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Different incubation temperatures affect viral polymerase activity and yields of low-pathogenic avian influenza viruses in embryonated chicken eggs

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Abstract

Various incubation conditions (35°C–38°C, 2d–7d) have been used in surveillance studies of the prevalence of avian influenza viruses in wild birds. Here, we studied viral polymerase activity and virus growth kinetics of low-pathogenic avian influenza viruses (LPAIVs) isolated from field samples [A/duck/Hong Kong/365/1978 (H4N6) and A/duck/Nanchang/-0480/2000 (H9N2)] during incubation at different temperatures (35°C, 37°C, and 39°C) in the allantoic cavity of 10-day-old embryonated chicken eggs (ECE). The higher incubation temperatures (37°C and 39°C) significantly promoted virus growth which is most likely as a result of greater viral polymerase activity (20%–60%) than that observed at 35°C and as much as 100% greater virus yield (as measured by hemagglutination assay) 2 days after inoculation. Our findings revealed that the optimal activity of viral polymerase complex resulting in highest yield of LPAIV field isolates could be obtained by incubation for 2 days in ECE at 37°C and 39°C.

Introduction

Wild aquatic birds are natural reservoirs of low-pathogenic avian influenza viruses (LPAIVs) [1, 35]. Infection of wild fowl is usually asymptomatic or mild [8], allowing direct or indirect transmission of influenza A viruses (IAVs) to domestic birds, mammals and humans [6]. Among the viral factors that contribute to the virulence and host range of IAVs are the viral polymerase proteins PB2, PB1, and PA [2, 10, 11, 24], which form together with NP and vRNA the viral ribonucleoprotein (RNP) complex. Ability to adapt to the cellular host conditions is a critical factor that contributes to virus replication. PB2 and PA have been shown to be responsible for a significant increase in polymerase activity [4, 20, 32]. PB2 also plays a role in host adaptation [9, 11, 27]. Amino acid residue 627 in PB2 appears to be one of the main determinants for cold sensitivity of the avian IAV and for viral pathogenicity in mammalian hosts [12, 23, 24, 33]. The presence of lysine at position 627 (K627), instead of the glutamic acid (E627) that is found in avian viruses, is crucial for high virulence in mice [11]. However, E627K mutation polymerase complexes with PB2 derived from the avian influenza virus retain transcriptional activity over a broad temperature range (30–42 °C), whereas those with PB2 derived from the human influenza virus lose their activity at elevated temperatures (39 °C or higher) [4].

While the effect of different temperatures on IAV replication have been well studied in various infection systems, including experiments in cultured cells and animal models [23–26], little is known about growth properties of LPAIVs in embryonated chicken eggs (ECE)

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at different incubation temperatures. Optimal incubation conditions for growing avian influenza viruses from field samples vary in a wide temperature and duration range. For example, the World Health Organization [36] recommends 35°C or 37°C for avian influenza as optimal temperatures for their replication in 10-day-old ECE during 2–3 incubation days [36]. The Organization for Animal Health [29] suggests influenza virus isolation from 9- to 11-day-old ECE at 37°C (range, 35°C–39°C) for 4–7 days [29]. In addition, the European Union (EU) Commission Decision 2006/437/EC approves IAV isolation by using 9- to 11-day-old ECE at 37°C with daily candling during 6 days' incubation [7]. Furthermore, field studies of wild birds and domestic poultry have also used a range of incubation conditions to isolate avian influenza viruses (Table 1).

To investigate whether temperature alone could significantly influence viral growth in ECE, we compared the effect of three incubation temperatures (35°C, 37°C, and 39°C) on the viral polymerase activity and replication rates of two LPAIVs isolated from wild ducks [A/duck/Hong Kong/365/1978 (H4N6) and A/duck/Nanchang/2-0480/2000 (H9N2)] incubated for 1–3 days in 10-day-old ECE. We used the predominant avian influenza subtypes, including H4N6, which is most frequently isolated in surveillance studies of migratory birds [5, 18, 37], and H9N2 as a comparative study, which is associated with mild respiratory disease outbreaks in domestic poultry [14, 28]. Our findings showed that viral polymerase activity differed significantly between lower and higher incubation temperatures in ECE. The viral polymerase complex of both viruses exhibited greater activity at the two higher temperatures (37°C and 39°C) than at 35°C, resulting in increased virus yield collected from allantoic fluids 2 days after inoculation.

