

RESISTANCE OF TURKEYS TO EXPERIMENTAL INFECTION WITH AN EARLY 2009 ITALIAN HUMAN INFLUENZA A(H1N1)V VIRUS ISOLATE

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We performed an experimental infection of 21- and 70-day-old meat turkeys with an early human isolate of the 2009 pandemic H1N1 influenza virus exhibiting an α -2,3 receptor binding profile. Virus was not recovered by molecular or conventional methods from blood, tracheal and cloacal swabs, lungs, intestine or muscle tissue. Seroconversion was detected in a limited number of birds with the homologous antigen only. Our findings suggest that in its present form, the pandemic H1N1 influenza virus is not likely to be transmitted to meat turkeys and does therefore not represent an animal health or food safety issue for this species.

Introduction

Following the emergence of the human pandemic influenza A(H1N1)v virus in spring 2009, questions about the circulation of this virus in an animal reservoir were raised by international organisations. In particular, three aspects appeared to be of relevance, namely implications on animal health, aspects of food safety, and epidemiological aspects related to animals being infected with a human virus and perpetuating a parallel channel of infection in the animal reservoir.

Turkeys (*Meleagris gallopavo*) are highly susceptible to type A influenza virus infection and have been infected in the past with viruses of swine origin [1-4]. In August 2009, infection of two turkey flocks in Chile with the human influenza A(H1N1)v virus was reported [5]. The genetic profile of the virus appeared to be closely related (similarity ranging between 99.7% and 100%) to the strain that was circulating in the human population in Chile at the time [6]. The aim of this experiment was to establish the susceptibility of turkeys of different ages to infection with the human virus and to assess whether it would be detectable in the blood or in tissues of meat birds following administration of a high viral dose.

Materials and methods

Animals

Commercially available turkeys were used in this study. The birds originated from a flock that was serologically negative for all avian influenza subtypes, including influenza A(H1N1)v, by agar gel immunodiffusion test (AGID) and enzyme-linked immunosorbent assay (ELISA) and negative for influenza A virus by real-time reverse transcription-PCR (RRT-PCR) on cloacal and tracheal swabs [7]. All animals were identified by means of wing tags and received feed and water *ad libitum*. Birds were housed in negative pressure, high

efficiency particulate air (HEPA) filtered isolation cabinets for the duration of the experimental trial.

Challenge virus and protocol

Challenge of turkeys was carried out with the influenza A virus isolate A/Italy/2810/2009(H1N1). The virus was isolated from a human case detected in Verona, Italy, in specific pathogen-free (SPF) embryonated hens' eggs via the amniotic cavity and was characterised according to chapter on swine influenza in the World Organisation for Animal Health (OIE) Manual of Diagnostics Tests and Vaccines for Terrestrial Animals [8]. The number of virus passages in SPF embryonated hens' eggs was limited to the minimum (two) in order to limit laboratory manipulation and adaptation.

The haemagglutinin (HA) and neuraminidase (N) genes of the virus obtained from nasal swabs of the patient and the HA of the virus obtained from the allantoic fluid after the second passage in eggs, were genetically analysed and sequences were deposited in the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID), accession numbers EPI181386, EPI181387 and EPI211620. The A/Italy/2810/2009(H1N1) virus isolate has 99.6% homology with influenza A/California/4/09. The HA gene of the strain grown in eggs, which was used for the infection, contains arginine (Arg) instead of glutamine (Gln) at position 226. This substitution is associated with a receptor binding affinity to α -2,3 sialic acid receptors which are typical of avian viruses and thus bind preferably to avian cells [9].

For viral titration, 100 μ l of 10-fold diluted A/Italy/2810/2009 virus was inoculated into five SPF embryonated hens' eggs and the median embryo infectious dose (EID₅₀) was calculated according to the Reed and Muench formula [10].

Molecular tests

Extraction of RNA

Viral RNA was extracted from 200 μ l of blood using the commercial kit 'NucleoPsin RNA II' (Macherey-Nagel) and from 200 μ l of phosphate-buffered saline (PBS) suspension of cloacal and tracheal swabs and homogenised organs using the 'High Pure RNA Isolation Kit' (Roche®) commercial kit.

