ANIMAL HEALTH SERVICE

Gezondheidsdienst voor Dieren
b.v.

SECTOR
RESEARCH AND DEVELOPMENT
POULTRY HEALTH

Study : Survival of Avian Influenza virus on eggs

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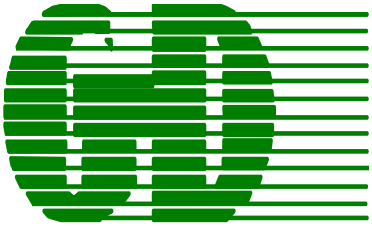


Internal report

Introduction

During the 2003 outbreak of the high-pathogenic H7N7 Avian Influenza Virus (AIV), an estimated 20 million table eggs (source ANEVEI) were preventively destroyed to avoid spread of the AI-virus and/or infection of consumers. The policy pursued by the Dutch government as regards table eggs from Protective/Surveillance (B/T) areas was stricter than prescribed in the European Directive. Belgian eggs from Belgian B/T areas could still be merchandized, (in accordance with the European Directive) while Dutch eggs from Dutch B/T areas were destroyed.

A desk study into the risk of spread of AI through eggs shows that very little information has been published about AI virus (AIV) in and on eggs. An article by Cappuci et al. (1985) on isolation of AIV from eggs from flocks that were acutely infected with high-pathogenic H5N2 states that in 30% of the eggs AIV could be isolated from the albumen and in 20% from the albumen-yolk mixture.

The desk study showed that AIV could be spread through the egg contents. However, it is not known how long the AIV present on the eggshell or in the egg would remain infectious. There is no information available whether eggs from an AI-free farm could be infected through cross-contamination with AIV-infected eggs. However, this knowledge is necessary to be able to pursue a founded policy regarding table eggs in the event of an AI outbreak. This information is not only important in the event of an outbreak of high-pathogenic AI, but also for an outbreak of low-pathogenic AI (LPAI).

A study has been carried out into the survival time at 4 temperatures of LPAI virus in and on the egg.

Material and method

Virus

The LPAI strain H5N2 (a/chicken/Belgium/150/99) was used to infect the SPF eggs in the flow cabinet. This recent isolate was obtained from the CODA, (Dr. T. van den Berg) of Brussels. This strain has an IVPI (intravenous pathogenicity index) of 0.0 and has no “risk sequence”. Allantoic fluid harvested from SPF eggs infected with the PAI H5N2 strain is used to infect or contaminate the eggs. This Positive Allantoic Fluid (PAV) contains $10^{7.1}$ EID_{50} virus per ml.

Virus isolation

The re-isolation is carried out according to a modified version of the internal GD procedure. This procedure was drawn up according to the regulations of CIDC Lelystad. The modification of the procedure referred to the number of passages used.
The SPF eggs had been hatched for 8 to 10 days. Death within 24 hours was considered as non specific. Allantoic fluid was collected from eggs that died later or still lived after six days and individually tested for haemagglutination.

RT-PCR AIV

For various samples RT-PCR AIV was used to establish whether a detectable quantity of AIV genome was present. The RT-PCR used for this purpose was the matrix RT-PCR by Fouchier *et al*. that is also used as screening test by the CIDC.

Test 1

The eggshell, albumen or yolk of 156 intact SPF eggs was infected with non pathogenic AI Virus (H5N2, Belgium) containing PAV ($10^{7.1}$ EID_{50}/ml). The eggshells of an other group eggs were infected with AI Virus containing droppings (homogenous mixture of equal parts SPF droppings and PAV).

The yolk and the albumen were infected by injecting 0.25 ml PAV ($10^{6.5}$ EID_{50}), after which the eggshells were resealed using paraffin wax.

To infect the eggshells, 0.25 ml PAV of the mixture of PAV (and droppings) was placed on the egg within a paraffin ring with a diameter of 2 cm, which had dried for 3 to 4 hours.

