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Heat Inactivation of Avian Influenza (H7N3) Virus In Experimentally Infected Chicken Meat

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Abstract: Introduction Avian influenza virus (H7N3) cause severe systemic disease in chickens and can be persisted in infected chicken meat that cause public health concern. Influenza viruses are heat liable but parameters for heat inactivation have not been known. **Methodology** The study investigated the quantitative heat inactivation of H7N3 virus from experimentally infected chicken meat. Twenty (20) sero-negative broiler chickens of 04 weeks age were divided into two group viz A (control) and B artificially infected by giving 0.1ml of 10^6 embryo infectious dose 50 (EID_{50}) (H7N3) virus through intranasal route. Birds were slaughtered on day 9 post infection and tissues (trachea, kidney, lungs and liver) were collected. Inactivation curves were determined at temperature 60, 61, 62, 63 and 64°C. **Results** Based on D values, time to inactivate H7N3 virus depends on viral titers and inversely related to temperature. This study investigated confirmed that H7N3 virus was effectively inactivated in chicken meat at 60°C in 4 minutes while at 64°C in 1 minutes. Moreover, protocols per log EID_{50}/g reduction in terms of D values at 60 and 64°C were noted 120 and 30 sec, the coefficient of regression ranged as 0.941 to 0.889.

Keywords: Avian influenza virus (H7N3), heat inactivation, EID_{50}, Dt values

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Introduction

Avian influenza virus (AIV) causes severe respiratory disease in poultry with high morbidity and mortality. AIV replicates in respiratory organs and then spread throughout the body by viremia and subsequent infection in internal organs such as brain, spleen, and skeletal muscles [1, 2]. Influenza virus caused mild disease in ducks whereas severe clinical manifestations occurred in chicken [3]. Despite of severe disease in poultry, replication, and presence of AI in the internal organs raised the question of whether AI viruses transmitted to other birds, mammals and humans via direct contact, faecal and oral route or by ingestion of infected meat or not.

Highly pathogenic (HP) influenza viruses persisted in infected chicken meat and in some cases; the zoonotic transmission has been associated with direct contact to AIV infected birds [4, 5]. Even though the consumption of undercooked or raw meat obtained from ill birds have been used as a vehicle for the transmission of the HP influenza virus in a small number of cases yet to a lesser extent foodborne transmission of the virus to humans has been supported epidemiologically. More specifically, H5N1 HPAI viruses have been recovered from chicken

thigh and breast meat, internal organs, and eggs produced by acutely infected chickens [6]. Likewise, consumption of influenza H7N3 virus infected meat caused severe infections in mammals and humans [7, 8].

Due to severe complications in poultry caused by HPAI which can be capable of zoonotic transmission, the World Organization for Animal Health recommended that poultry meat and products from countries with HPAI viruses have been treated for viral inactivation before export to other countries [9]. The heat inactivation of influenza viruses in poultry meat and their products revealed that thermal inactivation could be an effective method for prevention of zoonotic infection [4, 10]. A preliminary study performed on a precise micro assay system developed for measuring heat inactivation of influenza virus in poultry meat experimentally. Nevertheless, quantitative method for AI virus inactivation in meat samples has not been established and adopted [4]. AI viruses are heat liable single stranded negative sense RNA viruses yet can be partially protected from heat inactivation methods if attached with organic materials [11, 12].

The frequency of viral outbreaks in poultry have increased the concern of viral transmission through ingestion of meat from infected poultry. Therefore, the study was conducted to investigate the quantitative heat inactivation of a representative H7N3 virus in infected chicken meat and evaluated the efficacy of food safety and cooking guidelines with respect to H7N3 virus.

