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Calogero Terregino, Maria Serena Beato, Elena Bertoli, Marzia Mancin, Ilaria Capua. Unexpected resistance of Italian LP and HP avian influenza viruses of the H7 subtype following prolonged exposure at 37°C. *Avian Pathology*, 2009, 38 (06), pp.519-522. 10.1080/03079450903350244 . hal-00540171

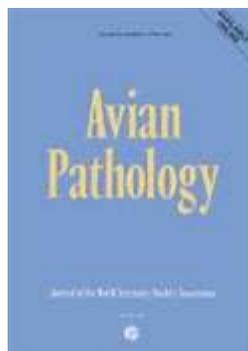
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Unexpected resistance of Italian LP and HP avian influenza viruses of the H7 subtype following prolonged exposure at 37°C

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2009-0051.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	28-Jul-2009
Complete List of Authors:	Terregino, Calogero; Istituto Zooprofilattico Sperimentale delle Venezie, Virology Dept. Beato, Maria Serena; Istituto Zooprofilattico Sperimentale delle Venezie, Virology Dept. Bertoli, Elena; Istituto Zooprofilattico Sperimentale delle Venezie, Virology Dept. Mancin, marzia; Istituto Zooprofilattico Sperimentale delle Venezie, Risk Analysis and Surveillance Systems in Public health Capua, Ilaria; Istituto Zooprofilattico Sperimentale delle Venezie, Virology Dept.
Keywords:	Avian influenza, H7 subtype, resistance, temperature

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Cavp-2009-0051.R2

Unexpected heat resistance of Italian LP and HP avian influenza A viruses of H7 subtype to prolonged exposure at 37°C

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Short Title: Italian LP and HP avian influenza A viruses

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Abstract

The increased attention of the international community to the occurrence of repeated outbreaks of avian influenza infections worldwide has highlighted several knowledge gaps in the field. Among these, within the EU funded project Fluresist, we addressed the resistance of selected H7 subtype strains at 37°C. In general terms resistance was high, although some strains were more resistant than others, remaining viable after 15 days at 37°C. These results should be considered when designing guidelines for outbreak management.

Introduction

Highly pathogenic avian influenza (HPAI) viruses pose a significant health threat to animals (mainly birds) and humans. These viruses generally emerge by mutation or recombination of low pathogenicity avian influenza (LPAI) viruses, which can be considered the precursors of the more virulent viruses. Both HPAI viruses and LPAI viruses of the H5 or H7 subtype are classified by the World Organisation for Animal Health (OIE) as Notifiable Avian Influenza (NAI).

Spread of NAI viruses in poultry is linked to many factors, among which several man-related activities, such as the movement of infected animals, contaminated materials, vehicles or staff, assume prominent roles. It follows, that the extent of spread and the potential for a given virus (or subset of related viruses) to infect an increasing number of farms is also related to the survival of that virus in the environment. Knowledge on the environmental resistance of a NAI strains could be useful to amend intervention strategies and support decision making processes during outbreaks.

Recent studies have been carried out on the environmental resistance of HPAI viruses belonging to the H5N1 subtype, indicating a significant degree of variability among strains (Ito et al., 2006; Songserm et al., 2006). To date no studies have been carried out on the

environmental resistance of Eurasian lineage viruses of H7 subtype. We have performed preliminary investigations on four strains, including HPAI and LPAI viruses, obtained from the 1999-2001 H7N1 and 2003 LPAI H7N3 epidemics in Italy.

Materials and methods

Viruses. Viruses were selected according to their pathogenicity (HPAI and LPAI isolates), period of isolation and availability of sequence data. The following viruses were grown in embryonated Specific Pathogen Free (SPF) fowls eggs: A/turkey/Italy/4580/99 (H7N1, HPAI) isolated in December 1999; A/turkey/Italy/1387/00 (H7N1, HPAI) isolated in February 2000; A/turkey/Italy/3675/99 (H7N1, LPAI) isolated in October 1999; A/turkey/Italy/4608/03 (H7N3, LPAI) isolated in August 2003.

