Persistence of highly pathogenic avian influenza virus (H7N1) in infected chickens: feather as a suitable sample for diagnosis

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Selection of an ideal sample is a vital element in early detection of influenza infection. Rapid identification of infectious individuals or animals is crucial not only for avian influenza virus (AIV) surveillance programmes, but also for treatment and containment strategies. This study used a combination of quantitative real-time RT-PCR with an internal positive control and a cell-titration system to examine the presence of virus in different samples during active experimental AIV infection and its persistence in the infected carcasses. Oropharyngeal/cloacal swabs as well as feather pulp and blood samples were collected from 15-day-old chicks infected with H7N1 highly pathogenic AIV (HPAIV) and the kinetics of virus shedding during active infection were evaluated. Additionally, several samples (muscle, skin, brain, feather pulp and oropharyngeal and cloacal swabs) were examined to assess the persistence of virus in the HPAIV-infected carcasses. Based on the results, feather pulp was found to be the best sample to detect and isolate HPAIV from infected chicks from 24 h after inoculation onwards. Kinetic studies on the persistence of virus in infected carcasses revealed that tissues such as muscle could potentially transmit infectious virus for 3 days post-mortem (p.m.), whilst other tissues such as skin, feather pulp and brain retained their infectivity for as long as 5–6 days p.m. at environmental temperature (22–23 °C). These results strongly favour feather as a useful sample for HPAIV diagnosis in infected chickens as well as in carcasses.

INTRODUCTION

Over the past decade, highly pathogenic avian influenza virus (HPAIV) has raised alarming concerns and has remained an unsolved problem not only for public health but also as a disease of economic importance, and it poses a continuous threat connected mainly with animal reservoirs (Capua & Alexander, 2004, 2006). Early detection is crucial in prevention and control of avian influenza virus (AIV) outbreaks, particularly when H5 and H7 subtypes are involved. Both these subtypes are compulsorily notifiable (OIE, 2009) as they could potentially mutate from low-pathogenic AIV to HPAIV (Stech et al., 2009; Webster et al., 1992). HPAIV causes multisystemic disease with high viraemia levels in poultry, where virus is detected not only in respiratory and enteric organs but also in internal organs (Spickler et al., 2008; Starick & Werner, 2003; Swayne & Beck, 2005). In susceptible poultry species, HPAIV infection leads to severe depression and high morbidity and mortality rates (Spickler et al., 2008; Swayne, 2007), causing important economic losses (Capua & Marangon, 2000; Tiensin et al., 2005).

Since the spread of H5N1 HPAIV outbreaks in South-East Asia in the late 1990s, surveillance programmes in poultry and wild birds have been established in many countries to facilitate early detection and to circumvent possible virus transmission to other animal species, including humans. Oropharyngeal or tracheal swabs and cloacal swabs or faeces have classically been used in AIV sampling (Busquets et al., 2010; Ellström et al., 2008; Keawcharoen et al., 2008; Sturm-Ramirez et al., 2005; Webster & Bean, 1978; Webster et al., 1992). Oropharyngeal and tracheal swabs, however, are less sensitive for detecting virus during early infections than when the animals are sick or dead (Das et al., 2008). The choice of appropriate samples for AIV surveillance programmes is essential for early detection of AIV infection.

Similarly, information on the persistence of AIV in infected carcasses may help in optimizing the systematic investigation of mortality peaks in wild birds that is conducted as part of surveillance programmes. Such information may form a basis for control measures during AIV outbreaks and/or limit AIV circulation, which is a risk factor in the generation
of new reassortant viruses with other AIV strains from other animals such as swine or even humans. Infectious AIV has already been found in different tissues of infected chickens (Serena Beato et al., 2006; Starick & Werner, 2003; Swayne, 2007), ducks (Tumpey et al., 2002), turkeys (Capua et al., 2000), swans and geese (Brown et al., 2008), and detected in the muscles of infected chickens (Swayne & Beck, 2005), turkeys (Toffan et al., 2008), ducks and quails (Antarsena et al., 2006). HPAIV has also been detected in commercial frozen duck carcasses of retained slaughter batches (Harder et al., 2009), which has increased concern about the food safety of poultry products by its zoonotic implications. Similarly, the transmission of H5N1 HPAIV from infected carcasses to other animals in HPAIV-endemic areas has also been reported in different carnivores such as domestic cats (Songsersen et al., 2006a) and dogs (Songsersen et al., 2006b), tigers and leopards (Keawcharoen et al., 2004) and red foxes (Reperant et al., 2008). Nevertheless, kinetic and systematic studies on the persistence of AIV in different tissues from infected carcasses have not been conducted. Furthermore, the influence of environmental factors, specifically temperature, on HPAIV persistence in poultry carcasses has only been studied previously to a limited extent (Senne et al., 1994). Indonesia, where H5N1 HPAIV persists endemicity, and sub-Saharan African countries, where this virus is broadly spread, have a constantly high environmental temperature (Gilbert et al., 2008).