The SPF droppings mixed with negative allantoic fluid that were used as negative verification, had also dried up after about 3 hours. Then the infected eggs were stored at 4 different temperatures (4°C (cold store), 9°C (refrigerator), 15°C (new EU rule) and 20°C (room temperature)) and at different moments (4 hours (zero measurement), 5 days (average merchandize time)), 11 days and 17 days sampled and used that same day for virus isolation for determining the presence of infectious AIV.

Schedule test 1:

Table 1. Determining survival of AIV in and on eggs (3 eggs at a time)

Number of eggs	Storage temperature	Storage duration	Eggshell (PAV)	Eggshell (PAV+ droppings)	Egg yolk	Albumen
12		4 hours	3	3	3	3
12 x 3	4°C	5 days	3	3	3	3
		11 days	3	3	3	3
		17 days	3	3	3	3
12 x 3	9°C	5 days	3	3	3	3
		11 days	3	3	3	3
		17 days	3	3	3	3
12 x 3	15°C	5 days	3	3	3	3
		11 days	3	3	3	3
		17 days	3	3	3	3
12 x 3	20°C	5 days	3	3	3	3
		11 days	3	3	3	3
		17 days	3	3	3	3

Sampling:

Shell: the eggshell (the section on which the PAV had been applied) was pulverised and then mixed with 10 cc virus medium (of 4°C) and incubated during 1 hour at 4°C. During this hour the mixture was agitated twice on the Vortex mixer. Afterwards it was centrifuged and the supernatant (after filtration through a 450 µm filter), 4x1 cc was used for virus isolation.

Albumen: 10 cc was collected out of the egg, homogenise and inject 4x1 cc for virus isolation.

Yolk: 10 cc was collected out of the egg, homogenise and inject 4x1 cc per egg for virus isolation. For the albumen as well as the yolk it was in most cases still visible where the PAV had been injected. In those cases that location was always included in taking the sample (of the 10 cc).

Test 2

This subsequent test was carried out to assess the repeatability of parts of test 1 and to gain (initial) understanding for the operative mechanism of AIV inactivation. A total of 6 aspects were investigated:

A. PAV was titrated at time zero and after drying-up period (see B, C, D and E).
B. PAV was applied on the eggshell (3 SPF eggs) as in test 1. After drying up (about 4 hours) the shell (including the egg membrane) was pulverised and used for virus isolation. Of these 3 eggs the albumen was also used for virus isolation.
C. PAV was applied on the eggshell (3 SPF eggs) as in test 1. After drying up (about 4 hours) the shell (including the egg membrane) was sampled using a humid swab and used for virus isolation.
D. In addition, 0.25 cc PAV was applied on a sheet of plastic and after drying up (about 4 hours) a humid swab was used to take a sample for virus isolation.
E. Three eggshells of SPF eggs were pulverised and each mixed with PAV (ratios eggshell, PAV and medium as in the first test). Directly after mixing (t = 0) some material was centrifuged, filtered and used for virus titration. In addition, material was incubated separately at 4°C (Vortexing twice during the period), after 1 hour the material was centrifuged, filtered and used for virus isolation. After the drying-up period of about 4 hours the mixture was used for virus isolation after centrifuging and filtering the supernatant.
F. In addition, PAV was mixed with paraffin and after about 4 hours it was sampled for virus isolation.

Results

When applying the PAV in the albumen or the egg yolk, 0.25 cc of liquid was administered. After the incubation period, 10 cc of the albumen or yolk was collected and after homogenisation, 4 cc was than used for virus isolation (4 eggs each infected with 1cc). Because it was not possible to examine the complete egg but only part of it, not all virus that had been introduced into the egg could be re-isolated again. In order to estimate how much virus survived in the total albumen or the entire yolk, the quantity found in the sample should be multiplied by the dilution factor. This correction is not relevant when we are particularly interested in the rate at which the virus becomes inactivated (the purpose of this study). The dilution factor can be estimated as follows.

