerns for public health that has emerged in recent times. Over the last decade a sharp increase in number of outbreaks in birds, especially poultry, has occurred with a high economic impact on the commercial poultry sector. Moreover fears about the potential zoonotic risk have concurred to reduce market demand for poultry products, both in affected and in non-affected countries increasing the crisis for the poultry industry and affecting employment rates in some countries (Doyle & Erickson, 2006).

The occurrence of major AI outbreaks between 1999 and 2004, caused by viruses of the H7 subtype, have stimulated the revision of the definition of avian influenza for statutory and trade purposes (Capua & Alexander, 2006). One of the main changes in current EU legislation and in OIE guidelines is that the use of vaccination is seen as a potentially useful aid in the control of AI infections.

The issue of trading meat, obtained from vaccinated animals, has been the subject of an international debate for a significant amount of time, and the consensus reached is that meat and products obtained from vaccinated animals can be traded, provided the animals can be shown not to be infected at the time of slaughter. Although several systems exist to differentiate infected from vaccinated animals (Capua & Cattoli, 2007) a risk that a vaccinated/infected flock may escape surveillance systems in place cannot be ruled out, particularly during the early phases of infection. This raises concerns for the role that potentially infected meat may play in the spread of infection to avian and non avian hosts.

In addition to the H7N1 and H7N3 epidemic waves of 1999-2003, ongoing surveillance programmes in Italy have shown that LPAI viruses of H7 subtype appear to be prevalent in the wild bird population and have also been detected in backyard flocks (Terregino *et al*, 2007). Field and laboratory evidence indicates that turkeys are particularly susceptible to AIV infections, (Capua *et*

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al., 2003; Tumpey *et al.*, 2004; McNally *et al.*, 2006) requiring minimal doses of field virus to develop infection.

HPAI virus infections in *Galliformes* species produce a systemic disease causing viraemia with viral replication and localisation in organs and muscles (Mo *et al.*, 1997; Perkins & Swayne, 2001; Starick & Werner, 2003; Swayne & Beck, 2005). In contrast LPAI virus infections usually cause milder conditions, which can be complicated by other pathogens (Capua & Marangon, 2000; Kishida *et al.* 2004). For LPAI viruses, viral replication is allegedly restricted to the respiratory and gastro-intestinal tracts (Alexander, 2000), but little is known on the ability of these viruses to replicate in other tissues and organs including muscles. For this reason we carried out an investigation to detect the presence of viral RNA and live virus in meat collected from vaccinated and unvaccinated turkeys experimentally infected with HP and LP AI viruses of H7 subtype, and performed additional tests on meat to establish its potential role in spreading infection through swill-feeding.

Materials and methods

Vaccine and vaccination scheme. Chickens and turkeys were immunized with a commercially available inactivated bivalent vaccine containing the following strains: H5N9 LPAI (A/chicken/Italy/22A/1998) and H7N1 LPAI (A/chicken/Italy/1067/1999). The vaccination scheme consisted of two doses of 0.5 ml administered by the subcutaneous route in the back of the neck at 19 days of age and three weeks later (at 40 days of age). Both chickens and turkeys were given the same vaccination protocol.

Serum samples collected from vaccinated birds before each vaccination and before challenge were tested by haemagglutination inhibition (HI) tests for antibodies to H7 subtype. The log₂ geometric mean titre (GMT) of the serum of vaccinated turkeys was calculated.

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Viruses. Experimental infection of turkeys was carried out with two H7N1 viruses: A/turkey/Italy/3675/1999 (LPAI) and A/turkey/Italy/4580/1999 (HPAI).

Viruses were titrated in 9- to 10-day-old embryonated eggs from specific pathogen free (SPF) domestic fowls, using 10-fold dilutions and 5 eggs per dilution and the EID₅₀ (median egg infectious dose) was calculated according to the Reed and Muench formula (Reed & Muench, 1938).

Animals. Commercially available day-old poults were used in this study. Experimental birds originated from a parent flock that was negative serologically by agar gel immunodiffusion test (AGID) and enzyme-linked-immunosorbent assay (ELISA) tests and by real-time reverse transcriptase polymerase chain reaction (RRT-PCR) on cloacal swabs were used. ~~One-day-old~~ SPF chickens were used in the swill-feeding experiment.

All animals were identified by means of wing tags and received feed and water *ad libitum*. Birds were housed in negative pressure, HEPA-filtered, isolation cabinets for the duration of the experimental trial.

Assessment of viral presence in muscles. Two groups of fifteen 6- to 7-week-old vaccinated poults and two groups 15 unvaccinated hatch mates were used in the experimental trial. At 50 days of age (10 days after the second vaccination) all experimental groups were given oro-nasally 100µl of challenge virus containing 10⁶ EID₅₀. For clarity, each of the two challenge viruses was given to one vaccinated and one unvaccinated group.

