Infectivity of H7 LP and HP influenza viruses at different temperatures and pH and persistence of H7 HP virus in poultry meat at refrigeration temperature

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\section*{Abstract}

The aims of this study were to assess the infectivity of highly pathogenic (HP) and low pathogenicity (LP) H7 AI viruses at different temperatures and pH values and to investigate the persistence of HP H7 virus in chicken, turkey and duck meat. The H7 viruses tested remained infectious at \(+4\) \(^\circ\)C and \(+20\) \(^\circ\)C for 200 and \(\geq 450\) days, respectively. At pH 5, H7 viruses retained their infectivity for a shorter period of time compared to pH 7. The infectivity of HP H7 was detected \(\geq 2\) months in meat maintained at \(+4\) \(^\circ\)C and was higher in chicken meat compared to turkey and duck meat. Results of this study show that higher temperatures and lower pH values both reduce virus infectivity and demonstrate that HP H7 virus can remain infectious in meat for extended periods of time.

\section*{Introduction}

Avian influenza viruses (AIVs) are members of the family \textit{Orthomyxoviridae}, are enveloped and have a negative sense, single stranded, segmented RNA genome.

Spread of AIVs in poultry is linked to factors such as the movement of infected animals, presence and movement of contaminated materials, vehicles, and personnel. The extent of spread is also linked to viral resistance to environmental factors such as temperature and pH.

In the environment, infectivity of AI viruses is dependent on parameters such as temperature, pH, salinity and relative humidity (De Benedictis et al., 2007, Stallknecht and Brown, 2009). In addition, the tenacity of AI viruses to physical and chemical factors increases in the presence of organic material (Lu et al., 2003). Further, the infectivity of AI viruses at different temperatures is variable from strain to strain (Brown et al., 2007; Paek et al., 2010; Terregino et al., 2009).

Great importance has been given to the study of physical and chemical factors influencing the survival of animal influenza viruses particularly for the sanitation of holdings following the emergence and spread of HPAI H5N1 virus. Increasing amounts of data are available on H5N1 HPAI infectivity under chemical and physical conditions (Lenes et al., 2010; Paek et al., 2010; Rice et al., 2007; Shahid et al., 2009; Wanaratan et al., 2010). The majority of studies investigate the effect of disinfectants on viral infectivity or the infectivity of HPAI viruses in water (Brown et al., 2009; Stallknecht et al., 2010) but few address the direct effect of chemical and physical factors on influenza virus infectivity (Dundon et al., 2007; Pizzuto et al., 2011; Terregino et al., 2009).

The widespread infection of farm animals with HPAI H5N1 throughout Asia, Africa and Europe has raised concerns about the safety of animal products for human consumption and for the risk of transmission of infection to animals through the feeding of swill. The introduction of HPAI into an HPAI-free country through trade of poultry commodities has been reported and has increased these concerns (Cobb, 2011). Highly pathogenic AI virus has been isolated from poultry meat (Beato et al., 2009; Das et al., 2008; Pantin-Jackwood et al., 2007; Swayne and Beck, 2005; Tofan et al., 2008) and thus infection of poultry with these viruses poses a risk of spread and transmission through movement and trade of this commodity. For example, the incursion of H5N1 HPAI in backyard chickens in Germany in 2007 was linked to exposure to contaminated duck meat and offal from commercial ducks with a subclinical
infection with HPAI H5N1 (Harder et al., 2009). The increasing international trade of poultry meat represents an opportunity for the spread of HPAI virus through this activity (Cobb, 2011).

Although the effect of heat and pressure treatments on HP H5 viruses in chicken meat has been investigated (Isbarn et al., 2007; Swayne, 2006; Thomas and Swayne, 2007; Thomas et al., 2008), data on the persistence of HP H7 viruses in poultry meat at refrigeration temperature is not currently available. Efforts to prevent spread of influenza infections through contact transmission via fomites and commodities require knowledge of virus stability in the environment and in poultry meat. The lack of knowledge in this area provided impetuous to generate information on H7 virus infectivity under different physical and chemical conditions and in poultry meat at refrigeration temperature.