Fragmented information on HPAIV detection and isolation from the tissues of infected chickens already exists (Serena Beato et al., 2006, 2009; Spickler et al., 2008; Starick & Werner, 2003; Swayne, 2007, Swayne & Beck, 2005); however, complete studies on HPAIV detection in several samples during active infection as well as at different times post-mortem (p.m.) have not been carried out. In addition, most recent studies have focused on the H5N1 strain (Swayne, 2006) and little information is currently available on the detection and persistence of other HPAIV subtypes in animal tissues.

The main objectives of this study were to characterize the shedding pattern of H7N1 HPAIV in chickens by quantifying and comparing viral loads in cloacal and oropharyngeal swabs and feathers during infection, and to establish the virus persistence patterns of H7N1 HPAIV in cloacal, oropharyngeal and brain swabs, feather pulp, muscle and skin from infected chickens at different times p.m. under two environmental temperatures to gain a better understanding of the epidemiology of AIV disease.

**RESULTS**

Clinical manifestations and mortality of HPAIV-infected chicks

At 2 days post-inoculation (p.i.), two chicks infected with H7N1 HPAIV at a dose of \(10^6\) 50 % egg lethal doses (ELD50) via the intranasal route died and the remaining \(n=13\) animals showed severe clinical signs, such as depression, inactivity, ruffled feathers and neurological signs. At 3 days p.i., nine chicks died and the remaining chicks \(n=4\) showed prostration, cloudy eyes and dyspnoea and were euthanized for ethical reasons. In the control group, all chicks survived without any clinical signs.

Kinetics of virus shedding in oropharyngeal and cloacal swabs, feather pulp and blood samples in HPAIV-infected chicks

Viral load could be detected by one-step quantitative real-time RT-PCR (RT-qPCR) in oropharyngeal swabs, feather pulp and blood from infected chicks as early as 1 day p.i. Oropharyngeal swabs showed the highest viral RNA load (around 5 \(\log_{10}\) viral RNA copies; Fig. 1) at this time. However, feather pulp manifested a much higher viral load than oropharyngeal or cloacal swabs from 2 days p.i. onward. The viral RNA load detected in feather pulp increased proportionally to blood viral load and reached a maximum peak (around 9 \(\log_{10}\) viral RNA copies) at 2–3 days p.i. Samples from control chicks were negative for the whole experimental period.

A comparison of the sensitivity of RT-qPCR with the quantitative method of virus titration in Madin–Darby canine kidney (MDCK) cells showed strong differences. The ratio of viral RNA copies to infectious virus particles was 4 or 5 \(\log_{10}\) viral RNA copies per infectious virus particle, with a higher ratio in cloacal swabs under the conditions of the study. Infectious virus was detected only in oropharyngeal swabs at 1 day p.i., in all samples at 2 days p.i., and in oropharyngeal swabs and feather pulp but not in cloacal swabs at 3 days p.i. (Fig. 1).

**Fig. 1.** Viral load expressed as \(\log_{10}\) viral RNA copies ml\(^{-1}\) and as \(\log_{10}\) TCID\(_{50}\) ml\(^{-1}\) from swab samples [oropharyngeal (OS) or cloacal (CS)], feather pulp (Fp) or total blood at 1, 2 and 3 days p.i. The positivity (number of birds in which AIV was detected) is indicated by numbers when the number of positive samples was <3. Asterisks indicate that the viral load in the Fp sample was statistically significantly higher (\(P<0.05\)) than that in the OS and CS samples. Results are shown as means ± SD.
Kinetics of virus persistence in HPAIV-infected carcasses at different temperatures

In order to evaluate the influence of temperature on virus persistence, infected carcasses were divided into two groups (five in each) and kept at 22–23 or 30–31 °C. Samples of skin, pectoral muscle, brain swab, feather pulp and oropharyngeal and cloacal swabs were collected from 0 to 6 days p.m. On day 0 (upon death), four of seven tested samples (skin, muscle, brain swab and feather pulp) were found to be positive, with the highest viral load reaching a mean value of 9.46 log_{10} viral RNA copies ml^{-1} [4.29 log_{10} 50 % tissue culture infective dose (TCID_{50}) ml^{-1}].