Of an average egg, 12% of the weight is eggshell. Of the other 88% roughly 64% (56% of the egg weight) is albumen and 36% (32% of the egg weight) is egg yolk. Administering 0.25 cc of liquid in the albumen of eggs with a weight of roughly 60 grams, results in a dilution of roughly 34 times (^{10}log step of 1.5) if the PAV would automatically spread out evenly over the entire albumen. Administering the same quantity in the egg yolk results in a dilution of roughly 22 times (^{10}log step of 1.3) if the PAV would automatically spread out evenly over the entire yolk. In reality it appears that this does not or not fully happen in either albumen or yolk, because in many cases signs of the “injection location” could still be seen. If all the injected virus would remain near the injection spot and could then be sucked up fully on sampling for virus isolation, the dilution would be 2.5x (10 cc sucked up, of that 4 cc placed in eggs for virus isolation). That is a ^{10}log step van 0.4. When administering 0.25 cc of liquid on eggshell, there will be little dilution, but additional medium will be added on filtering. The estimated dilution factor will be roughly 10x (^{10}log step of 1.0).

Test 1
The results are listed in the figures 1 up to and including 7. The zero measurement at 4 hours after infection of the eggs is shown in the figures as 0.17 (4/24 = 0.17).

Figure 1

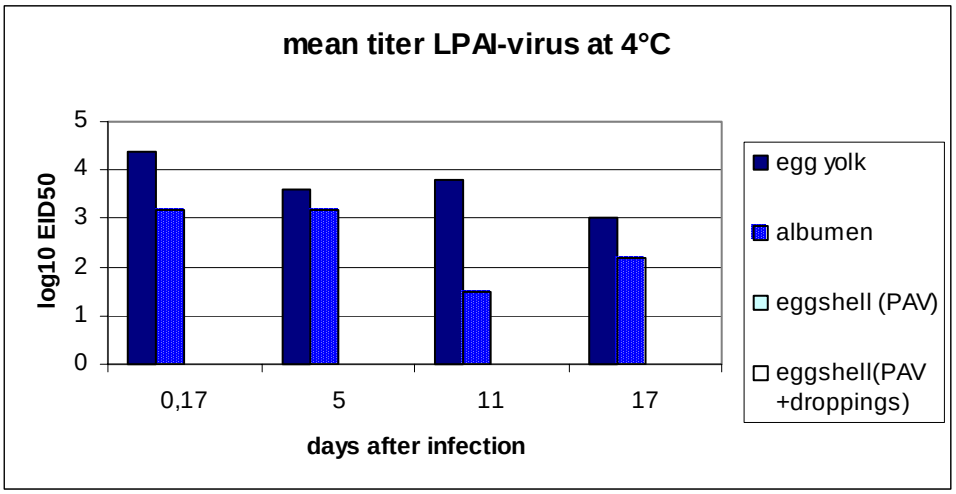


Figure 2

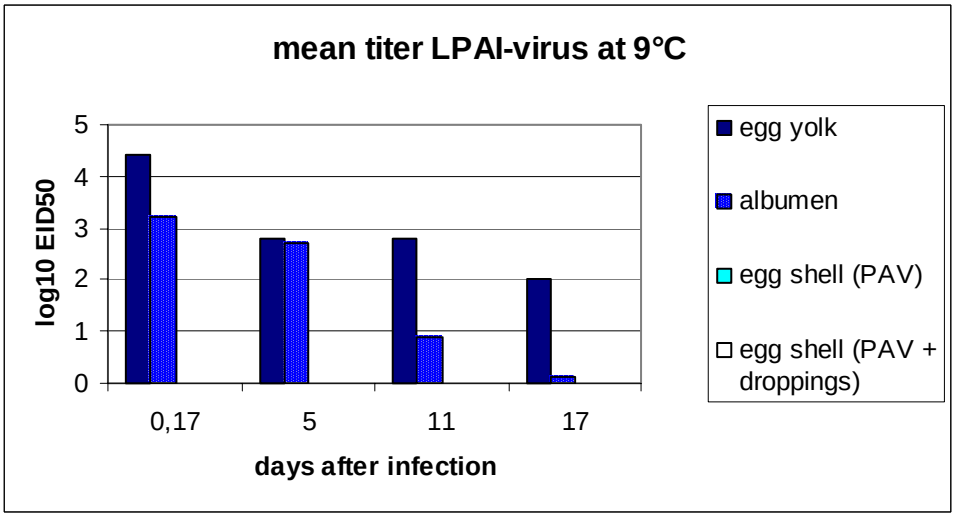


Figure 3

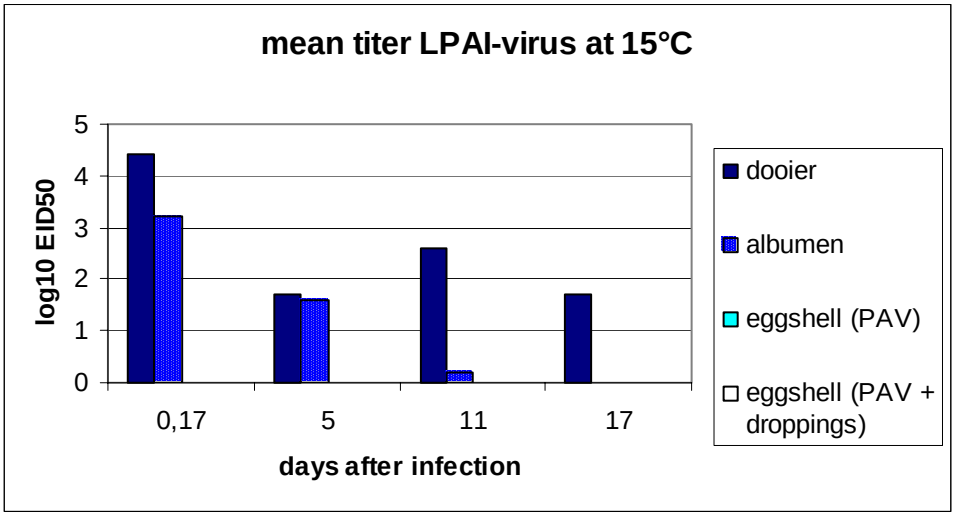


Figure 4

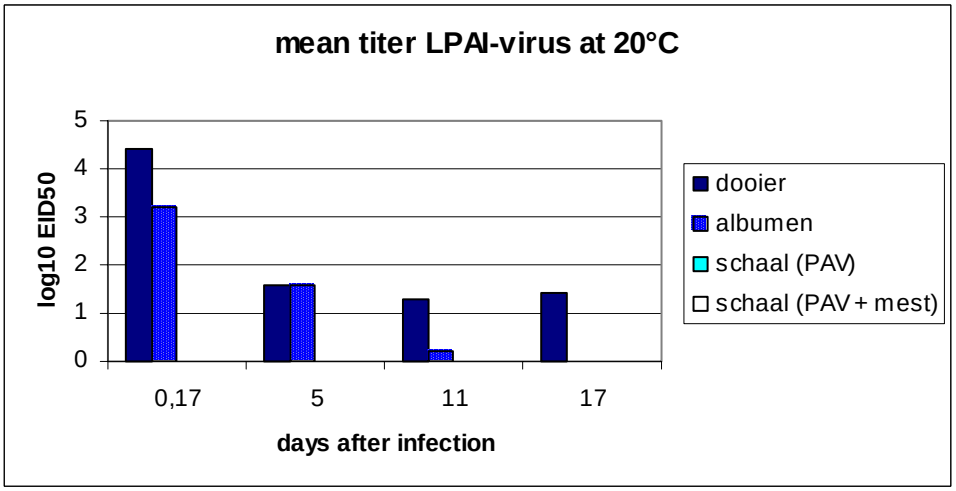


Figure 5

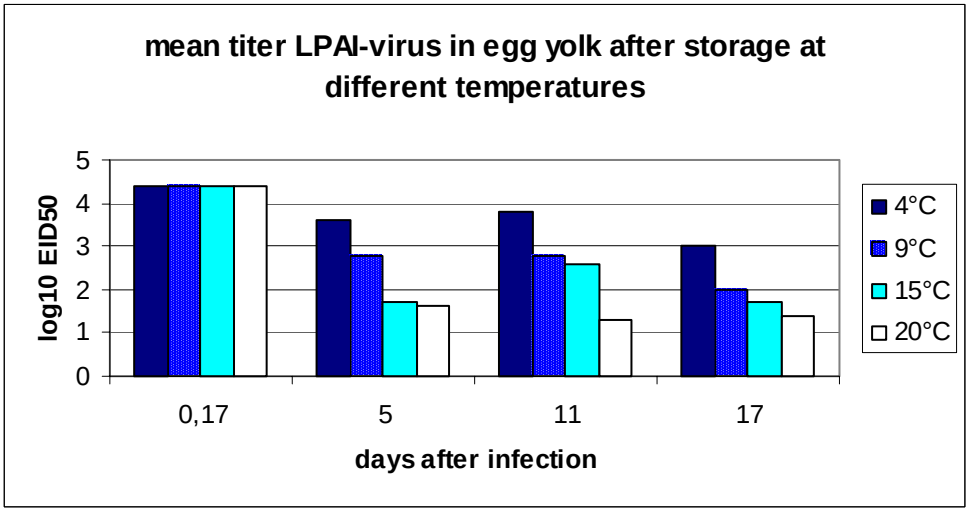


Figure 6

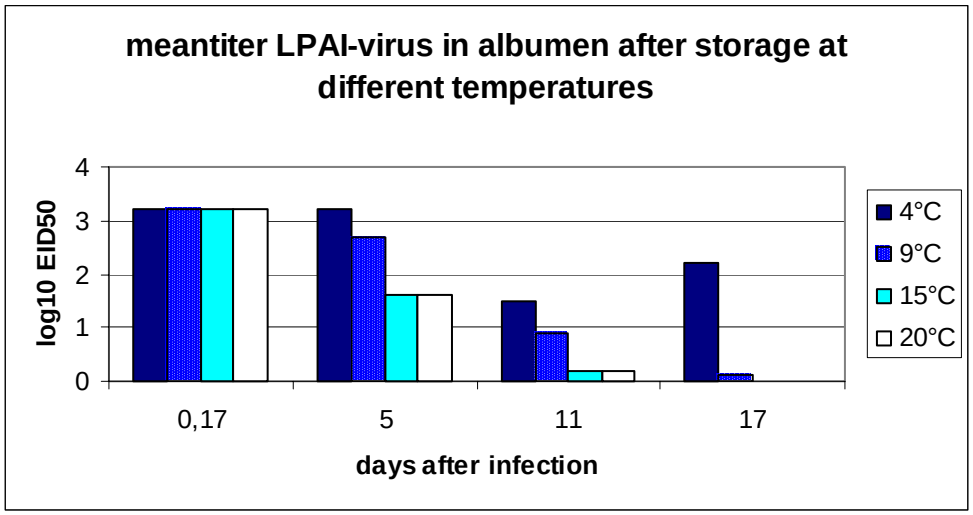
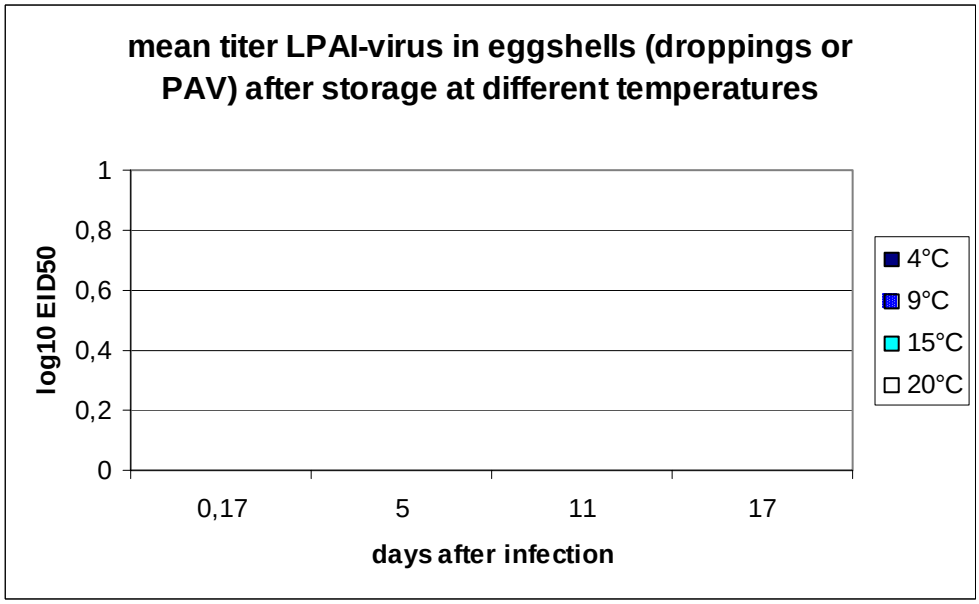


