建立新疫苗之動物實驗模式平台
Establishment of Animal Models Used for Vaccine Development

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研究成果報告

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摘要

97 年度我們已進行多項重要新型流感動物攻毒模式之建立及效力評估，包括：1.建立新型流感病毒 H5N1 於小鼠之攻毒平台；2.兔子動物模式之建立；3.雪貂動物模式之建立; 4. 抗 H5N1 病毒單源抗體之製備。H5N1 病毒已完成兩株病毒（A/Duck/China/E319-2/2003；A/VN/2004）之對小鼠 LD_{50} 測試，顯示兩株病毒具不同之毒力，其中 A/VN/2004 為強毒株，其 LD_{50} 約為稀釋至 10^{-4}，而 A/Duck/China/E319-2/2003 病毒對哺乳類動物為弱毒株，LD_{50} 約為 $10^{7.7}ELD_{50}$。完成之小鼠攻毒模式於 97 年 1-10 月已協助疾管局、中研院、國衛院等三單位進行 12 次新型流感疫苗效力試驗。而雪貂動物模式因實驗用雪貂受限於進口檢疫規定延至 11 月 14 日才得進口，但我們已先行利用 4 隻寵物雪貂完成 H5N1 病毒抗體檢測及病毒感染後之病毒分佈，臨床症狀及死亡率分析等技術之建立，未來可做為新疫苗測試模式。另關於抗 H5N1 單源抗體之製備，我們已完成 16 株單源抗體篩選，包括抗 HA、NA、NP 及 NS1 細胞株。其中抗 HA 抗體 YY1、33A 及 Y5 具病毒中和活性，而 YY1 與 33A 具雞紅血球凝集抑制活性。我們已完成使用 Baculovirus 表現各基因蛋白包括 HA、NA、NP 及 NS1 做為單源抗體專一性確認標準。

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關鍵詞：家禽流行性感冒；高病原性禽流感； 次單位疫苗； 動物模式；單源抗體

Abstract

In 2008, we have performed several important animal models for the development of avian influenza H5N1 vaccines, including: (1) the virus challenge model of avian influenza in mice; (2) to establish rabbit animal model for vaccine study; (3) to establish the ferret model for determining the vaccine efficacy following virus challenge; (4) to produce the monoclonal antibodies against H5N1 virus. We have finished the test of viral LD_{50} of two virus isolates in mouse models, indicating that A/Duck/China/E319-2/2003 and A/VN/2004 viruses contained various levels of virulence in mice. The A/VN/2004 virus is a highly virulent strain in mice. The LD_{50} was tested to be 10^{-4} dilution. However, the A/Dk/CHN/E319-2/2003 virus was a low virulent strain for mice with LD_{50} equal to $10^{7.7}$ EID_{50}. The mouse model has been used to help three units, including CDC, NHRI and Academia Sinica, determining vaccine efficacy. On the establishment of ferret model,

due to the limitation of ferrets imported from USA requiring the quarantine procedure, we have using 4 pet ferrets to practice the handling techniques and to characterize viral characteristics following infection. The experimental level of ferrets will be transported to this institute at Novenber 14th. For the production of monoclonal antibodies against H5N1 virus, we have obtained 16 strains of hybridoma, which secret antibodies against the HA, NA, NP and NS1 proteins. The antibodies such as YY1, Y5 and 33A contained activity to neutralize H5N1 virus; and the YY1 and 33A contained activity to inhibit hemaglutinin for chicken RBC. The identification of monoclonal antibodies recognizing viral components were determined by using proteins expressed in baculovirus system.

Keywords: Avian influenza; Highly pathogenic avian influenza; Subunit vaccine; Animal model; Monoclonal antibodies

緒言

水禽主要為鴨、海鷗及其他水鳥被認為是 A 型流感病毒的天然保毒者(Webster et al.,1992; Horimoto and Kawaoka,2001)，病毒通常已適應於這些宿主並隨著宿主演化，通常病毒於水禽身上增殖是受限的不會引起典型的臨床症狀。一般也没有品系間之傳播現象發生,但 H5N1 亞型病毒卻有能力直接感染家禽類且偶而會感染人類引起嚴重而致命的呼吸道疾病。自 1997 年起，H5N1 病毒首先在香港人類引起致命的感染，而後在中國、泰國、越南、印尼、寮國、土耳其、埃及、伊拉克等多國連續引起爆發感染 (Claas et al.,1998; Subbarao et al.,1998; Viseshakul et al.,2004; CDC,2006)。分子特徵顯示人類分離株的所有 8 段基因都是禽類來源者，顯示人類來源的 H5N1 病毒可能是直接從禽類傳染者 (Subbarao et al., 1998; Suarez et al., 1998; Viseshakul et al.,2004)。從 2003 年起 H5N1 病毒已連續在亞洲禽類中循環並建立起常在性感染 (Chen et al., 2005; Chen et al., 2006)，演化成不同的 H5N1 病毒並經由禽類運輸及候鳥遷移在歐洲及非洲國家擴大傳播到禽類及人類(WHO,2007)引起巨大的經濟損失及威脅人類生命。雖然 H5N1 病毒尚未在台灣發現，但一株 H5N1 病毒 (A/Duck/China/E319-2/2003) 在 2003 年已被從走私之紅面番鴨分離出。我們在這實驗中使用雞及小鼠之動物模式決定分子特徵及評估病毒之病原性(Lee et al., 2007)。