Methods

Experimental Infection in Chicken

The biologically characterized low pathogenic Avian influenza virus, A/Chicken/SPVC 26/03 (H7N3) of 10^6 EID_{50} stored at -84°C, at Sindh Institute of Animal Health (SIAH), Karachi was used in this study. Twenty (20) sero-negative broiler chickens of 04 weeks old were bought from Hatchery, divided into 2 groups (n=10) viz. group A (control) and group B (infected). All the experimental birds reared and maintained at experimental station of the SIAH. The birds were fed commercial poultry feed and watered at *ad libitum*. Birds were inoculated with virus inoculums of EID_{50} at a dose of 0.1ml/bird through the intranasal route as described by Reed & Muench [13]. Severely sick birds were slaughtered, and tissue samples of trachea, kidneys, lungs and liver were collected. Approximately about 01 gram of each tissue/organ was collected and EID_{50} titers were calculated. After that tissue samples were used for heat inactivation of virus.

Animal Ethics:

The current research work was approved by Animal Ethics Committee of Sindh Agriculture University Tandojam.

Heat Treatment of Meat Homogenates And Thermal Inactivation of AIV.

Tissue homogenates of the trachea, kidney, lungs and liver with EID_{50}/g titers i-e; 4.4, 4.9, 3.7 and 3.2 log10 respectively were dispensed in eight micro tubes of 225 µl, labelled as T1-T8. Each tube contains 120 µl which were exposed to 60, 61, 62, 63 and 64°C for 1, 2, 3, 4, 5, 6, 7 and 8 min. in a gradient thermocycler (Multigene USA). After that the samples were 10-fold (10^{-1}) diluted in normal saline and inoculated in 9 days old embryonated chicken eggs to determine the infectivity of virus [13].

Statistics

The current study represented the influenza virus inactivation through survival curves predicted D values (time required to reduce 1 log10 EID_{50} of virus) at a specified temperature. The viral titers plotted on a curve represented the infective particles in a mean log10 which is equal to median. The D values calculated from equation of linear regression of virus titers verses time at specified temperature from following equation.

$$LogN = LogN_0 - \frac{t}{D} \qquad z = \frac{(Tf-Ti)}{log(\frac{D_1}{D_2})}$$

Where N is number of virus survival logs, N_0 is number virus at initial time, t is time and $-\frac{1}{D}$ is decimal reduction in virus. Moreover, z is temperature for decimal reduction of virus, Tf is final temperature, Ti is initial temperature, D_2 is decimal reduction at final and D_1 is decimal reduction time at initial. The lowest limit of detection was set as 2 log10 EID_{50}/g. Regression is the curves were expressed in coefficient of regression (R^2) and y is the equation of linear regression.

Results

Thermal Inactivation of H7N3 in Meat From Experimentally Infected Chicken.

Because the virus persisted in the meat of infected chickens, so meat homogenates of trachea, kidney, lungs, and liver were pooled in a single tube with $log10^{4.4}$ EID_{50}/g. Thermal inactivation revealed that the virus was reduced to the lowest detection limit of ≤2 EID_{50}/g at 60, 61, 62, 63 and 64°C in -5, 4, 3, 2 and 1 min respectively Table 1.

Table 1 Thermal inactivation of H7N3 virus in artificially infected chicken meat

Time (min)	Decimal reduction (Log 10) at different Temperature				
	60°C	61°C	62°C	63°C	64°C
0	4.4	4.1	3.1	4.05	4.05
1	3.9	3.3	3	2.9	≤ 2
2	3	2.8	2.7	≤ 2	0
3	≤ 2.5	≤ 2.5	≤ 2	0	0
4	≤ 2	≤ 2	0	0	0
5	0	0	0	0	0

The results revealed that the coefficients of regression (R^2) and the D-values were calculated from the curves of linear regression as listed in Table 2. It was observed that inactivation curves were biphasic and inversely proportional to temperature. Virus was inactivated in 120 seconds when exposed to 60°C and inactivated in 30 sec on exposure to 64°C. Moreover, the R^2 values ranged from 0.941 to 0.889.