In this study we investigated the effect of temperature (+4 and +20 °C) and pH (5 and 7) on the infectivity of two H7 AI viruses, one HP and one LP, obtained from the international virus repository at National Reference Laboratory for AI (Italy). Moreover, we aimed at evaluating the infectivity of virus in meat, maintained at refrigeration temperature, from chickens, turkeys and ducks experimentally infected with HP H7 virus. Data on virus infectivity of influenza viruses will improve the assessment of the potential transmission of these viruses via fomites and poultry meat.

Results

Infectivity of H7 viruses at +4 and +20 °C

Data on virus infectivity at +4 and +20 °C are shown in Fig. 1A and B, respectively. At +4 °C the HP H7 virus was detected up to 210 days while the LP H7 virus was detectable up to 270 days. At the end of the observation period (60 days), for viruses maintained at +20 °C, the LP H7 virus titre ranged from log10 2.7 to 2.9, while the HP strain had no detectable infectivity. Based on results of the bootstrap linear regression models applied for the evaluation of the decrease of virus titres over time, the 90% reduction time (time in days to reduce the virus titre by 1 log10 EID50) was 27 and 36 days at +4 °C and 7.5 and 13 days at +20 °C for the HPAI and LPAI, respectively (Table 1). Considering a unique model for the experiment at +4 °C (R² = 0.9192) and at +20 °C (R² = 0.9654) the infectivity of both H7 viruses was time dependant (p < 0.01) with the HP virus showing a faster decrease in virus titre than the LP virus for each temperature.

The overall model, including the temperature as an explicative variable (R² = 0.9386), showed also that the duration of infectivity was reduced at +20 °C compared to +4 °C for all viruses tested and for the HP virus at each experimental condition (p < 0.01). The decrease in infectivity over time differed between the HP and LP viruses; the HPAI viruses were less tenacious than LPAI viruses at both temperatures as indicated by the statistical analysis.

The bootstrap linear regression models resulted appropriate for the data as the points in a residual plot are randomly dispersed around the horizontal axis (data not shown).

Evaluation of virus infectivity at pH 5 and 7

Results on virus infectivity at pH 5 and 7 are shown in Fig. 2A and B. Virus titres of the two H7 viruses used in the study, calculated at time point zero, were: 10^5.5 EID50/0.1 ml for HP and 10^6.5 EID50/0.1 ml for LP virus. The pH of negative and infectious allantoic fluid was stable throughout the study. The estimated persistence i.e., the time needed to reduce virus infectivity by 90% was higher for viruses exposed at pH 7 (Table 2) and ranged between 1 and 2 days for pH 5 and between 2 and 5 days for pH 7. Statistical analysis showed that the decrease in virus titre for each virus, at both pH values, was time dependant (p < 0.01). At pH 5, the regression model (R² = 0.6542) indicated that the LP virus titre was higher than the HP virus at each time point and that the decrease in virus titre over time was temperature dependent. At pH 7, the regression model (R² = 0.7185) showed that the virus titre was higher for the HP virus than the LP virus.

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**Figure 1.** Comparison of virus infectivity of H7 HP and LP viruses at +4 °C (A) and at +20 °C (B). Titres are expressed as Log10 EID50.

**Table 1** Duration of infectivity for low and highly pathogenic H7 avian influenza viruses at +4 and +20 °C. In the bootstrap linear regression model \( Y \) represented the virus titre and \( X \) the observation time.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Virus</th>
<th>Bootstrap linear regression model</th>
<th>( R^2 )</th>
<th>Estimate persistence *&lt;br&gt; (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td>A/turkey/Italy/1387/00 (HPAI)</td>
<td>( y = 7.3223 - 0.0368X )</td>
<td>0.9143</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>A/turkey/Italy/3675/99 (LP)</td>
<td>( y = 7.3347 - 0.0275X )</td>
<td>0.9239</td>
<td>36</td>
</tr>
<tr>
<td>+20</td>
<td>A/turkey/Italy/1387/00 (HPAI)</td>
<td>( y = 7.3618 - 0.1308X )</td>
<td>0.9648</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>A/turkey/Italy/3675/99 (LP)</td>
<td>( y = 7.2908 - 0.0768X )</td>
<td>0.9475</td>
<td>13</td>
</tr>
</tbody>
</table>

* Time (days) required to reduce the starting concentration by 90% (1 log10).
Comparing data obtained for viruses at pH 5 and 7 in a unique model ($R^2=0.7257$), both viruses retained their infectivity to a higher level at pH 7 than at pH 5 with a statistically significant difference ($p<0.01$) at both temperatures tested (Fig. 2A and B). Interestingly, temperature had little effect on virus infectivity at pH 5 and 7 as indicated by the statistical model applied.