Materials and Methods

Stock viruses

The strains A/duck/Hong Kong/365/1978 (H4N6) (A/HK/365/78 [H4N6]) and A/wild duck/Nanchang/2-0480/2000 (H9N2) (A/NAN/2-0480/00 [H9N2]) were obtained from the St. Jude Children's Research Hospital repository, propagated in ECE, and used as stock viruses. For propagation, 0.1 ml of virus stock solution was mixed with an equal volume of phosphate-buffered saline containing antibiotics (penicillin G, 4,000 U/ml; streptomycin sulfate, 800 U/ml; polymyxin B, 400 U/ml; gentamicin sulfate, 0.1 mg/ml), inoculated with 0.2 ml of this inoculum into 10-day-old ECE and incubated for 3 days at 35°C as previously described [13]. Stocks of both viruses were raised in the allantoic cavities of chicken embryos. Eggs were chilled at 4°C overnight or for 4 h before harvesting. The harvested allantoic fluids were stored in aliquots of 250 µl at –80°C. Bacterial contamination was monitored by streaking blood agar plates and incubating them for 24 h at 37°C. After the first passage, both viruses had a hemagglutinin (HA) titer of 512; the 50% egg infectious dose (EID₅₀) values were 10^{8.75} for A/HK/365/78 (H4N6) and 10^{8.50} for A/NAN/2-0480/00 (H9N2).

Virus growth at different temperatures

Fertilized White Leghorn chicken eggs were used for all experiments. The eggs were set in a 39°C incubator with 50% relative humidity. Eggs were automatically turned every 6 h. The 10-day-old embryonated chicken eggs were candled for embryo viability, and the eggs with dead embryos were discarded. Intra-allantoic inoculation was done through a hole on one side of the eggs at a dose of 0.1, 1, or 10 EID₅₀/ml of stock virus in 0.1 M phosphate buffer (pH=7). Each egg received 0.2 ml of inoculum, and viral growth was investigated at three incubation temperatures (35°C, 37°C, and 39°C). Before incubation, the inoculated eggs were divided into two groups for different methods of harvesting allantoic fluid. In the first group (n=108 [including three controls per investigation]), viral growth was monitored by

harvesting allantoic fluid daily from the same IAV-infected egg during the 3-day incubation. To control for the possibility that daily harvesting might injure the embryos, the second group of ECE was divided into three batches of 108 eggs (including three controls per day of examination) and chosen randomly after 24, 48, and 72 h. Mock-infected ECE were created by inoculating sterile PBS into the allantoic cavity. To help rule out nonspecific death, the eggs were candled 12 h after inoculation (a.i.). All investigations were done in triplicate. Influenza-infected eggs from both groups and mock-infected ECE were incubated at 35°C, 37°C, or 39°C. To monitor the viability of the embryos, we candled the ECE every 12 h during the 3 days. The harvested allantoic fluid of IAV-infected ECE of both groups were stored in aliquots of 250 µl at -80°C. Bacterial contamination was monitored by streaking blood agar plates and incubating them for 24 h at 37°C. To study the influence of different incubation temperatures on the spread of viral infection in other cell layers of the chicken embryos, we tested the amniotic fluid. This investigation was performed with randomly chosen eggs from the second group. After harvesting allantoic fluid, we collected amniotic fluid from the same egg and stored it in aliquots of 250 µl at -80°C. Bacterial contamination was monitored by streaking blood agar plates and incubating them for 24 h at 37°C. The harvested allantoic fluids from both groups were tested for virus growth by EID₅₀ and hemagglutination assay, and the results were compared. The amniotic fluids from the second group were investigated for virus products using a hemagglutination assay. The reference temperature for all experiments in this study was 35°C. All assays were performed in triplicate.

Detection of viral genes

RNA was extracted from virus-infected allantoic fluid using the QIAGEN RNeasy Mini Kit (Carlsbad, CA) as instructed by the manufacturer. Allantoic fluid was tested for IAV using previously described universal primers specific for the HA and NA genes (Hoffmann et al., 2001). One-step PCR was performed using the QIAGEN RT-PCR Mini Kit (Carlsbad, CA).

