DETECTION OF DISTRIBUTION OF AVIAN INFLUENZA H5N1 VIRUS BY IMMUNOHISTOCHEMISTRY, CHROMOGENIC *IN SITU* HYBRIDIZATION AND REAL-TIME PCR TECHNIQUES IN EXPERIMENTALLY INFECTED CHICKENS

Chanpen Chamnanpood[1,2], Donruedee Sanguansermsri[2], Sutatip Pongcharoen[3] and Phanchana Sanguansermsri[2]

[1]Veterinary Research and Development Center (Lower Northern Region), Department of Livestock Development, Phitsanulok; [2]Faculty of Medical Science, Naresuan University; [3]Faculty of Medicine, Naresuan University, Phitsanulok, Thailand

Abstract. Ten specific pathogen free (SPF) chickens were inoculated intranasally with avian influenza virus subtype H5N1. Evaluation revealed distribution of the virus in twelve organs: liver, intestine, bursa, lung, trachea, thymus, heart, pancreas, brain, spleen, kidney, and esophagus. Immunohistochemistry (IHC), chromogenic *in situ* hybridization (CISH), and real-time polymerase chain reaction (PCR) were developed and compared for detection of the virus from the organs. The distribution of avian influenza H5N1 in chickens varied by animal and detecting technique. The heart, kidneys, intestines, lungs, and pancreas were positive with all three techniques, while the others varied by techique. The three techniques can be used to detect avian influenza effectively, but the pros and cons of each technique need to be determined. The decision of which technique to use depends on the objective of the examination, budget, type and quality of samples, laboratory facilities and technician skills.

Keywords: H5N1, avian influenza, distribution, chickens

INTRODUCTION

Avian influenza virus (AIV) subtype H5N1 is a highly pathogenic avian influenza (HPAI). HPAI contains polybasic amino acids at a cleavage site for hemagglutinin glycoprotein (HA); the HAs are cleaved intracellularly by ubiquitously occurring proteases and therefore have the capacity to infect various cell types and cause systemic infection (Stieneke-Grober *et al*, 1992; Steinhauer, 1999). HPAI can kill 90-100% of a poultry flock. Epidemics of HPAI can spread rapidly, devastating the poultry industry and resulting in severe trade restrictions (Iowa State University, 2010). Infected animals die suddenly with distribution of the virus throughout various organs. The diagnosis of the H5N1 virus is based on a combination of clinical signs, gross pathology and laboratory results (OIE, 2009). Real-time polymerase chain reaction (PCR) is normally used

Correspondence: Phanchana Sanguansermsri, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand.
Tel: 66 (0) 89708 8384; Fax: 66 (0) 5596 4770
E-mail: phanchana@hotmail.com

for rapid and accurate results and can be a good quantitative test. Because of the expensiveness of real-time PCR machines, other tests, such as immunohistochemistry (IHC) and chromogenic *in situ* hybridization (CISH) are used instead.

Real-time PCR has been applied effectively to diagnose clinical samples in less than 3 hours (OIE, 2009). Many researchers have used real-time PCR to detect avian influenza virus, especially subtype H5N1 (Spackman *et al*, 2002; Lee and Suarez, 2004; Payungporn *et al*, 2006; Chen *et al*, 2007; Gu *et al*, 2007; Lu *et al*, 2008; Kalthoff *et al*, 2008; Spackman and Suarez, 2008; Witoonsatian *et al*, 2008; Wu *et al*, 2008). IHC has also been used to detect avian influenza virus antigen (Rimmelzwaan *et al*, 2001; Gu *et al*, 2007; Kalthoff *et al*, 2008; Chen *et al*, 2009) comparing the results with hematoxylin and eosin staining on histopathology sections (Kuiken *et al*, 2003; Perkins and Swayne, 2003; Klopfleisch *et al*, 2006, 2007; Teifke *et al*, 2007; Vascellari *et al*, 2007). CISH has become used more often to diagnose H5N1 because molecular studies have become more popular (Gu *et al*, 2007; Vascellari *et al*, 2007; Chen *et al*, 2009).

We studied the detection of avian influenza virus in the organs of experimentally infected chickens, comparing IHC, CISH, and real-time PCR, to determine the most appropriate technique and the best to submit for laboratory detection.