The bootstrap linear regression models were appropriate for the data as the points in a residual plot are randomly dispersed around the horizontal axis (data not shown).

### Virus persistence in poultry meat

Meat samples collected from chickens showed the highest virus titre, followed by turkey, then duck. Infection of ducks was more difficult because infection was achieved in only four of the five challenged ducks. At time point zero, virus titres in duck meat were low ($\log_{10} 1.9–2.7/g$). The HP H7 virus was detected in chicken meat, at $+4^\circ C$, for 135 days while virus was detected for 90 days in turkey meat (Fig. 3). Despite the low virus titres in duck meat at time point 0, virus was isolated from meat samples until day 75 (Fig. 3).

The linear mixed model for longitudinal data showed that species, days of exposure to condition and their interaction were significant ($p<0.01$). Therefore, virus persistence in meat samples decreased proportionally with exposure time ($p<0.0001$). Statistical analysis showed that, independent from virus titre at time point zero in meat samples of the species tested, virus titres decreased less in chicken meat followed by duck and turkey meat, and that the decrease was statistically significant ($p<0.001$). This indicates that persistence of the H7 HPAI virus under study is higher in chicken meat than in turkey or duck meat irrespective of the initial virus titres.

The model used showed a compound symmetry structure for variance–covariance which assumes the equal correlation of residuals. The residuals analysis showed that the proposed model was valid (data not shown).

### Discussion

Data presented herein provide additional information on infectivity of HP and LP H7 AI viruses at two temperatures inclusive of $+4^\circ C$ and $+20^\circ C$. Both HP and LP H7 AI viruses at two pH (5 and 7) each tested at $+4^\circ C$ and $+20^\circ C$, and persistence of HP H7 viruses in meat stored at $+4^\circ C$ from chickens, turkeys, and ducks.

Both HP and LP H7 viruses retained infectivity for a shorter period of time at $+20^\circ C$ (estimated infectivity 13 days) compared to $+4^\circ C$ (36 days) showing a less estimated infectivity than other H7 viruses (Stallknecht et al., 2010). At both temperatures the LP H7 virus retained infectivity for a longer period of time compared to the HP H7 virus. Our data showed that the decrease in infectivity for the viruses tested at $+4^\circ C$ and $+20^\circ C$ was both time- and temperature-dependent as already suggested (Stallknecht and Brown, 2009).

The compendium of work on the effect of temperature on infectivity of AIVs and paucity of data on the effect of temperature and pH on infectious allantoic fluid or water not suspended in organic materials make it difficult to compare data available in the literature. The observation that infectivity of AIVs is temperature-dependent and that infectivity is maintained better at $+4^\circ C$ was shown when AIVs diluted in water remained infectious for more than a year (Brown et al., 2009). It was also shown that,
when HP H5N1 virus was mixed with faeces, the virus remained infectious for > 40 days at +4 °C (Shortridge et al., 1998) but was inactivated after 1 day at +23 °C (Wood et al., 2010). In feathers stored at +4 °C HP H5N1 virus was recovered for up to 160 days but for only 10–15 days when stored at +20 °C (Yamamoto et al., 2010). Collectively these studies, although carried out on different matrices, confirm our findings on higher tenacity of H7 AI at +4 °C.

From an experimental point of view, Shahid et al. (2009) and Paek et al. (2010) both conducted studies similar to the present one with allantoic fluid but used HP H5N1 virus. Shahid et al. (2009) reported similar results to our study on viral persistence at +4 °C; in fact the viral infectivity of a Pakistani H5N1 HPAI virus was detected for more than 100 days but was completely inactivated at +28 °C virus following 1 day of exposure. Paek et al. (2010) showed that 3 Korean H5N1 HPAI viruses remained infectious for a prolonged period of time compared to the H7 viruses under study; viable virus was recovered after 250 days of exposure at +4 °C and between 250 and 300 days at +20 °C.