Hemagglutination titration

The Hemagglutinin (HA) of newly formed virus particles in the harvested allantoic and amniotic fluids was titrated according to the WHO protocols [36] by using serial 2-fold dilutions of the fluids in 96-well microtiter plates with 0.5% chicken red blood cells.

Determination of EID₅₀

Half-log serial dilutions of virus (100 µl) were incubated in the allantoic cavities of 10-day-old ECE at 35°C for 48 h. HA was then titrated in the harvested allantoic fluid (Swayne et al., 1998). EID₅₀/ml was calculated for each day by the method of Reed and Muench (Reed and Muench, 1938).

Dual luciferase assay of viral polymerase activity

Transit LTI (Panvera, Madison, WI) was used according to the manufacturer's instructions to transfect subconfluent monolayers of human embryonic kidney epithelial cells (293T) with 1 µg of a firefly luciferase reporter plasmid flanked by human PolI promoter (pPolI A-luci) and murine terminator sites, which produced a viral RNA (vRNA)-like RNA transcript. Cells transfected with 0.02 µg of Renilla luciferase-expressing plasmid served as a loading control. After 24 h, cells were infected with virus-containing allantoic fluid at a multiplicity of infection of 1. Cells were lysed in 500 µl of passive lysis buffer (Promega, Madison, WI) 9 h post-infection (p.i.). Luciferase activity was assayed using a dual luciferase assay reagent kit (Promega) and a BD Monolight 3010 luminometer (BD Biosciences, Franklin Lakes, NJ). Viral polymerase activity was expressed as the ratio of firefly luciferase signal to Renilla luciferase signal

Statistical analysis

A backward/forward stepwise multivariate analysis [3] was performed to compare the effect of the different incubation temperatures, virus inoculation doses, and incubation periods on replication of the two virus subtypes. The model was simplified and evaluated using parametric bootstrapping and likelihood ratio tests [17]. Results of a univariate analysis using ANOVA for each time point (temperature, length of incubation, and inoculation dose) were compared by the Turkey-Kramer multiple comparison method [16]. All data were expressed as the mean $\pm$ SE. $P \leq 0.05$ was considered to indicate a statistically significant difference. All statistical analyses used the program R 2.10.0 provided by the Institute for Statistics and Mathematics, Vienna University of Economics and Business (<http://www.r-project.org/>).

Results

Viral polymerase activity in embryonated chicken eggs at different incubation temperatures

Because polymerase complexes play an important role in host adaptation [9–11, 27], we examined the effect of different incubation temperatures (35°C, 37°C, and 39°C) on the polymerase activity of the two avian influenza strains isolated from wild ducks. To this end, 293T cells were transfected with a reporter plasmid expressing viral RNA-like RNA encoding firefly luciferase gene. Renilla luciferase served to control for background activity. After 24 h, the cells were infected with virus harvested from ECE allantoic fluid 2–3 days a.i. The luciferase signal (corresponding to viral polymerase activity) was measured 9 h p.i. (a single viral replication cycle). The highest luciferase signals were assigned a value of 100% activity to allow comparison. The RNPs of both viruses showed significantly higher polymerase activity at 37°C and 39°C than at 35°C ($P = 0.05$; Figure 1), with the exception of the virus subtype H4N6 harvested 72 h a.i. The polymerase activity of A/HK/365/78 (H4N6) harvested 48 h a.i. differed significantly after incubation at 35°C and 37°C ($P = 0.004$), 35°C vs. 39°C ($P = 0.01$), and 37°C and 39°C ($P = 0.01$), while virus harvested 72 h a.i. had significantly different polymerase activity after incubation at 37°C vs. 35°C ($P = 0.01$) (Figure 1). A/NAN/2-0480/00 (H9N2) harvested 72 h p.i. showed significantly different polymerase activity after incubation at 35°C and 39°C ($P = 0.02$; Figure 2).