On days 1, 2, 3, 4 and 5 post infection (p.i.) blood was collected from each bird from the wing vein, mixed with anticoagulant (Alsever's solution 1:1) and the establishment of viraemia was evaluated by RRT-PCR. If blood samples yielded positive results, up to three birds from each group

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presenting viraemia were killed humanely on the day of testing. When blood samples yielded negative results three turkeys were killed humanely randomly. In case of any death, organs were collected on the day of death. Lungs, superficial and deep pectoral muscles and thigh muscles were collected from each killed or naturally deceased turkey.

AI transmission-feeding test. For this experimental trial, two groups of seven-week-old SPF chickens and two groups of turkeys of the same age were used. One group of chickens and one group of turkeys were vaccinated as described above and two left unvaccinated.

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At 50 days of age, experimental groups of birds were fed with a meat homogenate prepared as described below and were observed daily for clinical signs. Tracheal and cloacal swabs were collected on days 3, 5 and 7 after administration of the homogenate. Serum samples were also collected on 7, 14 and 21 days post-administration of the homogenate.

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The infective meat homogenate was prepared with meat samples positive by virus isolation (VI) collected from unvaccinated turkeys infected with the HPAI virus. Muscles that yielded the highest viral titres were homogenised together with sterile quartz sand. The meat homogenate was further titrated in embryonated SPF eggs to ascertain the infectious dose. Two grams of the infective meat homogenate containing $10^{3.6}$ EID₅₀/0.1g were administered to each animal by the oral route.

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Virus Isolation. Organs collected were weighed (1g) and homogenised with sterile quartz sand and phosphate buffer solution (PBS) containing antibiotics and 20% glycerol (v/v) to make a 1:10 (w/v) suspension. Blood samples, collected for the evaluation of viraemia, were diluted 1:10 in PBS containing antibiotics to avoid the embryo toxicity of the anticoagulant. Tracheal and cloacal swabs were immersed in 1 ml of PBS containing antibiotics. Virus isolation and titration were performed according to the EU diagnostic manual (EC, 94/2005).

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Real Time RT-PCR. Organs were weighed and 0.1g homogenised with sterile quartz sand in 1 ml of sterile PBS to make 1:10 (w/v) dilution.

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Extraction of RNA. Two-hundred microlitres of PBS suspension from homogenated organs, blood and swabs were used to extract the RNA using a commercial kit (High Pure™ RNA extraction kit; Roche). RNA was eluted in a final volume of 60 µl containing 20 units Rnase Inhibitor (Applied Biosystems) and stored at -80 °C.

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Real-Time RT-PCR (RRT-PCR). Thirty microlitres of RNA solution were retrotranscribed with random hexamers in a final volume of 60 µl following the instructions of the kit (High Capacity® cDNA Archive kit; Applied Biosystems). Published primers and probes (Spackman *et al.*, 2002) targeting the M gene of type A influenza virus were applied for PCR, namely forward primer M+25, reverse primer M-124 at the optimized concentration of 300 nM each, the specific fluorescent label probe, M+64, was used at the final concentration of 250 nM. cDNA was amplified in a final volume of 25 µl using TaqMan® Universal PCR Master Mix. The PCR reaction was performed in a ABI Prism 7700 SDS apparatus (Applied Biosystem) with the following protocol: 2 minutes at 50 °C and 10 minutes at 95 °C followed by 40 cycles at 95 °C for 10 sec and 60 °C for 1 min. Samples with a threshold cycle value (Ct) ≤ 35 were considered positive for influenza type A viral RNA.

Serology. Type and subtype specific antibodies were detected by HI tests and a competitive ELISA, developed in-house, which utilizes a monoclonal antibody against the nucleoprotein (NP) of type A influenza viruses. For the HI test, the detection of antibodies to the H7 and H5 subtypes of AI was performed using 4 haemagglutinating (HA) units of the antigens H5N9 (A/chicken/Italy/22A/1998) and H7N1 (A/chicken/Italy/1067/1999).

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Results

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Assessment of viral presence in muscles

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Unvaccinated turkeys infected with HPAI virus. Due to the clinical condition and subsequent high mortality rate post challenge, it was not possible to follow the experimental design for this group, and to collect samples until day 5 post-challenge. All unvaccinated, HPAI-infected turkeys showed severe clinical signs starting from day 1 p.i. with 100% mortality by day 4 p.i. (Table 1). On day 1 p.i., 2 of 14 blood samples (1 turkey was found dead) yielded positive results by RRT-PCR. On day 2 p.i., 7 of 11 blood samples were positive and on day 3 p.i. blood from all the surviving birds yielded positive results (Table 1 and 2). Virus isolation confirmed these results. Virus was detected by RRT-PCR and virus isolation from lung and breast muscle samples of all dead and killed turkeys, and from thigh muscles of all but 2 birds killed humanely on day 1 p.i (Table 2). The amount of virus varied between 10¹ to 10^{4.38} EID₅₀/0.1g in muscle tissues and from 10¹ to 10^{5.8} EID₅₀/0.1ml in blood (Table 3).