Working stocks of the H7 viruses were obtained by propagating them in 10- to 11-day-old embryonated SPF fowls eggs via the allantoic cavity. The eggs were incubated at 37 °C in an atmosphere of approximately 62% humidity with a forced air circulation to enable embryo development.

Allantoic fluid with a strong haemagglutinating activity harvested from dead embryos was pooled and clarified by centrifugation at 3,000 *g* for ten minutes and stored at -80 °C in cryo-vials each containing 0.5 ml.

A ten-fold dilution series was made with each working stock, for each dilution 0.1ml was inoculated into the allantoic cavity of each of five embryonated SPF fowls eggs and the median egg infectious dose (EID₅₀) calculated using the Spearman-Kärber formula (Lennette, 1964).

Experimental design. For each of the selected viruses 51 cryo-vials containing infectious allantoic fluid were thawed. Forty eight of them were incubated at 37° C in a heating block and the remaining 3 tubes were used to estimate the initial viral titres (point zero). Three tubes from each viral batch at 37°C were collected at 6, 12, 24, 48 hours and subsequently every 24 hours up to 15 days. The viral suspension contained in each tube was titrated in embryonated SPF fowls eggs. For virus titration, five embryonated eggs were inoculated via the allantoic cavity with 0.1 ml of a tenfold-diluted sample obtained after the heat treatment.

Inoculated embryonated eggs were candled daily. Allantoic fluid of dead embryos or embryos incubated for 7 days were harvested and assayed for haemagglutinating activity according to standard procedures (CE, 2006). Two blind passages were performed before dismissing a sample as negative. The D_t value, time necessary to reduce the infectious titre by 10^1 EID₅₀/0,1 ml (1 log) was calculated for each virus (Swayne & Beck, 2004).

Statistical analysis. The viral titres (EID₅₀), calculated at each time point, were represented graphically in order to compare data for each of the viruses tested. A regression model was applied to verify the influence of the two variables: incubation time and virus strain on the decrease of viral titres. The Wald test was used to identify whether the decrease of infectious titres differed in a statistically significant manner among viruses (Azzalini, 2001). The statistical analysis was performed by using the Stata software (Stata, 2005).

Results

The titres of the viruses, calculated at time point 0, were: $10^{7.7}$ EID₅₀/0.1ml for A/turkey/Italy/4580/99 (H7N1, HPAI); $10^{7.3}$ EID₅₀/0.1ml for A/turkey/Italy/1387/00 (H7N1, HPAI); $10^{7.1}$ EID₅₀/0.1ml for A/turkey/Italy/3675/99 (H7N1, LPAI); $10^{6.1}$ EID₅₀/0.1ml for A/turkey/Italy/4608/03 (H7N3, LPAI).

The decrease in viral titre following heat exposure of the four strains under examination is plotted in figure 1.

A decrease of 1 log₁₀ was detected between 48 and 72 hours for A/turkey/Italy/1387/00 and between 24 and 48 hours for A/turkey/Italy/4580/99 (both HPAI). These HPAI viruses had titres ranging from 10^{1.5}-10^{2.0} EID₅₀/0.1ml to 10^{2.5}-10^{3.0} EID₅₀/0.1ml after 10 days showing a decrease of approximately 5 logs and were still viable after exposure to 37°C for 15 days.

For both LPAI viruses titres decreased by 1 log between 48 and 72 hours. A/turkey/Italy/3675/99 showed the highest resistance among the viruses tested with a decrease of only 3.6-3.7 log after 10 days at 37°C. In contrast, A/turkey/Italy/4608/03 had the lowest titre after 10 days (10^{0.3} EID₅₀), a decrease of almost 6 logs. At the end of the observation period (15 days) LPAI strain A/turkey/Italy/4608/03 (H7N3 LPAI) had no detectable infectivity, while A/turkey/Italy/3675/99 (H7N1 LPAI) still showed a mean titre of 10^{1.45} EID₅₀ (Table 1).