Differences in viral polymerase activity levels affect the amount of newly synthesized virions in embryonated chicken eggs

Luciferase assays showed that more viral polymerase activity was found in infected ECE incubated at higher temperatures (37°C and 39°C). Because viral polymerase activity has been shown to influence the outcome of infections in cultured cells and avian hosts [21, 22], we determined the effect of different incubation conditions on the amount of newly synthesized virions by hemagglutination assay. Overall, the highest HA titers were observed in allantoic fluid from ECE incubated at 37°C and 39°C for 1 and 2 d. There was a 1- to 2-log greater HA titers at 37°C than at 35°C and a 2- to 3-log greater HA titer at 39°C than at 35°C at 1 and 2 d p.i. (Table 2).

The results of inoculation with 1 and 10 EID₅₀/ml were similar in allantoic fluid harvested by the two methods. All samples of both viruses have tested positive at three different temperatures during 1–3 days of incubation. Of the samples from eggs inoculated with 0.1 EID₅₀/ml, a single sample of A/HK/365/78 (H4N6) was positive at 35°C (Table 2). This positive sample was obtained only by the continual harvesting method (first group), in which the viral growth was monitored by harvesting allantoic fluid daily from the same infected egg. However, we found no statistically significant differences in the mean HA

titers of this virus at different temperatures ($P = 0.38$). None of the harvested allantoic fluids of mock-inoculated ECE generated any virus-specific products.

To assess whether incubation time and inoculation dose influence the effect of incubation temperature, we performed univariate and multivariate analyses. When analyzed individually, all three incubation temperatures ($P = 0.01$), time ($P = 0.03$), and the inoculation dose of 10 EID₅₀ ($P = 0.05$) significantly influenced viral growth. Multivariate analysis revealed that the three variables interact ($P = 0.05$). We next analyzed the influence of the variables according to virus strain. On day 1 a.i., the inoculation dose of 10 EID₅₀ yielded a significantly greater HA titer in both viruses when incubated at 35°C vs. 37°C ($P = 0.04$) and at 35°C vs. 39°C ($P = 0.05$). On day 2 p.i., the HA titer of A/HK/365/78 (H4N6) inoculated with 10 EID₅₀ was significantly greater after incubation at 37°C vs. 35°C ($P = 0.03$), and that of A/NAN/2-0480/00 (H9N2) was significantly greater at 39°C vs. 35°C ($P = 0.05$). No significant difference was observed in HA titers by to harvest method (first vs. second group) ($P = 0.77$); temperature and harvest method ($P = 0.19$); or time, temperature, and harvest method ($P = 0.96$).

To study the influence of different incubation temperatures on the spread of viral infection in other cell layers of the ECEs, we harvested and tested amniotic fluid of the second group. No virus replication was detected beyond the site of allantoic inoculation.

Higher polymerase activity enhances virus replication rates in embryonated chicken eggs

Higher viral polymerase activity at 37°C and 39°C resulted in increased HA titers, indicating an elevated amount of newly synthesized virions. However, in contrast to other forms of virus quantification, this assay does not give any measure of viral infectivity. Therefore, we studied the growth kinetic of each strain by determining the EID₅₀ 48 h and 72 h after incubation at all three temperatures. After 48 h, both viruses replicated to higher levels at 37°C and 39°C than at 35°C, consistent with the results of hemagglutination and polymerase assays (Figure 2). After 72 h, A/HK/365/78 (H4N6) titers did not differ at 35°C and 39°C but were lower at 37°C, while the titers of A/NAN/2-0480/00 (H9N2) were highest after incubation at 39°C and lowest at 35°C (Figure 2). After 48 h, titers of A/HK/365/78 (H4N6) virus were significantly different at 35°C and 37°C ($P = 0.01$), 35°C and 39°C ($P = 0.001$), and 37°C and 39°C ($P = 0.02$; Figure 2). There was also a significant difference between titers of A/NAN/2-0480/00 (H9N2) virus incubated at 35°C vs. 39°C at 48 h p.i. ($P = 0.05$) and 72 h p.i. ($P = 0.03$; Figure 2). The results showed that both viruses replicated better at higher incubation temperatures (37°C and 39°C).