Using RT-qPCR, viral RNA could be detected in all samples collected up to 6 days p.m. at both temperatures, with the exception of some cloacal and oropharyngeal swabs from chick carcasses at 30–31 °C. AIV RNA copies were also undetectable in some cloacal swabs obtained from carcasses kept at 22–23 °C from 5 days p.m. onwards (Fig. 2). When infectivity was examined by using the MDCK titration system, HPAIV viability was clearly higher at 22–23 °C than at 30–31 °C (Fig. 2). At 30–31 °C, most of the samples (muscle and cloacal swabs) showed no infectivity or maintained infectivity only at low levels (brain and oropharyngeal swabs) at 1 day p.m. Feather pulp and skin retained a few infectious viruses at 2 and 3 days p.m., respectively. In contrast, infectivity at 22–23 °C differed, as virus could still be collected from muscle and oropharyngeal swabs at 3 and 4 days p.m., respectively. Interestingly, skin, feather pulp and brain swabs maintained infectious virus for 5 days p.m. or longer at 22–23 °C. Again, the lowest viral persistence was observed in cloacal swabs for only 1 day p.m. (Fig. 2).

![Graphs showing viral load over time](image)

**Fig. 2.** Viral load expressed as log_{10} viral RNA copies ml^{-1} [feather pulp (Fp), brain (BS) and oropharyngeal (OS) and cloacal (CS) swabs] or log_{10} viral RNA copies g^{-1} (skin and muscle), or as log_{10} TCID_{50} ml^{-1} (Fp, BS, OS and CS) or log_{10} TCID_{50} g^{-1} (skin and muscle) from H7N1 experimentally infected carcasses at 22–23 and 30–31 °C for 6 days p.m. The positivity (number of birds where AIV was detected) is indicated by numbers when the number of positive samples was lower than the total number of birds. Asterisks indicate that the amount of viral RNA at 22–23 and 30–31 °C showed a statistically significant difference (P<0.05).
At 22–23 °C, the decrease in number of viral RNA copies with time (from 0 to 6 days p.m.) reached around 1.1 log_{10} reduction in skin and muscle, 1.5 log_{10} reduction in feather pulp and oropharyngeal swabs and as high as 2.26 log_{10} reduction in brain swab. At 30–31 °C, the decrease in viral RNA copies with time in infected carcasses was accelerated: approximately 2.6 log_{10} reduction in feather pulp, approximately 2.8 log_{10} reduction in skin and oropharyngeal swabs, almost 3.6 log_{10} reduction in brain swabs and approximately 5 log_{10} reduction in muscle. Based on the influence of temperature (from 22–23 to 30–31 °C) on viral genome persistence in infected carcasses, the samples could be grouped into two main categories: skin, oropharyngeal and brain swabs showed significant differences (P<0.05) regarding the two temperatures from 3 days p.m. The same significant differences between these temperatures were observed in breast muscle and feather pulp (Fig. 2), but only at 5 days p.m. The viral RNA levels detected in cloacal swabs showed strong variations with time at both temperatures.

**Comparison of feather pulp with traditional samples used for AIV detection**

Viral RNA load in feather pulp was statistically significantly higher (P<0.05) than the traditional samples used for AIV detection (oropharyngeal and cloacal swabs) from 1 day p.i. to 6 days p.m. at both temperature ranges used (22–23 and 30–31 °C) (Fig. 3).

![Fig. 3](image)

**Fig. 3.** Viral load expressed as log_{10} viral RNA copies ml⁻¹ from swabs [oropharyngeal (OS) and cloacal (CS)] or feather pulp (Fp) for 9 days p.i. The arrow shows the time of death. Asterisks indicate that the viral load in the Fp sample was statistically significantly higher (P<0.05) than that in the OS and CS samples. The dotted line indicates the limit of detection.