Table 2 Experimental D-value of H7N3 virus from infected chicken meat

Temp	Trachea		Kidney		Lungs		Liver	
(°C)	D-value (sec)	R^2	D-value (sec)	R^2	D-value (sec)	R^2	D-value (sec)	R^2
60	120	0.941	120	0.942	120	0.941	120	0.889
61	120		90		120		90	
62	90		90		90		90	
63	60		60		60		30	
64	30		30		30		30	

Plotting the linear regression of Dt verses temperature; two different types of inactivation curves were generated such as monophasic and biphasic. Monophasic began with a decline in Dt value with increase in temperature which was observed in trachea and lungs whereas biphasic curve that showed immediate reduction in Dt with increase of temperature which was observed kidney and liver (Figure 1).

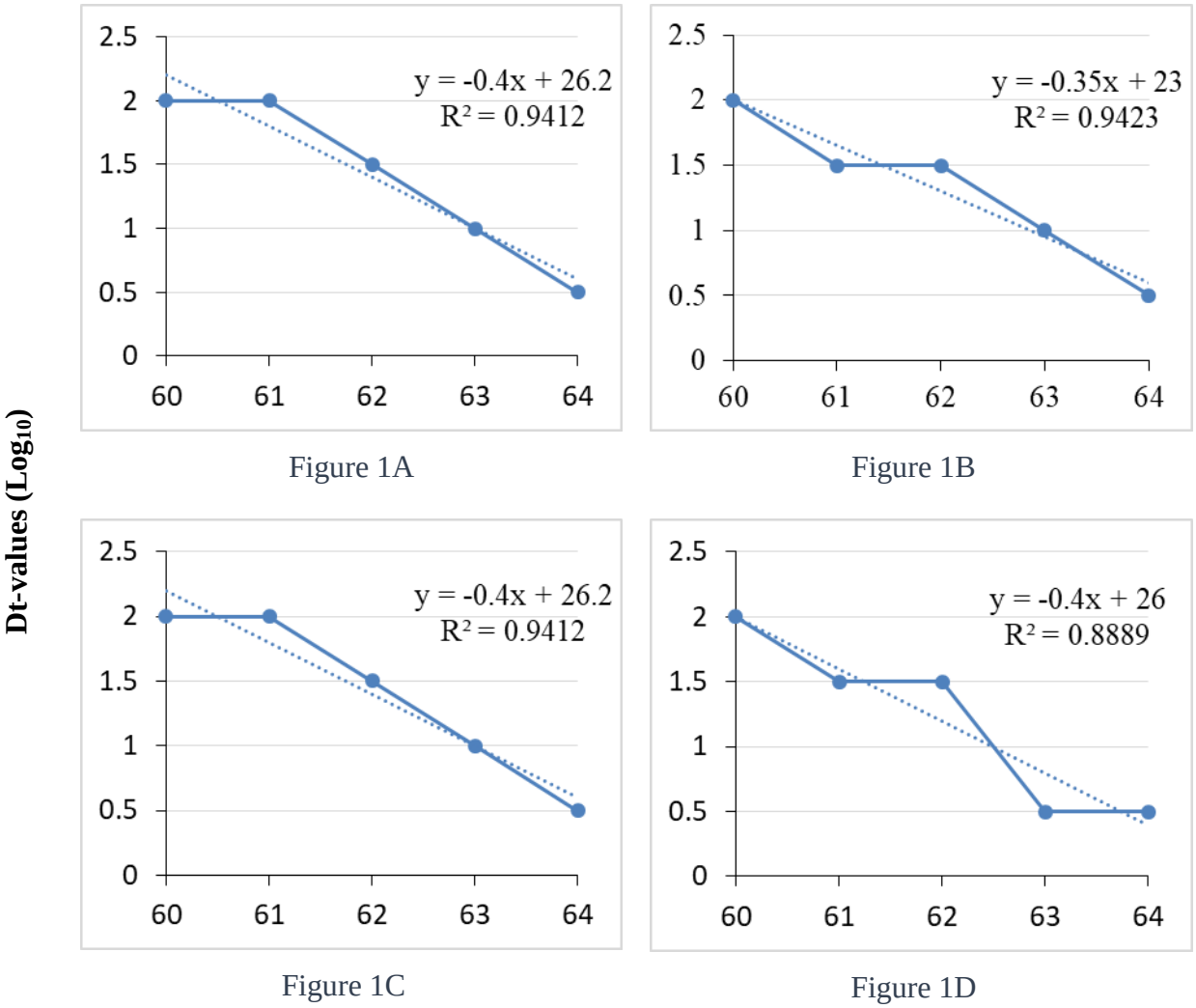

Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1 Inactivation curves of LP H7N3 (Dt values) verses temperature. Figure 1A, B, C and D represents the trachea, kidney, lungs, and liver of chicken infected with H7N3 virus; Y-axis represents Dt values while X-axis represents temperature. Y is regression equation and R^2 is the coefficient of regression.

Inactivation of virus verses time.

Inactivation curves were generated based on the reduction of log10 viral titre verses time, represented in Figure 2. Biphasic curves with delayed decline to the lowest detection limit was noted. It was observed in plateau of a curves that mostly