自 1997 年起，H5N1 病毒已在人類引起超過 200 人以上致命感染 (WHO,2007)。在 2003 年之前，H5N1 病毒的爆發感染只限於亞洲國家如中國、印尼、泰國及越南。那時以疫苗政策控制疫情是被禁止的或認為是無法接受的風險。後來病毒活性增加並透過候鳥或家禽運輸從亞洲散播到歐洲及非洲(Chen et al., 2004; Guan et al., 2002; Webster et al., 2002)。這種不斷增加的風險已隨候鳥每年遷移而改變，因

此使用疫苗政策控制不斷增加的爆發感染變成可能的選項。目前已有中國、越南及印尼使用疫苗控制家禽疫情。而商業上可買到的疫苗目前只有 H5 及 H7 病毒製成之不活化疫苗（Capua et al., 2003; Liu et al., 2003; Ellis et al., 2004)。但以 H5N1 病毒製造人用或動物用之不活化疫苗對人及禽類有較高之病原性且病毒需於 BSL-3 實驗室操作，有增加散佈之危險性。因此次單位疫苗由 H5N2、H5N3、H1、H2、H3、H7 及 H9 病毒之 HA 抗原製成者過去也都經評估過（Crawford et al.,1999; Lu et al.,2001)。這些研究顯示 HA 抗原不論是製成 DNA-plasmid 或 HA 次單位疫苗都可引起極好的保護效果抵抗病毒的攻擊致病。另外，次單位疫苗最大之好處是可用診斷試劑與田間感染者區分（Capua et al.,2003; Lee et al.,2004; Liu et al.,2003)。因此次單位疫苗的開發也許可用於控制爆發感染，用於限制病毒的持續排毒，減少經濟損失。

目前先進國家不論人用或動物用 H5N1 疫苗之研發都已被列為國家之最優先政策。但 H5N1 新型流感疫苗之研發涉及動物攻毒實驗，其實驗室之規格相當高。本所因擁有合格之 BSL-3 攻毒實驗室及訓練良好之操作人員，因此適合進行禽流感 H5N1 病毒之攻毒試驗。本計畫主要為建立各種動物模式平台協助人用新型流感疫苗之開發。

材料與方法

一、小鼠動物模式之建立：

主要係在Balb/c品系進行，一般向國科會實驗動物中心購買4-5週齡小鼠。測試病毒毒力係將病毒以MEM培養液稀釋從10^{-1}~10^{-7}，每稀釋階8隻以每隻100 µl 從鼻腔滴入感染，小鼠需先行以Ether麻醉，感染後之小鼠每日進行體溫及體重量測，並記錄死亡率。每隻小鼠並觀察發病後之臨床症狀。本試驗於本所之BSL-3實驗室Isolator內進行。

二、病毒來源及特性分析：

攻毒用H5N1病毒係採用本所2003年之金門分離株A/DK/CHN/E319-2/2003及中央研究院基因體中心自香港實驗室引入之病毒株包括：A/VN/2004、A/HK/1997、A/HK/2003病毒。輸入之病毒係由疾管局以MDCK細胞增殖後再分讓至本所，其中A/DK/CHN/E319-2/2003病毒特徵已發表於期刊(Vet. Microbiology, 20007)。這些病毒分別存放於BSL-3實驗室作為Balb/c小鼠及雪貂測試病毒之LD50劑量用。

三、雪貂動物模式之建立：

由於雪貂感染流感病毒及禽流感病毒後呈現與人類感染相似的臨床呼吸

道症狀並引起死亡率，已被認為是新型流感病毒研究最具代表性之哺乳類動物模式（Belser et al., 2007；van Riel et al., 2007；Suguitan et al., 2006）。因此本計畫擬進口20隻實驗用等級之雪貂供流感疫苗研發。另已於尚未進口前先行採購4隻寵物用雪貂做為實驗操作技術之建立。雪貂之麻醉可用Ketamine及Telazol共同注射（0.2 ml）。雪貂之H5N1病毒攻毒需於BSL-3實驗室內進行並飼養於具負壓系統之Isolator內觀察。攻毒用H5N1病毒係採用A/DK/CHN/E319-2/2003及A/VN/2004病毒株。攻毒後測試病理變化如：血球數之變化及病毒於呼吸道內洗出液之病毒含量。另感染後2及4天之雪貂各臟器亦作成10倍乳劑於SPF雞胚胎中測試病毒含量。