Figure 7



Egg yolk

At all storage temperatures AIV could still be isolated from the egg yolk up to 17 days after infection (Figures 1 to 4). The reduction in quantity of virus that could be isolated (the virus titre) was temperature-dependent. The reduction progressed more rapidly at increasing temperatures (Figure 5).

Albumen

At all storage temperatures AIV could still be isolated from the albumen up to 11 days after infection and at the 2 lowest temperatures also up to 17 days after infection (Figures 1 to 4). The reduction of quantity of virus that could be isolated (the virus titre) was temperature-dependent. The reduction progressed more rapidly at increasing temperatures (Figure 6).

Eggshell

On determining the survival period of AIV on (brown) SPF eggs no more infectious virus could be detected at 4 hours after applying (and drying) the PAV (pure or mixed with droppings) on the eggshell (figure 7). We did find a positive RT-PCR AI in the (filtered) liquid of the pulverised infected eggshells.

Test 2

This subsequent test was carried out to assess the repeatability of parts of test 1 and to gain (initial) understanding of the operative mechanism of AIV inactivation. The results of the 6 investigated aspects were as follows.

A. No reduction was observed in the virus titre of the PAV itself between the start and the end of test 2. At times 0 and 3 hours ^{10}log titres were found of ≥ 5.25 and ≥ 5.5 respectively. The dilution medium was negative.
B. Just like in test 1, in Test 2 it was not possible to isolate the PAV after drying in on the eggshell. It was not possible to isolate virus from the albumen of these eggs either.
C. On sampling the eggshell using a humid swab after the PAV had dried in, it was not possible to isolate virus either.
D. The virus isolation (both titration steps, all eggs) was positive on the swab of the PAV that was applied and had dried in on the plastic sheet. This was unlike the swabs of the PAV that had been applied on the eggshells (see C).
E. From the mixture of PAV with pulverised eggshell, AI virus could be isolated both directly and after 4 hours (both 2 titre steps carried out in which all eggs were positive for AIV, so no titre reduction could be measured).
F. The contact with the paraffin did not show in activation of the virus.

Discussion

LPAI virus ($10^{6.5}$ EID_{50}) introduced to the egg yolk or albumen could be re-isolated from the egg during an extended period (11 to 17 days). The quantity of virus that could be isolated dropped after a longer storage time, while this reduction was stronger in albumen than in yolk. At higher temperature the quantity of (infectious) virus that could be isolated dropped more rapidly than at lower temperatures.

Both in test 1 and in test 2, $10^{6.5}$ EID_{50} AI-virus that had been applied on the eggshell (as pure allantoic fluid or mixed with droppings (test 1)) could no longer be re-isolated after the drying period of 3 to 4 hours. It was not possible to isolate virus from the albumen of these eggs either. When already pulverised eggshell was mixed with PAV, it was possible to isolate virus (easily) after 4 hours (drying time). It can be concluded from these facts that the finding that it was not possible to isolate the virus from the eggshells after the drying period on the eggshell can not only be explained by inactivation of the virus as a result of contact with the eggshell (lime) alone. Just drying in the PAV does not cause (full) inactivation, as appears from the result of the swabs of the dried PAV on the plastic sheet. May be if the combination of drying and the (chemical) contact with eggshell (95% $CaCO_3$) is the cause of the rapid inactivation on the virus. Further research will be necessary to gain more insight into this process.

The results indicate that AIV present in eggs can remain present in infectious form during a prolonged period. AIV that ends up on an eggshell appears to be inactivated (very) rapidly.