the virus was inactivated in 180 seconds and sometimes it requires 240 seconds. More specifically, time for viral inactivation (Dt values) was directly related to virus titer per gram while inversely related to temperature (Table 2). However, the inactivation of virus was varied with tissue type of meat to be infected as the results revealed that the virus was promptly inactivated in liver than other tissues.

Discussion

Presence of AI in carcass and internal organs of infected poultry is of great concern because movement of infected poultry may spread virus to new locations, likewise virus can be transmitted to other birds through ingestion of raw meat and its products if not disposed properly. AI was first time introduced in Pakistan in early 90's after that the outbreaks have throughout the country, it might be due to spread of virus through movement of birds and persistence of AI in

infected carcass. Experimental study revealed that AI H5N1 has been transmitted to white leghorn through ingestion of infected chicken meat having $log10^{7.8}$ EID_{50} per hen yet H5N2 has not been transmitted through ingestion of infected meat having $log10^{3.6}$ EID_{50} per hen [10]. Moreover, domestic cats had been infected by ingestion meat from H5N1 infected chicken carcasses [14].

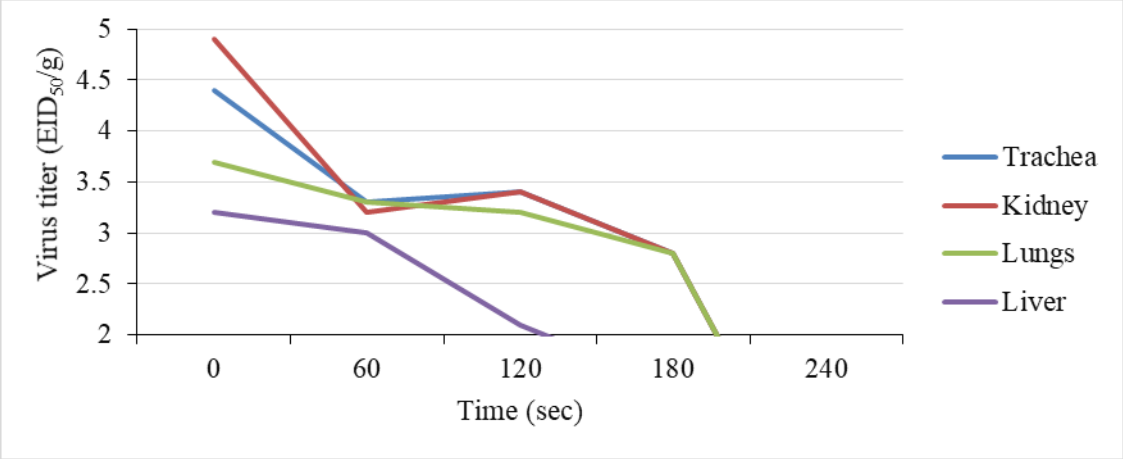

Figure 2 Inactivation of LP H7N3 verse time.