四、單源抗體之製備：

本所過去已使用A/DK/CHN/E319-2/2003病毒在Balb/c小鼠測試LD_{50}及做各種病原性實驗。因此使用感染後恢復之小鼠脾臟細胞與Myeloma細胞融合並選殖可分泌抗H5N1抗體之融合瘤細胞。至於可分泌抗體之融合瘤細胞係採用間接螢光法篩選，於96well細胞培養盤中培養MDCK細胞並感染約200 $TCID_{50}$之H5N1病毒16小時，做為抗原盤，將融合瘤細胞培養上清液加入作用1小時後再以FITC標示之抗鼠IgG作用檢測特異螢光。

結果

一、家衛所BSL-3實驗室之建立：

家衛所自 2003 年因 SARS 爆發感染，承接國科會計畫建立生物安全等級第三級（Bio-safety level 3；BSL-3）實驗室以供 SARS 疫苗研發，已建構完成一間 BSL-3 實驗室(Fig. 1)，實驗室具衡定之負壓系統、進口之 Isolator 及完全保護之實驗人員防護系統，適合國內非人類靈長類動物之攻毒實驗，並已於 2005 年通過衛生署疾病管制局之查驗，成為國內兩個可從事第三級病原之攻毒實驗室。此段時間本所的實驗人員亦在國內及國外接受第三級實驗室之專業訓練，並真正嘗試以第三級病原如禽流感(H5N1)及呼吸道病原如新城病病毒（Newcastle disease virus；NDV）演練與測試，其目的可了解呼吸道病毒之感染特徵與散播途徑，並可承擔第三級病原之實驗工作。由於 BSL-3 實驗室之建立除用於本所之開發研究外並協助人用疫苗之研發效力實驗。

二、小鼠攻毒模式之建立：

使用4-6週齡之小鼠建立對H5N1病毒之攻毒模式，結果顯示：A/DK/CHN/E319-2/2003病毒之LD_{50}約為$10^{7.7}$ ELD_{50}，感染後各臟器經用雞胚胎分離病毒後証實病毒只存在呼吸道系統包括肺與氣管，而於其他之臟器包括腎、肝、心、脾、腦及胰臟則無法檢出活病毒(Table 1)。另感染發病之小鼠呈現

呼吸急促、背部聳立、體重急速下降及體溫下降等臨床症狀，一般感染之小鼠若於第3-5天體溫恢復則可耐過感染，若體溫降至30℃以下則最後會死亡(Fig. 2)。而體重方面於感染後3-6天呈現最低，耐過者可慢慢恢復，體重持續下降者最後亦死亡(Fig. 3)。另A/VN/2004病毒在小鼠之LD_{50}約為稀釋10^{-4}，顯示病毒對小鼠具較高之病原性與致死率。而香港輸入之A/HK/1997及A/HK/2003病毒經疾病管制局增殖後分讓本所，其病毒之HA力價分別為128及64倍，但在小鼠攻毒二次皆不具任何致病性及致死率(Table 2 and 3)。可能原因為病毒在細胞長期繼代導致或力價不足。

小鼠動物模式建立後自97年1-10月已協助衛生署疾病管制局、中央研究院基因体中心及國家衛生研究院進行總共12次新型流感疫苗效力試驗，其效力測試結果因部分單位未提供相關免疫資訊，因此僅顯示疾管局之三次攻毒結果（Table 4, 5, 6)。

三、雪貂攻毒模式之建立：

由於實驗用雪貂需自美國進口，其採購進度受限於本國動植物防疫檢疫局之檢疫規定需至97年11月14日動物才會運底家畜衛生試驗所，並需進行約1個月之檢疫及建立相關之生理資料。因此於5-8月期間我們已採購4隻6-7月齡實驗用雪貂進行生理條件之建立及技術之操作練習(Fig. 4)。正常雪貂體溫未受病毒感染時維持於36.0-38.9℃，體重於6-7月齡時約540-860克重，血清經用H5N1病毒檢測結果HI值皆≦1:2。而雪貂感染$10^{9.5}$ ELD_{50} H5N1病毒（A/DK/CHN/E319-2/2003）後死亡率約為25%（1/4)。感染之雪貂呈現活動力降低、體溫可上昇至41℃維持約2天後又恢復正常(Fig. 5)，而死亡發生於体溫上昇期間，至於體重於感染後也呈現明顯下降約降低40-80克重。感染之雪貂病毒分佈也局限於呼吸道系統包括肺及氣管(Data not shown)。