Our data would also suggest that increasing temperatures will decrease the time necessary to achieve virus inactivation and that strain variability also accounts for differences seen in response to temperature (Brown et al., 2009; Shortridge et al., 1998; Stallknecht and Brown, 2009; Wood et al., 2010; Yamamoto et al., 2010).

Results showed that the effect of pH had the greatest influence on virus infectivity and that temperature had a minor effect on virus infectivity than pH as demonstrated by the statistical analysis. We observed that pH values below 7 had a negative effect but at pH 7 the viruses were relatively stable at both +4 and +20 °C. This is in agreement with previous studies (Brown et al., 2009; De Benedictis et al., 2007; Stallknecht et al., 2010). At pH 7, the HP H7 virus was more stable than the LP H7 virus at both temperatures.

Following exposure for 6 days at pH 5, both H7 viruses maintained at +4 °C remained infectious. When comparing the survival of the HP and LP viruses at pH 5 and 7 the two viruses tested showed a different pattern; the virus titres of the HP viruses were higher than the HP H7 at pH 5 but not at pH 7. However, the titre of both viruses were higher at pH 7 than pH 5 thus both viruses were less tenacious at pH 5 than shown in previous studies (Stallknecht and Brown, 2009), suggesting that variation in pH tolerance is strain dependent. Further, the HA molecule of AI viruses in acidic conditions undergo conformational changes, which degree varies according to the strain (Korte et al., 1999; Puri et al., 1990) which may explain the different characteristics of HP and LP viruses at pH 5 and 7.

It has been reported that H7 LP viruses lost 100% of their infectivity after 5 min at pH 2, but exposure to pH 5, 7, 10, and 12 for 15 min had no effect on their infectivity (Lu et al., 2003). Further, Muhammad et al. (2001) have shown that an H7N3 AI virus following 48 h of exposure at pH 1, 3, 10 and 14 lost its infectivity. A recent study showed that acidic or basic pH values (between 3 and 12) did not inactivate H5N1 viruses from Thailand (Wanaratana et al., 2010). Similarly, Shahid et al. (2009), showed that infectivity of a Pakistani H5N1 HPAI virus was maintained at pH 5 but not at pH 7 following 24 h of exposure. Another study showed comparable data to our study: of the 12 LPAI viruses tested, the majority persisted longer at pH values between 7.4 and 8.2 and the infectivity decreased at pH values less than 6.6 (Brown et al., 2009). An additional critical factor that influences the effect of pH on virus infectivity is salinity which was not measured in our study but might have played a role (Brown et al., 2009; Stallknecht et al., 1990).

Comparing the two experiments carried out at +4 °C (one assessing only the effect of temperature and the other the effect of temperature and pH), tested viruses showed different tenacity and data may appear contradictory. One possible explanation could be that the experiment at pH 7 and +4 °C was carried out on diluted allantoic fluid with PBS which produced a difference of composition of matrices tested in terms of salinity, protein content and pH making the 2 experiments not perfectly comparable. Considering that the pH of allantoic fluid prior dilution with PBS was 8.4 we cannot assume that pH 7 is the optimal condition to maintain the infectivity. Previous studies showed that some AIVs may retain their infectivity longer at slightly basic conditions, pH 7.4–8.2 and even 8.6 (Brown et al., 2009).

Detection of avian influenza viruses in poultry meat has been reported following natural and experimental infection (Beato et al., 2009; Beato and Capua, 2011; Nazir et al., 2011).

In our study, persistence of the HP H7 virus in poultry meat was retained for a prolonged period of time, suggesting that HPAI virus can be preserved in infected carcasses at low temperature. Further, we showed that irrespective of the initial virus titre, presence of HP virus in chicken meat was higher than for turkey or duck meat. Our data confirms field evidence that HP virus may remain infectious at low temperatures in duck meat (Harder et al., 2009) and is also in line with previous findings that show H4, H5 and H6 LPAI viruses maintain their virus infectivity for a prolonged period in contaminated duck meat (virus mixed with meat) at 0 °C while infectivity decreased at +20 and +30 °C (Nazir et al., 2011).