MATERIALS AND METHODS

Animal inoculation

Ten specific pathogen free (SPF) chickens were inoculated intranasally with $10^{4.5}$ EID_{50} 100 l A/duck/Phitsanulok/NIAH6-5-0001/2007(H5N1) avian influenza virus. The experiment was done in an animal biosafety level 3 laboratory at the National Institute of Animal Health, Department of Livestock Development, Bangkok, Thailand. The chickens were monitored for clinical signs of infection. Dead chickens were examined for pathologic lesions. Organs examined were the livers, intestines, bursa, lungs, trachea, thymus, heart, pancreas, brain, spleen, kidneys, and esophagus. They were collected and kept at 4ºC or -80ºC for real-time PCR and fixed in 10% buffered formalin for IHC and CISH examination.

Immunohistochemistry (IHC)

Monoclonal mouse anti-influenza NP was used to detect the primary antibody and EnVision™+/HRP, mouse (K-4001) DAKO was used to detect the secondary antibody.

Preparation and pretreatment of tissue samples. Formalin fixed paraffin embedded (FFPE) tissue sections were sliced at 3-4 microns thickness and placed on 3-amino-propyltriethoxysilane treated slides. The sections were dried at 25ºC and processed by histopathological techniques. In brief, the sections were heated to 43ºC, deparaffinized with xylene for 5 minutes twice, rehydrated with 100% ethanol for 5 minutes twice, soaked in methanol H_2O_2 for 30 minutes, and dipped in 70% ethanol. Then they were washed with distilled water (DW) for 5 minutes and kept at -20ºC until further study.

Proteinase K pretreatment. The tissue slides were incubated at 37ºC with 0.05% proteinase K for 15 minutes; then, the slides were rinsed with phosphate buffered saline (PBS) for 5 minutes twice and dried at room temperature (RT).

Blocking of nonspecific reaction. Five percent skim milk in PBS was added to the slides and incubated at 37ºC for 30 minutes, then rinsed with PBS.

Immunohistochemical step. The tissue slides with AIV, non-AIV, and unknown samples were soaked with monoclonal mouse anti-influenza NP diluted 1:2,000 in 1% bovine serum albumin (BSA) at 4ºC overnight and washed with PBS for 5 minutes 3 times. Secondary antibody from DAKO EnVision (K-4001) was added to the slides for 30 minutes and washed with PBS for 5 minutes 3 times and dipped in DW twice. 3-amino-9-ethylcarbazole (AEC) was added on the slides as substrate for 3 minutes. The slides were dipped in DW twice, counterstained with hematoxylin for 20 seconds, soaked in tap water for 10 minutes, and dipped in DW; then, the slides were mounted with glycergel, covered with cover glasses, and examined under a light microscope.

Chromogenic *in situ* hybridization

Preparation and pretreatment of tissue samples. The tissue samples for CISH were prepared in the same way as the IHC samples.

Proteinase K pretreatment. The tissue slides were incubated at 37ºC for 15 minutes in a solution containing 50 g/ml proteinase K in proteinase K buffer (Na-EDTA 18.61 g, Tris HCl 15.76 g in 1 liter DW, pH 8.0); then, the slides were rinsed with DW for 5 minutes and dried at room temperature (RT).

Endogenous enzyme pretreatment. Endogenous alkaline phosphatase in the proteinase K treated samples was eliminated with 20% cool acetic acid for 20 seconds; then, the slides were rinsed with DW for 5 minutes and dried at RT.

Preparation of probe. A 756 base dUTP labeled with digoxigenin by PCR digoxigenin labeling mix (Roche Diagnostics, Mannheim, Germany) was used for probe preparation. Primers were from avian influenza H5, diagnosed by PCR technique at the Veterinary Research and Development Center (Lower Northern Region), Thailand. The forward and reverse primers were 5′ ACA ATA ATA CCA ACC AAG AAG AT-3′, and 5′ CTT CCA TCT TCT TGT TTA AAT TT-3′ respectively. The H5 hemagglutinin gene from A/chicken/Phichit/NIAH6-4-001/2006 (H5N1) cloned in pHW 2000 was used as the template.

***In situ* hybridization step.** The tissue slides of AIV, non-AIV, and unknown samples were denatured by adding 100-200 l/slide of hybridization buffer, placed on a hot plate at 90ºC for 10 minutes, and then on ice for 10 minutes.

Twenty microliters of probe was added to each slice before the cover glass was placed. Hybridization was conducted at 37ºC overnight in a moisture chamber.