四、抗H5N1病毒單源抗體之開發：

使用受A/DK/CHN/E319-2/2003病毒感染恢復之Balb/c小鼠開發單源抗體，我們已獲得16株融合細胞株可分泌包括：抗HA，NA，NP及NS1之抗體(Fig. 6)。抗HA抗體包括YY1及33A甚至有高力價之病毒中和活性及HI活性，適合做為標準診斷用抗體或開發成診斷試劑(Table 7)。此兩抗體使用dot blotting分析甚至可與大部分之H5N1、H5N2及H5N3病毒作用，而Y5抗HA抗體則只專一性與H5N1病毒作用(Fig. 7)，具有病毒中和活性但不具HI活性。其他抗NA、NP及NS1抗體目前尚未完成定性。至於單源抗體對病毒蛋白之位置確認係用Baculovirus表現之各別蛋白進行測試。

討論

人用新型流感（H5N1）疫苗之開發常使用之動物模式包括哺乳類動物之小鼠、雪貂及獼猴。小鼠動物模式由於容易操作且實驗期程短因此較常被使用。然因雪貂受H5N1病毒感染後呈現與人類感染相同之臨床症狀，因此被認為更適合作為人用疫苗開發之動物模式。

本計畫已建立完成小鼠及雪貂之攻毒模式。其中小鼠模式自97年1月開始已協助國內人用新型流感疫苗研發之效力測試。目前可使用之病毒包括有A/DK/CHN/E319-2/2003及A/VN/2004兩株病毒。而A/HK/1997及A/HK/2003兩株病毒雖含有對雞紅血球HA之活性及對MDCK細胞之感染性，但已先後兩次於小鼠測試皆不見任何致死力價。推測可能之原因為兩株病毒可能於細胞中長期繼代導致，或其他不知原因。因此兩株香港分離株H5N1病毒不適合作為攻毒之病毒株。

至於A/DK/CHN/E319-2/2003及A/VN/2004病毒於小鼠呈現之病原性顯然是有差異的，例如A/VN/2004病毒之LD_{50}約為稀釋10^{-4}其病毒力價＜10^{4} $TCID_{50}$以下，顯示應為對哺乳類之強毒株。而A/DK/CHN/E319-2/2003病毒對小鼠之LD_{50}為$10^{7.7}$ EID_{50}，呈現對哺乳類之低致死率及病原性，但仍維持對家禽之極高病原性。這些結果加上2003-2005年間因為SARS爆發感染所建立之ＢＳＬ-３實驗室極適合協助國內人用或動物用疫苗研發單位進行新藥研發之動物攻毒試驗。

而雪貂攻毒試驗本年度本所亦已建立，由於雪貂之動物取得不易且價格昂貴，因此進口受拖延。但採用寵物雪貂檢測其對H5N1病毒之HI抗體力價均呈現陰性，適合作為初步之感染特性分析，結果顯示雪貂對H5N1病（A/DK/CHN/E319-2/2003）只達25%之死亡率，因此A/DK/CHN/E319-2/2003病毒於雪貂模式不適合以LD_{50}做為攻毒之劑量標準。是否A/VN/2004病毒強毒株雪貂可有較高之死亡率需待進口之實驗用雪貂測試後才能了解。若兩株病毒對雪貂之致死率皆不達100%則未來攻毒之標準擬採用呼吸道之沖洗液之病毒含有量減少作為疫苗開發之效力標準。

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Table 1. Virus titers in mouse and chicken tissues infected by A/Dk/CHN/E319-2/2003 virus

Dose(ELD_{50}) Per animal	Infected animal	Mortality	Average tissue titer(ELD_{50}/g of tissue)								
			Trachea	Lung	Kidney	Liver	Heart	Bursa	Spleen	Brain	Pancreas
$10^{8.6}$	Mouse	8/8	$10^{7.0}$	$10^{7.7}$	-	-	-	ND	-	-	ND
$10^{7.9}$	Mouse	8/8	$10^{7.0}$	$10^{5.5}$	-	-	-	ND	-	-	ND
$10^{7.6}$	Mouse	3/8	$10^{5.5}$	$10^{4.7}$	-	-	-	ND	-	-	ND
$10^{6.6}$	Mouse	0/8	$10^{4.4}$	$10^{5.5}$	-	-	-	ND	-	-	ND
$10^{5.6}$	Mouse	0/8	$10^{4.4}$	$10^{4.7}$	-	-	-	ND	-	-	ND
$10^{7.6}$	Chicken	5/5	$10^{7.8}$	$10^{6.5}$	$10^{4.7}$	$10^{5.5}$	$10^{7.5}$	$10^{6.5}$	$10^{5.7}$	$10^{6.7}$	$10^{4.7}$