Various carnivores, including domestic cats, dogs, tigers, and leopards have been infected through ingestion of meat from AIV H5N1 infected birds during outbreaks [15, 16]. Although, little knowledge available whether LP H7N3 transmitted through consumption of infected meat or not.
Presence of virus and its titers in meat depends on subtype of virus, route of infection, time post infection and the breed of infected animal or birds and its immunity. Yet the reported titers of AI H5N1 in experimentally infected SPF eggs were $Log10^{7-8}$ EID_{50} [17]. In present study, intranasal inoculation, LP H7N3 virus detected from trachea, kidney lungs and liver. Preheat titers of H7N3 virus in trachea ($10^{4.4}$), kidney ($10^{4.9}$), lungs ($10^{3.7}$) and liver ($10^{3.2}$ EID_{50}/g of meat). Generally, LP couldn't be present in infected meat; yet respiratory secretions and faecal contaminations be the potential source of dissemination. Previous studies revealed that LP AI was isolated from body cavity, but virus isolation was negative in thigh and breast meat. It might be due to less amount of virus to be detected [10]. Presence of virus in meat depends on the concentration of virus in the secretions and the volume of faeces that contaminated the meat. However, the number of viral particles were very high in the experimentally infected meat used in the present study. Experimentally infected chickens with HP A/chicken/Korea/ES/2003(H5N1), the infective viral titers were calculated as $10^{8.0}$ and $10^{7.5}$ EID_{50}/g in uncooked thigh and breast meat [17].
The present study revealed that the cooking temperature effectively inactivated the LP AI virus in infected meat samples, the virus was inactivated in 4 to 1 minute at a temperature of 60 to 64°C (Table. 2). So, the cooking is considered, to be the method for viral inactivation. AI viruses are heat sensitive and lose their infectivity when exposed to 60°C for 10 minutes in chorioallantoic fluid and at 61ºC for 20s in egg products [10]. Likewise, the survival AI viruses increased with attachment with living body contents, it was observed that heat inactivation of the HP H5N2 virus required more time at specific temperature than that of the LP H7N2 virus from fat free egg contents. The HP H5N2 virus was inactivated on exposure of temperature from 55 to 59ºC in 18.6 to 0.4 minutes whereas the LP H7N2 virus on temperature from 55 to 60ºC in 2.9 to 0.5 minutes respectively [18].
The results obtained from survival curves of D value showed that LP virus was inactivated in 120 sec at 60ºC while in 30 sec at 64ºC and coefficient of regression ranged from 0.941-0.889. after 30 sec, survival curves were not constructed because lower level of virus couldn't be detected (Table 3, Fig 1). The survival curves might vary with subtype of AI and meat of infected specie. At high temperature the virus was inactivated in short time as described by Thomas [19] that high

pathogenic A/chicken/Korea/ES/2003 were inactivated effectively in infected chicken meat exposed to 70 or 73.9 ºC for less than 1sec. Moreover, the 95% inactivation of virus was predicted the D-values at lower temperature 57ºC in 241.2 sec while at 61ºC in 33.1 sec and coefficient of regression ranged from 0.88 to 0.85 [17].

Conclusion.

Based on the results of the present study it is concluded that H7N3 virus is heat liable and can be inactivated through cooking temperatures. Moreover, the virus was inactivated by heating meat for at least 30 seconds at 64°C. However, ingestion of AIV contaminated meat may remain a risk for transmission in birds, animals, and humans. The results also revealed that direct or indirect ingestion of undercooked meat is unhygienic for human health.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

The study was conducted as per Animal Ethics Guidelines laid down by Animal Ethics Committee of Sindh Agriculture University Tandojam.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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