Discussion

A number of studies have sought to enhance influenza virus replication in ECE by using different incubation conditions. It was unknown whether different incubation temperatures could affect the viral polymerase activity in ECE, resulting in altered virus yields. We demonstrate that incubation of infected ECE at higher temperatures (37°C and 39°C) increased the activity of viral RNP complexes, leading to enhanced productive replication rates of the two avian influenza viruses used in our study. These findings help not only to determine the optimal incubation conditions for LPAIV in ECE, but also explain the underlying molecular mechanism.

Avian influenza viruses isolated from ducks were used in this study because duck populations are the most representative natural reservoir of LPAIV [1]. Moreover, we used viruses isolated during widely different years (1978 and 2000) to reveal temporal changes in viral replication in ECE. The selection of our incubation temperatures was based on temperatures recommended for avian influenza virus isolation by the WHO, the OIE, and

the EU Commission Decision 2006/437/EC [7, 29, 36] and the temperature at which avian influenza usually replicates (~39°C–41°C) in the intestinal tracts of birds (Massin et al., 2001). To precisely study the effect of different incubation temperatures on viral replication in ECE, various doses of stock virus were chosen for allantoic inoculation.

There is increasing evidence that high viral polymerase activity enhances the replication of avian influenza virus in mallard ducks [21]. In accordance with this finding, our polymerase activity assay showed higher activity at higher temperatures when cells were infected with either virus. In addition, virus yield (as measured by HA titer and EID₅₀) was at least twice as great at higher temperatures. This supports the finding [31] that 38°C is the optimal temperature for influenza virus RNA polymerase activity *in vitro*, and the detection of viral HA and NA proteins of the strain A/FPV/Rostock/34 (H7N1) was greatest between 38°C and 40°C. In addition, avian influenza is likely to be better adapted at higher temperatures than at 35°C, because it replicates primarily in the avian intestinal tract around 41°C [23].

Increased replication of LPAIV at 37°C and 39°C in ECE could also be explained by the activity of trypsin-like proteases, which are present in the extracellular (allantoic and amniotic) fluid of ECE [19, 30]. It is possible that these proteases cleave the HA of LPAIVs preferentially at temperatures above 35°C, resulting in increased viral binding to the epithelial cells and enhanced virus replication in 10-day-old ECE. Indeed, the simultaneous inoculation of 10-day-old ECEs with LPAIV (H3N2, H4N6, H5N1, and H5N2) and trypsin has been found to increase the virus yield (as measured by hemagglutination units) by 2 to 8 logs, as compared with controls without trypsin [34]. On the other hand, it has been reported that the allantoic fluid of 14-day old ECE with strong protease activity degraded the HA protein of avian influenza [15].

Additionally, we analyzed whether different incubation temperatures might affect the spread of viral infection in host cells of the amniotic cavity after allantoic inoculation in ECE. The infectious viral particles were restricted to the allantoic epithelium and did not invade the amniotic cell layers. This is consistent with the findings of Rott et al. [30] that non-pathogenic influenza strains multiply in cells of the allantoic cavity. It should be noted that despite the advantages of ECE (the short time consumption and low cost of virus replication), the results of viral growth in this system cannot always be duplicated exactly and therefore indicate only tendencies. This is due to the uncontrollable factors, particularly the inherent variations in the immune maturity and behavior of embryos or batches of ECE, in the virulence of influenza viruses, and in virus-host interaction.

Taken together, our findings show that avian influenza virus can be isolated from ECE within 48 h by incubation at 37°C or 39°C, whereas 72 h is required if ECE are incubated at 35°C. This finding may reduce the time required to isolate avian influenza virus from field samples.

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Figure 1. Viral polymerase activity of low-pathogenic avian influenza viruses

Viral polymerase activity (expressed as the ratio of firefly to Renilla luciferase signal) was measured 9 h p.i. with 10 EID₅₀/ml of virus (passage 2). Results are the mean $\pm$ SE from triplicate experiments. At 48 h p.i., A/HK/365/78 (H4N6) polymerase activity was significantly greater (*) after incubation at 37°C vs. 35°C ($P=0.004$), 39°C vs. 35°C ($P=0.01$), and 39°C vs. 37°C ($P=0.01$); at 72 h p.i., values were significantly greater at 35°C vs. 37°C ($P=0.01$). At 72 h p.i., A/NAN/2-0480/00 (H9N2) values were significantly greater after incubation at 39°C vs. 35°C.