ND=not done

“-”indicates no virus titers

Table 2. The LD_{50} test of three AI viruses imported from Hong Kong

Strain / Titer	mortality		
	A/HK/97-104207	A/HK/03-27173	A/VN/04-3028
10^{0}	0/6	0/6	6/6 (3-5 D)
10^{-1}	0/6	0/6	6/6 (4-6 D)
10^{-2}	0/6	0/6	6/6 (2-3 D)
10^{-3}	0/6	0/6	6/6 (5-7 D)
10^{-4}	0/6	0/6	3/6 (8-9 D)
10^{-5}	0/6	0/6	0/6
10^{-6}	0/6	0/6	0/6
10^{-7}	0/6	0/6	0/6

Balb/c mice aged at 5 wks old
Infected with 100 μl by intranasal route

Table 3. The LD_{50} test of two HK strains and NIBRG-14 virus

Strain / Titer	Mortality (HA titer)		
	A/HK/97-104207	A/HK/03-27173	NIBRG-14
10^{0}	0/6 (1:128)	0/6 (1:64)	6/6 (2-3 D)
10^{-1}	0/6	0/6	6/6 (5-7 D)
10^{-2}	0/6	0/6	4/6 (4 D)
10^{-3}	0/6	0/6	0/6
10^{-4}	0/6	0/6	0/6

Balb/c mice aged at 5 wks old
Infected with 100 μl by intranasal route
NIBRG-14 virus transferred from NHRI

Table 4. The result of protection efficacy on mouse model.

標號	病毒株	免疫劑量（μg/dose）	Balb/c 老鼠	Challenge 結果 (survive)
1	07Flu01（RG14）	4	6	2/6
2	07Flu01（RG14）	2	6	2/6
3	07Flu01（RG14）	1	6	1/6
4	07Flu02（RG14）	4	6	2/6
5	07Flu02（RG14）	2	6	2/6
6	07Flu02（RG14）	1	6	1/6
7	07Flu03（RG14）	4	6	4/6
8	07Flu03（RG14）	2	6	1/6
9	07Flu03（RG14）	1	6	2/6
10	對照組-saline	22	6	0/6

1. 免疫時程 2/26-3/18，共進行兩次免疫。
2. 第一次免疫後隔兩週再進行第二次免疫，後一週委託淡水家畜衛生試驗所進行攻毒試驗。
3. 樣品 07Flu01（RG14）、07Flu02（RG14）、07Flu03（RG14）三株病毒分別稀釋爲 4、2、1 μg/dose，每一病毒之每一濃度各別以六隻 Balb/c 老鼠進行免疫並以 saline 爲對照組。
4. The mice were challenged with A/CHN/E319-2/03 virus containing 8 LD50 at March 18th. The infected mice were observed to April 1st.

Table 5. The result of vaccine protection efficacy on mouse model.

標號	疫苗株	免疫劑量（μg/dose）	Balb/c 老鼠隻數	Challenge 結果 **（存活數/總數）（存活率% ）**
1	08Flu01（RG23）	2	11	4/11 (36%)
2		1	11	2/11 (18%)
3		0.5	11	5/11 (45%)
4	08Flu02（RG23）	2	11	3/8 (37.5%)
5		1	11	1/9 (11%)
6		0.5	11	1/9 (11%)
7	對照組-saline		9	1/6 (17%)

1.免疫時程 8/19-9/9，共進行兩次免疫。
2.第一次免疫後隔兩週再進行第二次免疫，後一週委託淡水家畜試驗進行攻毒試驗。
3.樣品 08Flu01（RG23）、08Flu02（RG23）、08Flu03（RG23）兩株病毒分別稀釋爲 2、1、0.5 μg/does，每一病毒之每一濃度各別以六隻 Balb/c 老鼠進行免疫,並以 saline 爲對照組。
4. Mice were challenged with the A/dk/China/E319-2/2003 virus containg 8 LD_{50} in 100 ul.

Table 6. The results of vaccine protection efficacy on mouse model.

標號	疫苗株	免疫劑量（μg/dose）	Balb/c 老鼠隻數	Challenge 結果 (存活數/總數)（存活率% ）
1	08Flu01（RG23）	4	7	5/7 (71.4%)
2		2	7	2/7 (28.5%)
3		1	7	3/7 (42.8%)
4	08Flu02（RG23）	3	7	6/7 (85.7%)
5		1.5	7	5/7 (71.4%)
6		0.75	7	2/7 (28.5%)
7	對照組-saline		8	0/8 (0%)

1.免疫時程 10/7-10/28，共進行兩次免疫。
2.第一次免疫後隔兩週再進行第二次免疫，後一週委託淡水家畜試驗進行攻毒試驗。
3.樣品 08Flu01（RG23）、08Flu02（RG23）二株病毒分別稀釋爲 4、2、1 μg/dose；3、1.5、0.75 μg/dose，每一病毒之每一濃度各別以 6 隻 Balb/c 老鼠進行免疫,並以 saline 爲對照組。