**DISCUSSION**

In the present study, the systematic detection and quantification of HPAIV H7N1 in several tissue samples and swabs from experimentally infected specific-pathogen-free (SPF) chickens at different times p.i. and p.m. by RT-qPCR and virus titration in MDCK cells is reported. The RT-qPCR and virus titration in MDCK cells used in our laboratory for AIV quantification proved to be valuable tools in the study of virus shedding. RT-qPCR has also been reported previously as a feasible alternative to the traditional AIV titration in embryonated chicken eggs (Lee & Suarez, 2004), a gold-standard procedure for the isolation of AIV despite being expensive and time-consuming (OIE, 2009). The differences in viral detection by RT-qPCR with respect to infectious virus detection by MDCK cell titration of the same samples can be explained by the fact that the RT-qPCR system detected viral RNA (vRNA), complementary vRNA (cRNA) and mRNA, whereas the MDCK titration system only detected vRNA that was properly encapsidated and with an intact envelope.

There have been few reports on HPAIV detection in skin and feathers (Yamamoto et al., 2007, 2008a, b). Recently, Yamamoto et al. (2008a, b) reported AIV detection in feather epidermal cells of H5N1-infected birds (domestic ducks and geese) at 2 days p.i. and suggested that feathers could be a potential source of infection. Similarly, H5N1 HPAIV has also been detected in feather follicles of gallinaceous species at 1 day p.i. (Perkins & Swaye, 2001). However, information on other HPAIV subtypes is scarce. In this study, we have provided evidence that high H7N1 HPAIV titres are present in feathers not only during active infection, but also for several days p.m.

One of the objectives of the present work was to compare samples used traditionally for AIV surveillance (oropharyngeal or tracheal and cloacal swabs) with other suitable and easily accessible tissues. In this study, H7N1 HPAIV was detected in feather pulp and in oropharyngeal swabs as early as 1 day p.i. Moreover, the highest viral load was recorded in feather pulp in the infected animals throughout the infection period from 1 day p.i. until the animal died. Regarding p.m. viral detection, feather pulp had a statistically significantly higher viral load than oropharyngeal and cloacal swabs until 6 days p.m. under both temperature conditions (22–23 and 30–31 °C). Although further studies with other HPAIV strains and doses are necessary, our results on virus detection during active infection as well as in infected carcasses strongly favour both oropharyngeal swabs and feather pulp as the most suitable samples for HPAIV diagnosis, and feather may even be the best sample for HPAIV detection p.m. In contrast, and in agreement with previous descriptions (Swaye & Beck, 2005), cloacal swabs showed the lowest viral load and their shedding pattern was also recorded as being more variable than that of oropharyngeal swabs and feather pulp. This viral load variability in faeces may be

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explained by the different virus distribution and the amount of sample collected. Moreover, lower infectious viral loads were obtained from cloacal swabs than from oropharyngeal swabs with similar viral RNA load, suggesting a detrimental effect of faeces in AIV viability.

During active viral infection, viral load detected in the feather pulp reached a maximum peak at 2–3 days p.i. and increased proportionally with viaemia levels. As feather pulp is highly vascularized, the elevated amounts of HPAIV may not be the only cause of an active replication in feather epidermal cells, as suggested previously (Yamamoto et al., 2008b); it may also be caused by the blood vascularizing these tissues. In infected carcasses, feather pulp was also one of the samples where the virus persisted for longer. Altogether, these results confirm and emphasize the importance of feather pulp in the dissemination and transmission of virus, which is not limited to active viral infection but also involves infected carcasses. In addition, feathers might represent a useful and easy alternative for sampling for laboratory diagnostics during surveillance or HPAIV outbreaks.

It is well-known that viral infectivity is related inversely to temperature once viruses are in the environment (Abad et al., 1994; Stallknecht et al., 1990a, b). After death, body decomposition occurs and accelerates as the environmental temperature increases. At 22–23 °C, although viral RNA load reductions of between 1 and 2.67 log10 were observed, high levels of viral RNA were still detected at 6 days p.m., particularly in muscle, skin and feather pulp. In the brain, the greatest decrease in AIV load was observed at 22–23 °C, possibly due to the rapid p.m. liquefaction of this particular tissue. At 30–31 °C, inactivation, particularly in muscle samples, was more accelerated. Nevertheless, taking into consideration the TCID50 values obtained in MDCK cell titration, muscular tissue from the carcasses was potentially able to transfer infectious virus for 3 days p.m., with other tissues (skin, feather pulp and brain) maintaining infectious virus for as long as 5–6 days p.m. at 22–23 °C, and these could act as mechanical vectors to infect other birds from the flock.