The slides were soaked in 2x saline sodium citrate (SSC) at RT for 15 minutes then at 37ºC for 5 minutes, followed by soaking in 1xSSC at 37ºC for 5 minutes, 0.5xSSC 37ºC for 1 hour, and DW for 5 minutes. Nonspecific binding of endogenous digoxigenin was blocked by adding 1% skim milk and incubating at RT for 1 hour in a moisture chamber. The slides were submerged in tris buffered saline (TBS) at RT for 5 minutes, the anti-digoxigenin conjugated alkaline phosphatase (Roche Diagnostics) was added at 100 l/slide, the incubated in a moisture chamber at RT for 1 hour, then submerged in TBS for 5 minutes 3 times. Nitroblue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate was added to the slides and they were kept in the dark at RT for 20 minutes. The reaction was stopped by submerging in detecting buffer at RT for 10 minutes, counterstained with nuclear fast red for 10 minutes and rinsed with detecting buffer at RT for 5 minutes. Permount and a cover

glass were then added and the slides were then examined under a light microscope.

Real-time polymerase chain reaction (Real-time PCR)

Real-time PCR was used to detect the viral load for the H5N1 virus in organ samples of chickens. Viral and structural protein RNA was extracted from the tissues using RNeasy Mini kit (Qiagen Hilden, Germany). Complementary deoxyribonucleic acid (cDNA) was prepared using a M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA) with Uni-12 random primer. A LightCycler 480® Instrument was used to process the test and a Lightcycler® 480 SYBR Green I Master (Roche) was used for amplifying the H5 hemagglutinin gene and structural proteins or house keeping genes. Glyceraldehydes-3-phosphate dehydrogenase enzyme, the main enzyme in glycolysis, was used for house keeping genes in the examination. All methods were performed as described by the manufacturers. The forward and reverse primers for detection of RNA from the house keeping genes were 5′ ACT ATC TTC CAG GAG CGT GAC-3′ and 5′ AGC ACC ACC CTT CAG ATG AGC-3′, respectively. The forward and reverse primers for detection of H5 avian influenza virus RNA were 5′ GAA TGG TAG ATG GTT GGT ATG G-3′ and 5′ GTT GAC CTT ATT GGT GAC TCC-3′, respectively. The primers were designed for routine use at the Veterinary Research and Development Center (Lower Northern Region), Thailand.

RESULTS

Clinical signs

One chicken which died suddenly after viral inoculation was not included in this examination. All nine chickens died within 24 hours post-inoculation.

Gross pathology

Lesions from the nine chicken carcasses inoculated with avian influenza viruses were very similar. Gross pathology showed widespread hemorrhages and edema as the predominant lesions. The lesions were swollen kidneys, lung hemorrhages, a swollen and hemorrhagic pancreas, cardiac hemorrhages and swollen and hemorrhagic thymus.

IHC

Distribution of avian influenza virus by IHC showed virus proteins were strongly positive in the lungs, spleen, heart, and kidneys. All nine chickens had +3 positive staining in the lungs. Five and four of the nine chickens had +3 staining in the spleen and heart, respectively. The other organs had +1 and +2 staining. Avian influenza virus was found in every organ of the animals (Table 1).

CISH

Positive signals with the CISH technique were inconsistent and variable. The heart, intestines, pancreas, and kidneys had +1 to +2 signals in eight, five, five and four of the nine chickens, respectively. Only three chickens had a +3 signal from lungs. Many of the organs of the nine infected chickens were negative with the CISH technique. The distribution of the avian influenza virus in each organ is shown in Table 1.

Real-time PCR

The heart, kidneys, intestines, and lungs were organs found positive with real-time PCR. The method used to obtain the results was an absolute quantification program which was normalized with the house keeping genes using the program in the LightCycler 480® real-time PCR Instrument. Distribution of avian influenza virus in each organ by real-time PCR is shown in Table 1.

Table 1
Distribution of avian influenza virus in organs of nine infected chickens examined by IHC, CISH and real-time PCR.