Table 7. Virus neutralization test of monoclonal antibodies

Virus strain / mAb	E319a	NIBRG14-M4b	PR8-M3c
YY1	>4,096x	28,512x	7,112x
Y5	2,048x	46x	46x
Y26	2,048x	<10x	<10x
Y10	0x	ND	ND
M112	0x	ND	ND
M86	0x	ND	ND

a.H5N1 influenza virus (A/Duck/China/E319-2/03)
b.NIBRG14-M4: H5N1 recombinant virus
c.PR8-M3: H1N1 recombinant virus

Virus control			(Unit:TCID50)	
160	16	1.6	0.16	0.016
+	+	+	+	−
+	+	+	−	−

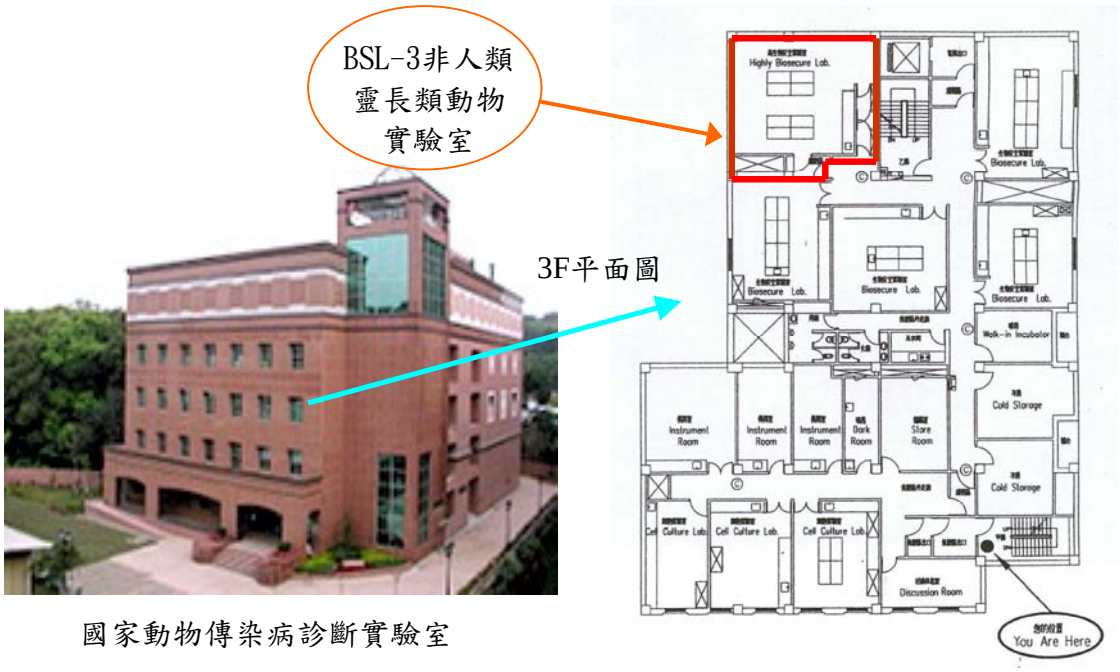


Fig. 1. The BSL-3 facilities and building established in Animal Health Research Institute. The BSL-3 lab was established in the National Animal Diagnostic building. The BSL-3 lab contains negative pressure system and human protection facilities.