The individual titres obtained for each virus and exposure time at 37 °C are shown in Table 1.

The decrease of infectivity for all viruses tested was time-dependent and the decrease was statistically significant for each virus under assessment (p<0.05).

It can be concluded that the H7N3 is the least resistant virus when exposed at 37°C followed by the HP viruses 1387/00 and 4580/99, the LPAI virus of H7N1 subtype being the most resistant.

Discussion

Data reported in the present paper show the survival curve of Italian H7 isolates following exposure at 37°C over a period of 15 days. Data generated from this study suggest that all four Italian H7 strains under examination are highly resistant to exposure at 37°C.

In the literature there are only few studies on environmental resistance of viruses of the H7 subtype, they are fragmentary and results were generated by applying a variety of protocols, which makes the comparison of results difficult. However previous studies have shown that LPAI strains of H7N2 subtype (A/chicken/PA/3972-1/97, A/chicken/PA/3972-2/97, A/chicken/PA/3779-1/97, A/chicken/PA/3779-2/97) with titres from 10^4 to 10^5 ELD₅₀ remained infectious after 30 minutes at 56°C in a water bath, but no infectivity could be detected after 60 minutes at the same temperature or after 10 minutes at 60°C (Castro et al., 1998; Lu et al., 2003). Data have been generated on the resistance at lower temperatures (17°C and 28°C) for other LPAI viruses isolated from wild birds indicating that AIVs can persist in distilled water for more than 100 days at 28°C and 200 days at 17°C (Stallknecht et al., 1990a) or up to 15 days at room temperature (Tiwari et al., 2006). Muhmmad *et al.*, (2001) reported that for a Pakistan AIV of H7N3 subtype at concentration of 4 haemagglutinating units infectivity could still be detected after incubation in peptone water (pH 7.0) at 37°C for 35 days.

The retention of detectable infectivity for over 10 days at 37°C for the Italian viruses may have contributed to the extended duration of the 1999-2000 HPAI outbreak (4.5 months) and to the re-emergence of the LPAI virus in late 2000 (Capua & Marangon, 2007).

Among the control strategies to limit the spread of AI infections in poultry farms the implementation of biosecurity measures is an important part of current EU legislation. Although other environmental factors such as the presence of organic material, pH or salinity of the medium (Beard et al., 1984, Stallknecht et al., 1990b, Lu et al., 2003, Swayne & Halvorson, 2003) certainly contribute to environmental resistance, the availability of data on resistance to temperature of AIVs may be useful to generate guidelines for appropriate

biosecurity measures and repopulation programmes in the field.

Acknowledgements

This work was funded by EU project “FLURESIST” “Avian influenza virus survival in poultry, manure and the environment”.

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Table 1. Infectivity of the four viruses under study measured in triplicate at different times after exposure at 37°C.