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Vaccinated turkeys infected with HPAI virus. No clinical signs were observed during the experimental trial. No virus was detected in blood and muscle samples by RRT-PCR or VI. Virus was detected in lung samples collected on days 2 and 3 p.i. by both RRT-PCR and VI (Table 4).

Unvaccinated turkeys infected with LPAI virus. Birds showed mild respiratory disease with depression and sinusitis starting from day 3 p.i. (Table 5). Results of virus isolation and RRT-PCR are presented in [Table 6](#). Two blood samples collected on day 2 p.i. were positive by RRT-PCR but only one was confirmed by VI. Despite this case of viraemia, no virus was isolated in muscles.

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Vaccinated turkeys infected with LPAI virus. No clinical signs were observed during the experimental trial and no virus was detected in organs and muscles by either VI or RRT-PCR.

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AI transmission-feeding test. No clinical signs were observed in any group fed on infectious meat. Neither unvaccinated nor vaccinated birds showed increased antibody levels. All tracheal and cloacal swabs collected throughout the experiment yielded negative results.

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Discussion

The results of the challenge study with H7N1 HPAI indicate that this strain causes viraemia and is able to reach turkey muscle tissues. This data is in agreement with the evidence reported that in *Galliformes* species HPAI viruses cause viraemia and systemic infection and virus can be detected in the muscle tissues of infected birds. Several studies have shown that HPAI viruses may be recovered from meat of chickens, turkeys (Perkins & Swayne, 2001; Kishida *et al.*, 2004; Swayne & Beck, 2005; Swayne, 2006;) and ducks (Tumpey *et al.*, 2002; Lu *et al.*, 2003; Tumpey *et al.*, 2003; Beato *et al.*, 2007) following both field and experimental infection.

 In addition to the existing data we evaluated the trend of viraemia, showing high levels of viable virus in blood during the first three days post infection with HPAI virus, which correspond to high levels of virus in muscles.

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With reference to the H7N1 LPAI virus, infection results reported here show the ability of this strain to cause viraemia in turkeys although in a limited number of birds. This information is in contrast with the general consensus that LPAI viruses cause a strictly localised infection without viraemia, and is in-keeping with the clinical condition developed by some birds following natural and experimental infection. There is only one precedent report of LPAI virus causing viraemia, Kishida *et al.* (2004) described the isolation of two H9N2 strains from imported chicken meat and bone marrow. In their investigation, Kishida *et al.*, were able to detect virus in blood, bone marrow and muscles of chickens infected experimentally with those viruses. Our findings differ from those obtained by Swayne & Beck (2005) who failed to detect LPAI viruses (H5N2 and H7N2) in the blood of chickens infected experimentally. However, this difference could be due to the strain variability and to host susceptibility.

In any case, despite the detection of viable virus from the blood, no virus was isolated from muscles of the infected turkeys, although positive RRT-PCR results suggest that virus can reach muscle tissue.

In the present paper we report the absence of detectable virus in the blood and in meat of vaccinated turkeys infected with both LPAI and HPAI viruses. This evidence suggests that vaccination is an effective tool for preventing viraemia and, as a consequence, preventing meat from infected turkeys being infective.

Under experimental conditions, transmission of HPAI infection to naïve or vaccinated birds through the administration of 2 grams of meat homogenate containing $10^{3.6}$ EID₅₀/0.1g, failed, and therefore no transmission of infection could be expected from meat of vaccinated birds which both by RRT-PCR and by VI appeared to contain no virus.

It is noteworthy that viable virus was recovered from the lungs of vaccinated turkeys challenged with both LPAI and HPAI viruses. This data is in agreement with studies conducted by Swayne & Beck (2005), who detected viable LPAI H7N2 subtype virus from fluid collected post evisceration following internal body cavity wash. These findings suggest that contamination of the

carcass with internal organ exudates could represent a means by which LPAI can be introduced into a country through legal trade. It would, in any case, be logical to assume that the titre of the contaminating virus would not be high enough to generate a secondary cycle of infection if it were introduced in the animal food chain as swill feeding, giving the low initial titres and the dilution effect occurring during processing.

Several experimental studies highlight the possibility of AI transmission through the consumption of untreated infective meat. Purchase *et al.* (1931) and Swayne & Beck (2005) showed that chickens fed on muscle tissues from HPAI infected birds can become infected. On the basis of these previous investigations we tested the role that untreated infective meat may play in the transmission of AI viruses to vaccinated and unvaccinated chickens and turkeys. The results obtained with the transmission-feeding test indicate that infection by feeding birds with infective meat was achieved in neither unvaccinated nor vaccinated birds and it can be suggested that a higher dose of virus is required to cause the infection by oral route.