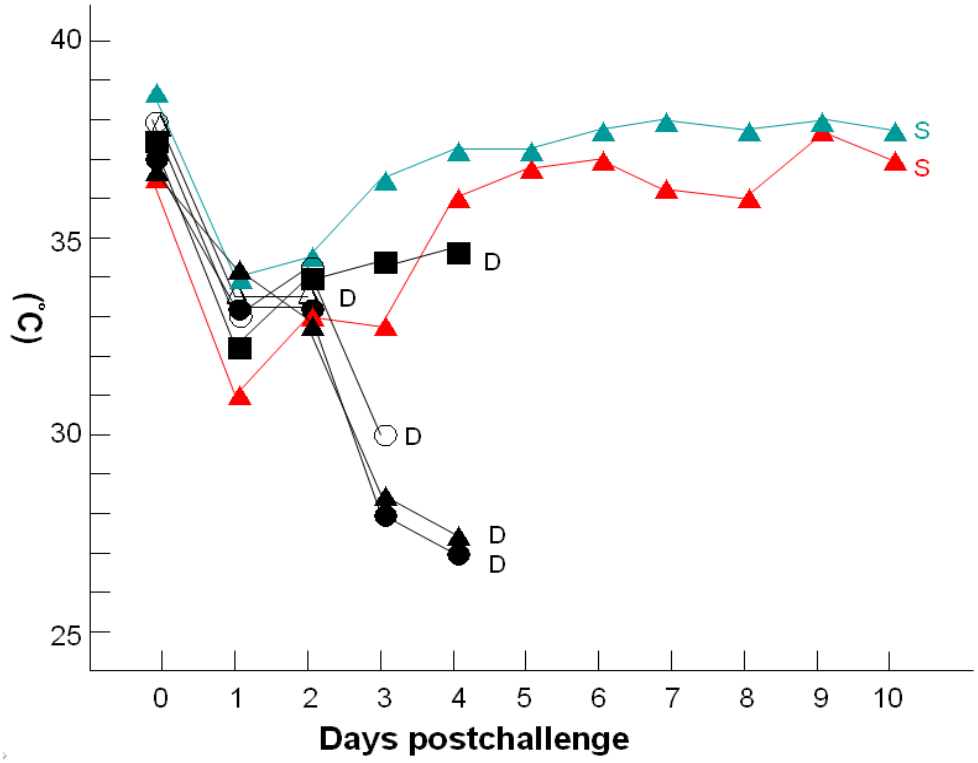


Fig. 2. The fluctuation of body temperature following the challenge by A/Dk/CHN/E319-2/2003 virus in mouse model. Each mouse was infected with 100 μl of virus. The infected mice were detected their body temperature each day and determined the mortality.

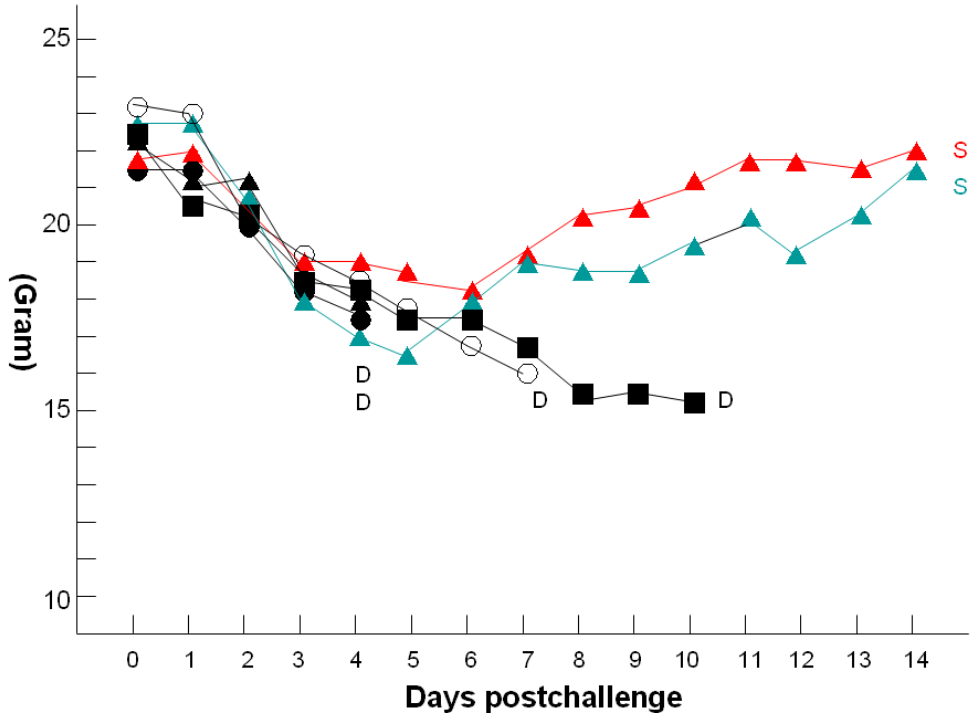


Fig. 3. The Change of body weight following the infection by A/Dk/CHN/E319-2/2003 virus in mouse model. Each mouse was infected with 100 μl of virus. The infected mice were detected their body weight each day.

Ferret cages

Pet ferrets

Fig. 4. Establishment of ferret model. The raised facilities for ferrets were established. Each cage can keep 1-3 ferrets. For virus inoculation of the ferrets, the animal must be maintained under anesthesia condition.