Infective carcasses might generate a secondary infection cycle by transmission if ingested by other animals. It has been reported previously that breast or thigh meat containing 7.3 log10 EID50 H5 HPAIV g−1 allowed direct virus transmission to other chickens eating the infected meat (Swanye & Beck, 2005). Although further transmission experiments would be necessary, the present results on virus persistence in external tissues (skin, feathers and muscle) indicate that oral transmission by eating infected carcasses a few hours or days after death could account for an estimable percentage of the whole viral transmission of HPAIV subtypes other than H5N1. They also emphasize the necessity for fast elimination of bird carcasses, not only on farms, but also in open-range facilities. Proper biosafety measures in all these activities must be adopted because of the high load of viable virus after chicken death and its high-level persistence in the carcass for several days.

**METHODS**

**Viruses.** The HPAI H7N1 strain A/Chicken/Italy/5093/99 used in the present study was isolated from a poultry outbreak in Italy in 1999 and was kindly provided by Dr Ana Moreno (Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, Bruno Ubertini, Brescia, Italy). This strain was replicated and titrated in 9-day-old SPF chicken embryonated eggs (OIE, 2009) and the virus titre was calculated by the Reed and Muench method (Villegas, 1998).

**Animal infection and sampling.** Twenty-five SPF chicks were hatched and subsequently placed in negative-pressure isolators under Biosafety Level 3 containment conditions at CReSA, Barcelona, Spain. The SPF chicks were split into two groups when they were 15 days old. One group of 15 chicks was inoculated by a natural route (intranasally) with 50 μl allantoic fluid containing 10^6.0 ELD50 virus. The remaining ten 15-day-old chicks, inoculated with the same volume of PBS, constituted the negative-control group. Animal care and all procedures were performed in accordance with the Ethics Committee regulations of the Universitat Autonoma de Barcelona. All birds were monitored daily for clinical signs and mortality.

After H7N1 HPAIV experimental inoculation in chicks, clinical signs and mortality were recorded. Three infected and two non-infected chicks were euthanized daily, and oropharyngeal and cloacal swabs, feathers (one or two) plucked from the pectoral area and whole blood in Alsever’s solution (Sigma) were collected from each bird every 24 h after inoculation until the chicks died. Chicks showing severe clinical signs were euthanized for ethical reasons. After death, ten chickens were divided between two plastic boxes (five in each) inside different isolators. One of the isolators was set up at 22–23 °C and the other at 30–31 °C. Every 24 h after death up to 6 days p.m., samples of skin, pectoral muscle, brain swabs, feathers and oropharyngeal and cloacal swabs were taken from each animal. Swabs, feather pulps and tissue samples obtained from live birds and carcasses were placed in 500 μl Eagle’s minimal essential medium (MEM) with 300 U penicillin (Lonzza) ml−1, 300 μg streptomycin (Lonzza) ml−1, 100 μg gentamicin (Lonzza) ml−1 and 100 μg kanamycin (Merit) ml−1, and stored at −80 °C until viral RNA quantification or virus titration. To extract feather pulp, the calamus was squeezed until pulp overflowed from the proximal umbilicus.

**AIV quantification.** Viral RNA quantification using RT-qPCR was carried out in all samples. Viral RNA was first extracted using a QIaamp Viral Mini kit (Qiagen) from blood, swabs and homogenized tissues (200 mg) and feather pulp. Amplification of a matrix (M) gene fragment was performed using primers and a TaqMan probe described previously (Spackman et al., 2002) at a concentration of 900 nM for each primer and 200 nM for the TaqMan probe, using one-step RT-PCR reagents (Applied Biosystems) and following the manufacturer’s instructions using a Fast 7500 system (Applied Biosystems). An internal positive control (IPC) (human RNA and specific probe and primers; Applied Biosystems) was included to check for false-negative results due to PCR inhibitors. Concentrations used for the IPC were 200 nM probe, 50 nM each primer and 2.5 × 10−2 fg human RNA μl−1. The IPC concentration of 2.5 × 10−2 fg μl−1 did not alter the cycle threshold (Ct) values of the M gene RT-qPCR assay.