Incubation Time	HPAI				LPAI			
	A/turkey/Italy/1387/00		A/turkey/Italy/4580/99		A/turkey/Italy/3675/99		A/turkey/Italy/4608/03	
	Titre of the initial solution $10^{7.3}$ EID ₅₀ /0.1 ml		Titre of the initial solution $10^{7.7}$ EID ₅₀ /0.1 ml		Titre of the initial solution $10^{7.1}$ EID ₅₀ /0.1 ml		Titre of the initial solution $10^{6.1}$ EID ₅₀ /0.1 ml	
0 hours	I° tube	$10^{7.3}$	I° tube	$10^{7.7}$	I° tube	$10^{7.1}$	I° tube	$10^{6.1}$
	II° tube	$10^{7.3}$	II° tube	$10^{7.1}$	II° tube	$10^{6.8}$	II° tube	$10^{6.1}$
	III° tube	$10^{7.3}$	III° tube	$10^{7.5}$	III° tube	$10^{7.0}$	III° tube	$10^{5.9}$
6 hours	I° tube	$10^{7.3}$	I° tube	$10^{7.3}$	I° tube	$10^{7.1}$	I° tube	$10^{6.0}$
	II° tube	$10^{7.1}$	II° tube	$10^{7.1}$	II° tube	$10^{6.8}$	II° tube	$10^{5.4}$
	III° tube	$10^{7.2}$	III° tube	$10^{7.3}$	III° tube	$10^{6.7}$	III° tube	$10^{6.1}$
12 hours	I° tube	$10^{6.5}$	I° tube	$10^{7.3}$	I° tube	$10^{6.7}$	I° tube	$10^{6.1}$
	II° tube	$10^{6.9}$	II° tube	$10^{7.1}$	II° tube	$10^{6.7}$	II° tube	$10^{5.4}$
	III° tube	$10^{6.9}$	III° tube	$10^{7.3}$	III° tube	$10^{6.9}$	III° tube	$10^{6.0}$
24 hours	I° tube	$10^{6.5}$	I° tube	$10^{6.7}$	I° tube	$10^{6.5}$	I° tube	$10^{5.3}$
	II° tube	$10^{6.5}$	II° tube	$10^{6.9}$	II° tube	$10^{6.7}$	II° tube	$10^{5.7}$
	III° tube	$10^{7.1}$	III° tube	$10^{6.8}$	III° tube	$10^{6.6}$	III° tube	$10^{5.5}$
2 days	I° tube	$10^{6.5}$	I° tube	$10^{6.7}$	I° tube	$10^{6.1}$	I° tube	$10^{5.3}$
	II° tube	$10^{6.5}$	II° tube	$10^{6.5}$	II° tube	$10^{6.3}$	II° tube	$10^{5.5}$
	III° tube	$10^{6.7}$	III° tube	$10^{6.7}$	III° tube	$10^{6.3}$	III° tube	$10^{5.2}$
3 days	I° tube	$10^{6.5}$	I° tube	$10^{5.2}$	I° tube	$10^{6.1}$	I° tube	$10^{4.7}$
	II° tube	$10^{5.7}$	II° tube	$10^{5.1}$	II° tube	$10^{6.1}$	II° tube	$10^{4.7}$
	III° tube	$10^{5.3}$	III° tube	$10^{5.6}$	III° tube	$10^{5.8}$	III° tube	$10^{4.3}$
4 days	I° tube	$10^{5.5}$	I° tube	$10^{4.7}$	I° tube	$10^{5.9}$	I° tube	$10^{3.5}$
	II° tube	$10^{5.7}$	II° tube	$10^{4.2}$	II° tube	$10^{5.4}$	II° tube	$10^{3.7}$
	III° tube	$10^{5.5}$	III° tube	$10^{3.7}$	III° tube	$10^{6.1}$	III° tube	$10^{3.9}$
5 days	I° tube	$10^{4.9}$	I° tube	$10^{3.5}$	I° tube	$10^{5.9}$	I° tube	$10^{3.2}$
	II° tube	$10^{4.5}$	II° tube	$10^{3.1}$	II° tube	$10^{5.3}$	II° tube	$10^{3.0}$
	III° tube	$10^{4.5}$	III° tube	$10^{3.1}$	III° tube	$10^{5.5}$	III° tube	$10^{2.7}$
6 days	I° tube	$10^{5.2}$	I° tube	$10^{2.8}$	I° tube	$10^{5.3}$	I° tube	$10^{2.3}$