No.	Organ	Method	Distribution of AIV in organs of nine chickens									
			1	2	3	4	5	6	7	8	9	Average
1	Liver	IHC	+1	+1	+2	+2	+1	+1	+2	+1	+2	+1
		CISH	+1	[a]	-	-	-	-	[a]	-	+1	-
		PCR[b]	$2.7x10^4$	[a]	$1.2x10^4$	$6.1x10^4$	$2.2x10^5$	$2.3x10^5$	$3.5x10^4$	$5.7x10^5$	$7.2x10^3$	$1.3x10^5$
2	Intestines	IHC	+1	+2	+1	+1	+1	+1	+1	+1	+1	+1
		CISH	-	-	-	+1	+1	+1	+1	+1	-	+1
		PCR	$7.5x10^5$	$7.3x10^4$	$3.4x10^5$	$2.4x10^5$	$5.1x10^5$	$4.6x10^5$	$8.8x10^5$	$2.2x10^5$	$7.0x10^5$	$4.6x10^5$
3	Bursa	IHC	+2	+1	[a]	+2	+1	+1	+1	+1	+1	+1
		CISH	-	-	-	-	-	-	-	-	-	-
		PCR	$2.7x10^4$	$1.8x10^5$	$1.5x10^5$	$1.3x10^5$	$6.8x10^4$	$7.5x10^4$	$4.6x10^4$	$7.3x10^4$	$4.9x10^5$	$1.4x10^5$
4	Lungs	IHC	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3
		CISH	-	+3	-	+3	-	-	-	+3	-	+1
		PCR	$1.9x10^5$	$2.7x10^5$	$4.5x10^5$	$1.7x10^5$	$3.3x10^5$	$2.1x10^5$	$2.6x10^5$	$4.0x10^5$	$5.0x10^5$	$2.6x10^5$
5	Trachea	IHC	+1	+1	[a]	+1	+1	+1	+1	+1	-	+1
		CISH	-	-	-	-	-	-	-	-	-	-
		PCR	$3.0x10^4$	$2.8x10^4$	$9.8x10^4$	$5.7x10^5$	$1.1x10^5$	$1.1x10^5$	$3.1x10^4$	$4.4x10^4$	$8.7x10^4$	$1.2x10^5$
6	Thymus	IHC	+2	+2	+1	+1	+2	+1	+1	+2	+1	+1
		CISH	-	-	-	-	-	-	-	+1	-	-
		PCR	$1.1x10^5$	$1.2x10^5$	$6.8x10^4$	$1.3x10^5$	$9.9x10^4$	$5.3x10^4$	$1.8x10^5$	$4.7x10^4$	$2.0x10^5$	$1.1x10^5$
7	Heart	IHC	+2	+3	+3	+2	+2	+2	+3	+3	+2	+2
		CISH	+1	+1	+1	+1	+2	+1	+1	-	+2	+1
		PCR	$3.4x10^5$	$2.4x10^6$	$1.4x10^6$	$9.2x10^6$	$1.2x10^7$	$6.3x10^5$	$6.5x10^5$	$4.1x10^5$	$2.2x10^6$	$3.3x10^6$
8	Pancreas	IHC	+1	+2	[a]	+1	+1	+1	+2	+1	+1	+1
		CISH	-	+1	-	+1	-	+1	+1	+1	-	+1
		PCR	$3.1x10^4$	$8.2x10^3$	$3.5x10^4$	$1.8x10^3$	$8.2x10^4$	$1.0x10^5$	$2.3x10^5$	$1.5x10^3$	$5.3x10^4$	$6.1x10^4$
9	Brain	IHC	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
		CISH	-	-	-	-	-	-	-	-	-	-
		PCR	$1.0x10^4$	$1.3x10^4$	$4.4x10^3$	$9.4x10^3$	$1.2x10^4$	$1.4x10^4$	$1.4x10^4$	$1.5x10^2$	$1.9x10^4$	$1.1x10^4$
10	Spleen	IHC	+3	+3	+3	+2	+2	+2	+3	+3	[a]	+3
		CISH	-	+1	-	-	-	-	-	-	-	-
		PCR	$4.5x10^3$	$5.5x10^3$	$1.2x10^4$	$2.8x10^2$	$6.6x10^3$	$2.1x10^3$	$4.3x10^2$	$4.6x10^3$	$5.6x10^2$	$4.0x10^4$
11	Kidneys	IHC	+2	+1	+1	+2	+1	[a]	+2	+1	+2	+2
		CISH	+2	[a]	-	+1	-	+1	[a]	-	+2	+1
		PCR	$2.8x10^4$	$1.1x10^6$	$2.3x10^6$	$1.2x10^6$	$1.8x10^5$	$2.4x10^6$	$3.4x10^5$	$5.1x10^4$	$7.1x10^5$	$9.1x10^5$
12	Esophagus	IHC	+2	+1	[a]	+1	+1	+1	+1	+1	+1	+1
		CISH	-	-	-	-	-	-	-	-	-	-
		PCR	$1.5x10^4$	$1.5x10^4$	$2.6x10^4$	$6.3x10^4$	$2.5x10^4$	$2.1x10^4$	$1.3x10^4$	$1.6x10^4$	$7.3x10^4$	$3.0x10^4$

[a]no result; [b]virus particles; - , none
+1 , few (1-20 positive signals); +2, moderate numbers (21-40 positive signals)
+3 , numerous (>40 positive signals)

Table 2
Distribution of avian influenza virus in organs from the averages of 9 infected chickens examined by IHC, CISH and real-time PCR.