Figure 2. EID₅₀ of the viruses 48 and 72 h after inoculation in chicken embryonated eggs ECE were inoculated with 10 EID₅₀/ml of stock virus (second passage). Values are the mean $\pm$ SE from triplicate experiments. At 48 h p.i., A/HK/365/78 (H4N6) values were significantly greater (*) at 37°C than at 35°C ($P=0.01$), at 39°C than at 35°C ($P=0.02$), and at 39°C than at 37°C ($P=0.001$). For A/NAN/2-0480/00 (H9N2) virus, values were significantly greater at 39°C than at 35°C at 48 h ($P=0.05$) and 72 h after inoculation ($P=0.03$).

Table 1

Overview of incubation parameters used for influenza A virus isolation

References	Incubation parameters ^a	
	Temperature (°C)	Time (days)
Cheng et al., 2010	35.0	2
Douglas et al., 2007	35.0	2–3
Hinshaw et al., 1978; Ottis and Bachmann, 1983; Kawaoka et al., 1988; Fouchier et al., 2003; Widjaja et al., 2004; Krauss et al., 2007	35.0	3
Süss et al., 1994	36.0	3
Shortridge et al., 1977; Guan et al., 2000; Wang et al., 2008; Wallensten et al., 2007 ^b ; Munster et al., 2005 ^b ; Munster et al., 2007 ^b ; Latorre-Margalef et al., 2009 ^b	37.0	2
Lei et al., 2007; Manin et al., 2010 ^c ; Savi et al.,	37.0	2–3
Stallknecht et al., 1990; De Marco et al., 2004; De Marco et al., 2005; Jahangir et al., 2008	37.0	3
Chen et al., 2006; Cattoli et al., 2007; Fereidouni et al., 2010; Pasick et al., 2010	37.0	1–6 1–7
Ip et al., 2008 ^b ; Dusek et al., 2009 ^b	37.2	3
Haynes et al., 2009 ^c	37.8	7
Siembieda et al., 2010	35.0–37.0	3–5
Hlinak et al., 2006; Müller et al., 2009	35.0–37.5	4–6
Goujgoulova et al., 2010 ^{b,c}	35.0–38.0	4
Wahlgren et al., 2008 ^c	37.0–38.0	3
Globig et al., 2006	38.0	5

^aIncubation parameters used in surveillance studies of influenza A virus prevalence in wild birds and domestic poultry. Viruses were inoculated into the allantoic fluid of 8- to 11-day-old embryonated chicken eggs [ECE].

^bField samples were screened by real-time PCR to ensure avian influenza genome before inoculation into ECE.

^cIncubation conditions of virus isolation in ECE were received by personal communication with a corresponding author.

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Table 2

Virus hemagglutination titers according to inoculation dose and incubation temperature^a

Inoculation dose	Virus isolate	Hemagglutination titer ^b									
		35°C					37°C				
		1dpi ^c	2dpi	3dpi	1dpi	2dpi	1dpi	2dpi	3dpi	1dpi	2dpi
10 EID ₅₀	Dk/HK/365/78/H4N6	16	128	512	64	256	1024	128	256	512	
	Wdk/NAN/2-0480/00/H9N2	128	1024	4096	512	2048	4096	1024	4096	4096	
1 EID ₅₀	Dk/HK/365/78/H4N6	8	128	512	32	256	512	32	512	512	
	Wdk/NAN/2-0480/00/H9N2	128	512	2048	512	4096	2048	1024	4096	2048	
0.1 EID ₅₀	Dk/HK/365/78/H4N6	2	8	32	<2	<2	<2	<2	<2	<2	
	Wdk/NAN/2-0480/00/H9N2	<2	<2	<2	<2	<2	<2	<2	<2	<2	

^aThe viral growth results of the first inoculation method (daily harvesting allantoic fluid from the same influenza A virus infected egg).

^bExpressed as the reciprocal of the mean virus dilution (triplicate) that yields complete agglutination of 0.5% chicken red blood cells.

^cDay post-inoculation.