	II° tube	$10^{4.3}$	II° tube	$10^{3.1}$	II° tube	$10^{4.9}$	II° tube	$10^{3.1}$
	III° tube	$10^{4.5}$	III° tube	$10^{2.9}$	III° tube	$10^{4.9}$	III° tube	$10^{2.9}$
7 days	I° tube	$10^{4.1}$	I° tube	$10^{2.3}$	I° tube	$10^{5.2}$	I° tube	$10^{2.5}$
	II° tube	$10^{3.7}$	II° tube	$10^{1.9}$	II° tube	$10^{4.9}$	II° tube	$10^{2.3}$
	III° tube	$10^{3.7}$	III° tube	$10^{2.5}$	III° tube	$10^{4.9}$	III° tube	$10^{1.5}$
8 days	I° tube	$10^{3.5}$	I° tube	$10^{2.3}$	I° tube	$10^{4.9}$	I° tube	$10^{2.2}$
	II° tube	$10^{3.6}$	II° tube	$10^{1.9}$	II° tube	$10^{3.0}$	II° tube	$10^{1.3}$
	III° tube	$10^{3.0}$	III° tube	$10^{2.5}$	III° tube	$10^{3.1}$	III° tube	$10^{1.7}$
9 days	I° tube	$10^{3.0}$	I° tube	$10^{2.2}$	I° tube	$10^{3.5}$	I° tube	$10^{1.0}$
	II° tube	$10^{2.5}$	II° tube	$10^{1.9}$	II° tube	$10^{3.5}$	II° tube	$10^{1.2}$
	III° tube	$10^{2.5}$	III° tube	$10^{2.5}$	III° tube	$10^{4.0}$	III° tube	negative
10 days	I° tube	$10^{3.0}$	I° tube	$10^{1.5}$	I° tube	$10^{3.5}$	I° tube	$10^{0.9}$
	II° tube	$10^{2.5}$	II° tube	$10^{1.9}$	II° tube	$10^{3.5}$	II° tube	negative
	III° tube	$10^{2.5}$	III° tube	$10^{2.0}$	III° tube	$10^{3.4}$	III° tube	negative
11 days	I° tube	$10^{2.1}$	I° tube	$10^{1.5}$	I° tube	$10^{3.3}$	I° tube	negative
	II° tube	$10^{1.5}$	II° tube	$10^{1.3}$	II° tube	$10^{3.1}$	II° tube	negative
	III° tube	$10^{2.0}$	III° tube	$10^{1.2}$	III° tube	$10^{3.1}$	III° tube	negative
12 days	I° tube	$10^{1.9}$	I° tube	$10^{1.1}$	I° tube	$10^{3.3}$	I° tube	negative
	II° tube	$10^{1.6}$	II° tube	$10^{0.7}$	II° tube	$10^{3.0}$	II° tube	negative
	III° tube	$10^{1.9}$	III° tube	$10^{1.0}$	III° tube	$10^{2.3}$	III° tube	negative
13 days	I° tube	$10^{1.5}$	I° tube	$10^{0.9}$	I° tube	$10^{2.7}$	I° tube	negative
	II° tube	$10^{2.0}$	II° tube	$10^{1.0}$	II° tube	$10^{2.7}$	II° tube	negative
	III° tube	$10^{1.9}$	III° tube	$10^{0.9}$	III° tube	$10^{2.5}$	III° tube	negative
14 days	I° tube	$10^{1.05}$	I° tube	$10^{1.15}$	I° tube	$10^{1.8}$	I° tube	negative
	II° tube	$10^{1.15}$	II° tube	$<10^{0.5}$	II° tube	$10^{2.0}$	II° tube	negative
	III° tube	$10^{1.1}$	III° tube	$<10^{0.5}$	III° tube	$10^{2.5}$	III° tube	negative
15 days	I° tube	negative	I° tube	$<10^{0.5}$	I° tube	$10^{1.3}$	I° tube	negative
	II° tube	$10^{0.75}$	II° tube	$<10^{0.5}$	II° tube	$10^{1.5}$	II° tube	negative
	III° tube	$<10^{0.5}$	III° tube	$<10^{0.5}$	III° tube	$10^{1.3}$	III° tube	negative

Figure 1. Comparison of survival curves of all viruses tested.