Although virus titres prior to exposure to temperature were lower in duck meat, virus was detected for more than 60 days which suggests that it is important to acquire knowledge on virus persistence survival in commodities.

**Conclusions**

Our study confirms that survival of H7 AI viruses can be influenced by the individual or combined effects of temperature, pH, and time of exposure. Results clearly show that the H7 AI viruses under study have a prolonged stability at low temperatures and that low pH levels reduce infectivity. Looking at data generated in the pH experiment, the variables (pH and temperature) which most influenced virus infectivity was pH (5 and 7) and not temperature (+4 and +20 °C) as demonstrated by the statistical analysis.

It is interesting to note that the LP H7 virus retained its infectivity at +4 and +20 °C for a longer period of time than HP H7 virus, suggesting that virus survival depends on the virus strain but not on virulence.

The results of our animal experiments show that there is a great variability in virus titre in the muscle of experimentally infected chickens, ducks and turkeys. Chicken meat appears to harbour viable HP H7 virus for a very long period of time (135 days), followed by turkey meat (90 days), then duck meat (75 days). These findings support control measures and trade restrictions presently imposed on holdings infected with HPAI viruses. The results of our investigations are in keeping with the international guidelines and national regulations which impose the destruction of carcasses in HPAI infected premises.

**Materials and Methods**

**Viruses**

Viruses were selected based on results of a previous study (Terregino et al., 2009) where virus infectivity of 2 HPAI and 2 LPAI H7 viruses was tested following exposure at +37 °C. The LP and HP viruses shown to be most tenacious at +37 °C were...
selected for this study. In detail, the following viruses were grown in 9-to-11-day-old Specific Pathogen Free (SPF) embryonating hen’s eggs (Charles River Laboratories, USA): A/turkey/Italy/3675/99 (H7N1, LPAI) and A/turkey/Italy/1387/00 (H7N1, HPAI).

The median embryo infectious dose (ELID₅₀) for each working stock of virus was determined by inoculating 0.1 ml of serially diluted (10-fold) stock virus into 5 SPF embryonating eggs per dilution. Endpoints were calculated using the Spearman–Karber method.

**Virus infectivity at temperatures of +4 and +20 °C**

Infectivity of H7 viruses at +4 °C was determined by placing vials containing 1 ml of infectious allantoic fluid in 1.5 ml polypropylene tubes (Eppendorf Biopur®, Germany) in a refrigerator, then routinely testing the fluids for a period of 1 year. During the first 2 months of the experiment, virus infectivity was assessed every two weeks, then monthly thereafter. The temperature of the refrigerator was monitored every 2 min using an electronic device (Labgard2, Aes Chemunex, France). Infectivity of H7 viruses at +20 °C was determined by placing vials containing 0.5 ml of infectious allantoic fluid in 0.5 ml polypropylene tubes (Eppendorf Biopur®, Germany) in a heating block for 60 days (Thermo Lyne, PBI International, Milan, Italy). Vials were sampled every 4 days for viable virus. Temperature was monitored using a specific software program of the heating block.

For each experiment (+4 and +20 °C), 3 tubes containing infectious allantoic fluid were used to determine the initial virus titre (point zero) and 3 tubes were collected at each time point to perform titrations in SPF chicken embryos.

**Evaluation of virus infectivity at pH 5 and 7**

Experiments on infectivity of H7 viruses at pH 5 and pH 7 were carried out by mixing (v/v) infectious allantoic fluid with PBS to achieve pH 5 and pH 7. The allantoic fluid had a pH of 8.3 and was diluted with PBS at pH 4.88 and 6.95, respectively (10% v/v). Following the dilution of AAF in PBS the pH was measured to confirm the targeted pH using a pH meter (Inolab pH Level 1, Sigma Precision s.r.l. Italy). Eighteen tubes per virus under study, containing 1 ml of the infectious allantoic fluid, were allocated in 1.5 ml polypropylene tubes (Eppendorf Biopur®, Germany). Vials were placed at +4 °C in a refrigerator or at +20 °C in a heating block (Thermo Lyne, PBI International, Milan, Italy) for 6 days. Each day 3 tubes were collected to test virus infectivity in SPF embryonating hen’s eggs. The temperature of the refrigerator was monitored every 2 min using an electronic device (software: Labgard2, Aes Chemunex, France).