The results reported in the present study suggest that vaccination prevents viraemia and viral localisation in turkey muscles and thus minimize the potential of meat as vehicle for transmission of HPAI and LPAI viruses of the H7 subtype.

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Table 1. *Clinical signs, schedule of death/sacrificing and time of appearance of viraemia by RRT-PCR of unvaccinated turkeys infected with HPAI virus A/turkey/Italy/4580/1999 (H7N1). In brackets viral titre expressed as EID₅₀/0.1ml.*

N° of turkey	Day Post-Infection			
	1	2	3	4
381	H/E	-	-	-
382	H/E viraemic (<10 ¹)	-	-	-
383	H	S viraemic (<10 ¹)	D	-
384	H	S	S viraemic (10 ^{4.3})	D
385	H	S	S viraemic (10 ^{3.8})	D
386	H	D viraemic (10 ^{4.5})	-	-
387	H	D viraemic (10 ^{5.8})	-	-
388	H/E viraemic (10 ^{1.25})	-	-	-
389	H	S	D	-
390	H	S viraemic (10 ^{3.6})	S viraemic (10 ^{1.8})	D
391	H	S	S viraemic (10 ^{4.6})	D
392	H	S viraemic (10 ^{2.4})	D	-
393	H	E viraemic (10 ^{3.5})	-	-
394	H	S viraemic (10 ^{1.7})	D	-
395	D	-	-	-

H: healthy; D: dead; S: sick; E: killed humanely

Table 2. *RRT-PCR and virus isolation results in unvaccinated turkeys infected with HPAI virus A/turkey/Italy/4580/1999 (H7N1)*

GROUP	Day post-infection				
	1	2	3	4	5
Virus isolation	Positive/Total				
Breast	4/4	3/3	4/4	4/4	nd
Thigh	2/4	3/3	4/4	4/4	nd
Lung	4/4	3/3	4/4	4/4	nd
Blood	2/14	7/11	4/4	nd	nd

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RRT-PCR	Positive/Total				
Breast	4/4	3/3	4/4	4/4	Nd
Thigh	2/4	3/3	4/4	4/4	Nd
Lung	4/4	3/3	4/4	4/4	Nd
Blood	2/14	7/11	4/4	nd	Nd

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Legend: nd= not done

Table 3: *RRT-PCR and virus isolation results in vaccinated turkeys infected with HPAI virus*

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A/turkey/Italy/4580/1999 (H7N1)

GROUP	Day post-infection				
	1	2	3	4	5
Virus isolation	Positive/Total				
Breast	0/3	0/3	0/3	0/3	0/3
Thigh	0/3	0/3	0/3	0/3	0/3
Lung	0/3	1/3	2/3	0/3	0/3
Blood	0/3	0/3	0/3	0/3	0/3
RRT-PCR	Positive/Total				
Breast	0/3	0/3	0/3	0/3	0/3
Thigh	0/3	0/3	0/3	0/3	0/3
Lung	0/3	1/3	2/3	0/3	0/3
Blood	0/3	0/3	0/3	0/3	0/3

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Table 4. *Clinical signs and schedule of sacrificing and time of appearance of viraemia by RRT-*

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PCR of unvaccinated turkeys infected with LPAI virus A/turkey/Italy/3675/1999 (H7N1).

N° of turkey	Day post-infection				
	1	2	3	4	5
341	H	E viraemic	-	-	-
342	H	H	E	-	-
343	H	H	S/E	-	-
344	H	E viraemic	-	-	-
345	H	H	S	S/E	-
346	H	E	-	-	-
347	H	H	S	E	-
348	E	-	-	-	-
349	H	H	S	S	E

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350	E	-	-	-	-
352	H	H	H	H	E
353	H	H	S/E	-	-
354	H	H	H	S	E
355	H	H	H	E	-
400	E	-	-	-	-

Legend: H: healthy; D: dead; S: sick; E: killed humanely

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Table 5: RRT-PCR and virus isolation results in unvaccinated turkeys infected with LPAI virus A/turkey/Italy/3675/1999 (H7N1)

GROUP	Day post-infection				
	1	2	3	4	5
Virus isolation					
	Positive/Total				
Breast	0/3	0/3	0/3	0/3	0/3
Thigh	0/3	0/3	0/3	0/3	0/3
Lung	0/3	2/3	3/3	0/3	0/3
Blood	0/15	1/12	0/9	0/6	0/3
RRT-PCR					
	Positive/Total				
Breast	0/3	3/3	2/3	0/3	0/3
Thigh	0/3	2/3	2/3	0/3	0/3
Lung	0/3	3/3	3/3	0/3	0/3
Blood	0/15	2/12	0/9	0/6	0/3

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