Standard curves and quantification were achieved by prior amplification of 99 bp of the M gene using the H7N1 strain RNA as template. The amplified M gene fragment was cloned into the pGEM-T vector (Promega) and transformed by heat shock into Escherichia coli competent cells (Invitrogen). The recombinant plasmid was purified using a QIAprep Spin kit (Qiagen) followed by digestion with Sau restriction enzyme (New England Biolabs) to obtain overhanging ends, and then converted to blunt ends using DNA polymerase I large (Klenow) fragment (Promega). In vitro-transcribed RNA was
generated from the T7 promoter (RiboMax kit; Promega). The residual template plasmid was removed by several RNase-free DNase I (Roche) treatments. The RNA transcript obtained was quantified spectrophotometrically at 260 nm (Qubit; Invitrogen). RNA copy numbers were calculated as described previously (Fronhoffs et al., 2002). Tenfold RNA transcript dilutions, ranging from 6 to 6 \times 10^7 molecules, were used to obtain standard curves.

A one-step RT-qPCR assay for the M gene was used to determine the viral RNA load, detecting vRNA, cRNA and mRNA. The limit of detection of the one-step RT-qPCR assay used in this study was as low as six viral RNA copies of in vitro-transcribed RNA per reaction, which was equivalent to 3.19 log_{10} copies (g tissue)\(^{-1}\).

In addition, viral RNA was serially diluted tenfold and a one-step RT-qPCR was performed to verify that the amplification efficiencies of the in vitro-transcribed RNA and the viral RNA were equivalent. Standard curves for these RNAs were generated by plotting their Ct value against the logarithm of the number of viral RNA copies. The efficiencies were considered equal if the difference between both slopes (Δs) of the standard curves was <0.1 (Gut et al., 1999). The Δs value obtained was 0.08, indicating equivalent retrotranscription and amplification efficiency, so the standard curve generated previously with in vitro-transcribed RNA was reliable and could be applied for AIV quantification as reported previously (Lee & Suarez, 2004). The viral RNA copies based on in vitro-transcribed RNA standard curves thus reflected the number of AIV RNA molecules, as the efficiency of retrotranscription and amplification was determined.

**Virus titration.** Virus infectivity from all collected samples except blood was assayed by titration in MDCK cells (ATCC CL34). Before titration, the cells were grown in Eagle's MEM with Earle's salts (Sigma) containing 2 mM l-glutamine, non-essential amino acids, 100 IU penicillin ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\) (all from Lonza) and 10% fetal calf serum (Euroclone). All samples were treated with 10 μg trypsin IX (Sigma) ml\(^{-1}\) at 37 °C for 30 min and then diluted tenfold in PBS and spiked onto PBS-washed MDCK cell monolayers grown in 96-well microtitre plates. Routinely, eight wells were infected for each tenfold dilution, and 20 μl inoculum was spiked into each well. After that, 150 μl Eagle's MEM with Earle's salts (Sigma) supplemented with 2 μg trypsin IX (Sigma) ml\(^{-1}\), 2 mM l-glutamate, non-essential amino acids, 100 IU penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\) (all from Lonza) was added per well and the plates were incubated at 37 °C and 5% CO\(_2\) for 6 days. Viral titration was performed by calculating the TCID\(_{50}\) ml\(^{-1}\) using the method of Reed and Muench (Villegas, 1998).

**Statistical analysis.** Prior to analysis, the numbers of viral RNA molecules, were used to obtain standard curves.

The values of viral RNA copies per sample were transformed to log_{10} values. For statistical evaluation, sas v9.1.3 software was used (SAS Institute). A repeated-measurement analysis of variance according to the general linear model procedure was used to evaluate differences in viral RNA load from oropharyngeal and cloacal swabs and feather pulps during infection of chicks and from samples collected post-mortem and kept at 22–23 and 30–31 °C at different intervals of time. Constant correlation was assumed between repeated measurements and the significance level for all analyses was set at 0.05. The normality of the residuals of the model was evaluated by a Shapiro–Wilks test.

**REFERENCES**


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