No.	Organs	IHC	CISH	Real-time PCR[a]	
1	Liver	+1	-	$1.3x10^5$	(2%)
2	Intestines	+1	+1	$4.6x10^5$	(8%)
3	Bursa	+1	-	$1.4x10^5$	(2%)
4	Lungs	+3	+1	$2.6x10^5$	(5%)
5	Trachea	+1	-	$1.2x10^5$	(2%)
6	Thymus	+1	-	$1.1x10^5$	(2%)
7	Heart	+2	+1	$3.3x10^6$	(59%)
8	Pancreas	+1	+1	$6.1x10^4$	(1%)
9	Brain	+1	-	$1.1x10^4$	(0%)
10	Spleen	+3	-	$4.0x10^4$	(1%)
11	Kidneys	+2	+1	$9.1x10^5$	(17%)
12	Esophagus	+1	-	$3.0x10^4$	(1%)

[a]virus particles; - , none ; +1 , few (1-20 positive signals)
+2 , moderate numbers (21-40 positive signals); +3, numerous (>40 positive signals)

The summary of the distribution of avian influenza virus in the nine infected chickens using IHC, CISH, and real-time PCR is shown in Table 2.

DISCUSSION

The distribution of avian influenza virus in the organs of the nine experimentally infected chickens varied by animal and evaluation technique. IHC, CISH and real-time PCR detect avian influenza effectively; CISH had a lower sensitivity in this study. The three techniques detect different parts of the virus. The IHC technique detects nucleoproteins of the viruses in cells; the CISH and real-time PCR techniques both detected viral RNA in cells but the real-time PCR includes amplifying and detecting house keeping gene processes. These different techniques lead to varying results. The heart, kidneys, intestines, lungs, and pancreas were positive with all three techniques.

These results are similar to those of Antarasena *et al* (2006), who evaluated avian influenza in chickens using an indirect fluorescent antibody (IFA) test and the organs most involved were the heart, lungs, intestines, and kidneys. Klopfleisch *et al* (2006) evaluated avian influenza virus using IHC in experimentally infected chickens and found the pancreas and thymus had a +2 level of infections and the lungs, intestines, adrenals, heart, liver, kidneys and spleen had a +1 level of infection. Vascellari *et al* (2007) using IHC and CISH found the pancreas and brain in infected Pekin ducks. Teifke *et al* (2007) found the avian influenza virus nucleoprotein in the pancreas, adrenals, liver and brain of naturally infected swans. Caroline *et al* (2009) found avian influenza antigens using IHC and PCR in the brain, pancreas, and upper respiratory tract of infected wild tufted ducks, while the influenza antigen was variably found in the liver, lungs, adrenals, kidneys and pe-

ripheral nerve ganglia. Londt *et al* (2008) inoculated Peking ducks experimentally with avian influenza virus and found that the highest viral loads in the heart and brain using IHC and real-time PCR.

There are many factors that could cause the variable results in distribution, such as the differentiation of the viruses, the infecting dose of the virus, the route of transmission, host sensitivity, variability among the individual animals, processing techniques and the reagents for the tests.

Our study found the distribution of avian influenza H5N1 in chickens varied by individual animal and detection technique. The heart, kidneys, intestines, lungs, and pancreas were positive with all three techniques, but the other organs had varying results. IHC is suitable for a routine avian influenza diagnostic laboratory because it does not need any sophisticated equipment or skills. CISH is used to detect nucleic acids in cells. The nucleic acids need to be abundant enough to detected the signals (Bracht, 2009). There are no amplifying processes with the CISH technique so it is likely to be a less sensitive test. CISH also needs additional molecular facilities which are more expensive. Real-time PCR is a rapid, quantitative, highly sensitive and specific test and more beneficial for outbreak control. The results are normalized with the house keeping genes, making it more accurate. However, real-time PCR needs expensive machines and well trained technicians.

In conclusion, the three techniques can be used to detect avian influenza effectively but each technique has pros and cons. The final decision of the technique employed needs to be based on the objective of the examination, budget, type and quality of samples, laboratory facilities and technician skills at the laboratory.

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