Real time RT-PCR (RRT-PCR)

Published primers and probes [7] targeting the Matrix (M) gene of type A influenza virus were used. The reverse primer M-124 was modified in order to have a perfect match with the M gene sequence of the influenza A(H1N1)v virus isolates. The forward, M-25, and reverse primers were used at the optimised concentration of 300 nM each, the specific fluorescent-labelled probe, M+64, was used at the final concentration of 100 nM. RNA was amplified in a final volume of 25 µl using a QuantiTect Multiplex® RT-PCR kit (Qiagen, Hilden, Germany). The PCR reaction was performed using the RotorGene 6000 (Corbett, Australia) apparatus with the following protocol: 20 minutes at 50 °C and 15 minutes at 95 °C followed by 40 cycles at 94 °C for 45 sec and 60 °C for 45 sec. All samples were also analysed using the RRT-PCR protocols for the M and HA genes recommended by WHO [11].

Serology

Type- and subtype-specific antibodies were detected by means of a commercial ELISA (ID-VET®) and AGID tests and by haemagglutination inhibition (HI) test according to the European Union (EU) diagnostic manual [12] using 1% chicken red blood cells. For the HI test, the detection of antibodies to the H1 subtype of avian influenza A virus was performed using four haemagglutinating units of the homologous antigens of the human H1N1v strain (A/Italy/2810/2009), an H1N1 strain of swine origin (A/swine/Italy/711/06) or an avian H1N1 strain (A/duck/Italy/1447/05).

Naïve animals were considered positive with a serologic titre of $\geq 4 \log_2$, as indicated by the EU guidelines.

Experimental design

Experiment 1: Evaluation of the presence of virus in blood, meat and viscera

A group of 10 70-day-old turkeys were oro-nasally infected with 100µl of the challenge virus containing 107 EID₅₀. 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 two birds presenting viraemia were killed humanely on the day of testing. When blood samples yielded negative results and no animals showed clinical signs, two turkeys were killed humanely on a random basis. In case of any death, lungs, intestine, superficial and deep pectoral muscles and thigh muscles were collected on the day of death.

Experiment 2: Evaluation of clinical signs, tracheal and cloacal shedding and seroconversion following experimental infection

A group of 12 21-day-old turkeys were used in this experiment. All animals were experimentally infected oro-nasally with 100µl of challenge virus containing 107 EID₅₀. Twice a day clinical signs were recorded. On days 2, 4, 6, 10 15 p.i. tracheal and cloacal swabs were collected from each bird. On day 14 and 21 p.i. blood samples were collected to evaluate seroconversion.

Results

Mild, non-specific clinical signs were observed in the 21-day-old birds a few days following administration of the challenge virus. These signs were considered to be non-specific because the birds did not exhibit the conjunctivitis, sinusitis or nasal discharge typical of low pathogenicity avian influenza infection. In both experimental groups, the virological and molecular results from all collected samples were negative. Seroconversion was detected in 41.6%, 8.3% and 33.3% of birds belonging to the younger age group by ELISA, AGID and HI tests (only with the homologous antigen), respectively. The results are presented in detail in the Table.

Discussion

The data reported here indicate that both 21- and 70-day-old turkeys are resistant to infection with early strains of the human influenza A(H1N1)v virus. Notwithstanding the high infectious dose and the mutation Arg to Gln in 226 of the HA gene, it was not possible to achieve infection or to detect virus in blood, respiratory and enteric organs or in muscles of experimentally infected birds. What is surprising is the evidence of seroconversion in a proportion of the infected poult. Since active infection was not achieved, it is likely that the seroconversion is related to the high viral dose administered. In any case, antibodies were detectable only with the homologous virus, thus indicating that intra-subtypic cross-reactivity was below HI detection limits.

Our findings indicate that unless the human influenza A(H1N1)v virus undergoes substantial changes, the risk that meat turkeys become infected with the virus is negligible. Therefore, there is no reason to be concerned about the animal health or food safety implications of this infection in this species.

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TABLE

Results of serological tests after infection (n=12)

Turkey (ID number)	14 days post infection			21 days post infection		
	HI*	ELISA	AGID	HI*	ELISA	AGID
71	n	p	d	n	p	d
72	1:64	p	d	1:32	p	d
73	n	n	n	n	n	n
74	1:16	p	d	1:16	d	d
75	n	n	n	n	n	n
76	1:4	p	d	1:8	p	d
79	n	n	n	n	n	n
80	n	n	n	n	n	n
81	1:32	p	p	1:64	p	p
82	n	n	n	n	n	n
83	n	n	n	n	n	n
84	1:16	p	n	1:16	p	n

AGID: agar gel immunodiffusion test; ELISA: enzyme-linked immunosorbent assay.

*Haemagglutination inhibition (HI) test performed with homologous antigen; n= negative; p= positive; d= doubtful.

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