At each time point, two tubes containing non-infectious allantoic fluid at pH 5 and pH 7, respectively, were included as a negative control.

**HPAI virus persistence in poultry meat**

**Birds**

Three-week-old commercial domestic Pekin ducks (Anas platyrhynchos) and turkeys (Meleagris gallopavo) as well as White Leghorn SPF chickens (Gallus gallus) were used in the study. All birds were identified by wing tags and given feed and water ad libitum according to their age and species.

Day-old commercial ducklings and turkey pouls originated from parent flocks virologically and serologically negative to AI. Hatchmates were also tested with a commercial competitive ELISA (IDscreen, ID.VET, Montpellier, France) directed to the nucleoprotein (NP) and shown to be serologically negative. Birds were housed in HEPA-filtered poultry isolators in BL3 animal facilities and were handled in strict accordance with the relevant national animal welfare bodies and approved by the local Animal Welfare Committee.

**Experimental protocol**

Groups of 5 birds were used for each species. Birds were infected oro-nasally with 100 μl of HP A/turkey/Italy/1387/00 H7N1 virus containing 10⁶EID₅₀. On day 3 post-infection, birds that did not succumb to infection were euthanized by cervical dislocation. Superficial and deep pectoral muscles were collected and processed for detection of viable virus. Lungs were collected and tested to verify infection in individual birds.

Superficial and deep breast muscle tissue from each bird was finely cut, homogenised and aliquots of 0.5 g each placed in 7 ml tubes (VWR International PBI) at +4 °C. A total of five aliquots per species for each time point were prepared. During the first week of exposure, aliquots were tested for virus daily, then at two week intervals thereafter up to day 150.

To test for residual virus, each tissue aliquot was homogenised with sterile quartz sand to obtain a 10% (w/v) suspension in PBS containing antibiotics and 20% glycerol. Suspensions were clarified by centrifugation and titrations performed in 9-to-11-day-old SPF embryonating hen’s eggs. Virus titrations were performed by inoculating each 5 embryos with 0.1 ml of 10-fold dilutions via the allantoic cavity. One aliquot per bird was used to estimate the initial (point zero) virus titre. Testing of muscle tissue continued until virus was no longer detected or the tissue became unsuitable for testing because of autolysis (approximately between day 75 and 150). Virus titres were calculated using the Spearmann–Karber method.

**Statistical analysis**

**Virus infectivity at temperatures of +4 and +20 °C**

To compare previous results obtained testing the same viruses at +37 °C a bootstrap linear regression model (Fox, 2002) was applied to verify the influence of the following variables: incubation time, virus strain, and their interaction on the decrease of virus titre for each temperature tested. The influence of temperature and interaction of temperature and virus was evaluated applying a bootstrap regression model as well (Fox, 2002).

**Virus infectivity at pH 5 and 7**

A bootstrap linear regression model (Fox, 2002) was utilized to verify the influence of the following variables: incubation time, virus strain and temperature and their interaction on the decrease of virus titre for each pH value tested. The influence of pH and combined influence of pH and temperature for each virus was evaluated applying a bootstrap linear regression model as well (Fox, 2002).

**Appropriate residual posterior analysis was performed to verify the validity of the proposed models for experiments at +4 and +20 °C and at pH 5 and 7. Statistical analysis was carried out by using the Stata 12.0 software (Anonymous, 2011a).**

**Virus persistence in poultry meat**

The linear mixed model (LMM) for longitudinal data was used to investigate the influence of species, exposure time and their interaction on virus infectivity. Different variance and covariance
matrices were evaluated to investigate any possible correlation among observations and to select the most suitable model. Appropriate residual posterior analysis was performed to verify the validity of the proposed model. Statistical analysis was performed by using the SAS 9.3 software (Anonymous, 2011b).

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