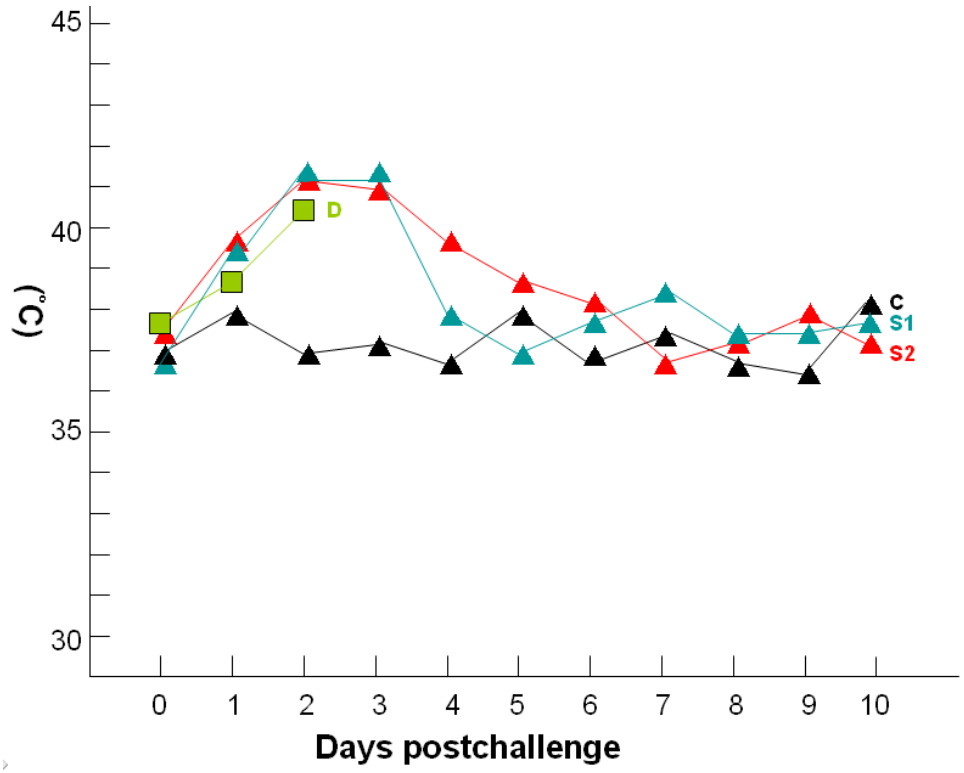


Fig. 5. The change of ferret body temperature challenged by A/Dk/CHN/E319-2/2003 virus. Each ferret was infected with 1 ml of virus containing $10^{9.5}$ ELD_{50} of virus by the nasal route. The D indicates that ferret is dead at that time.

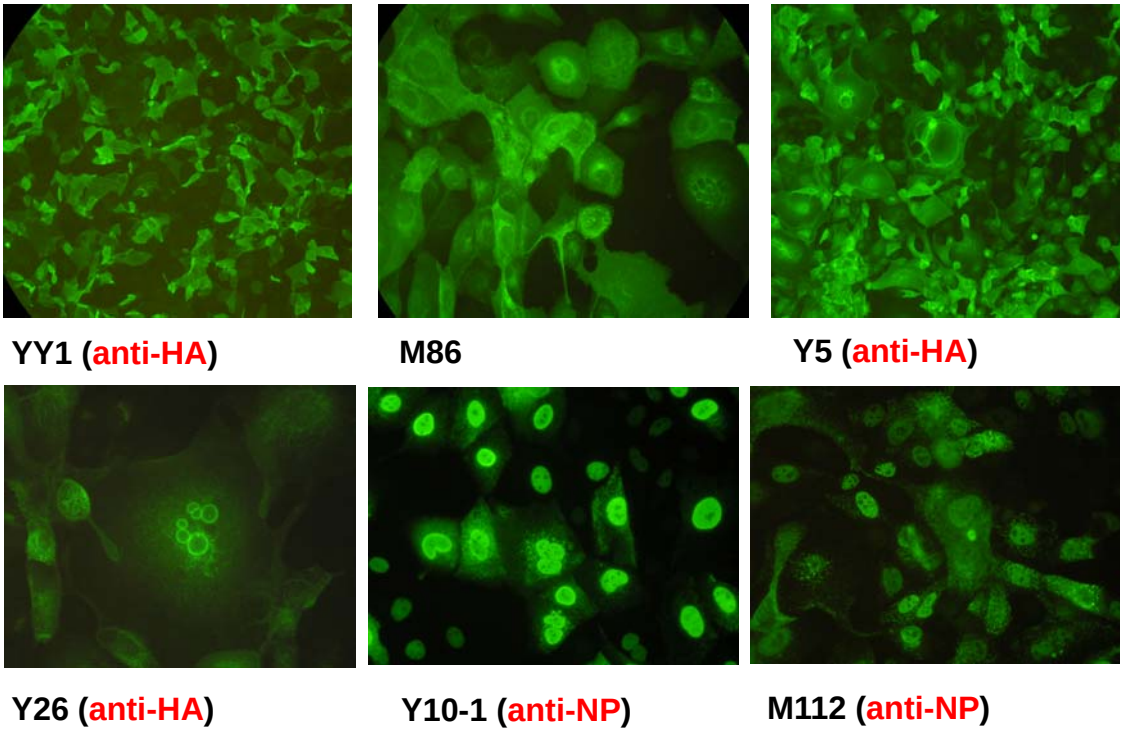
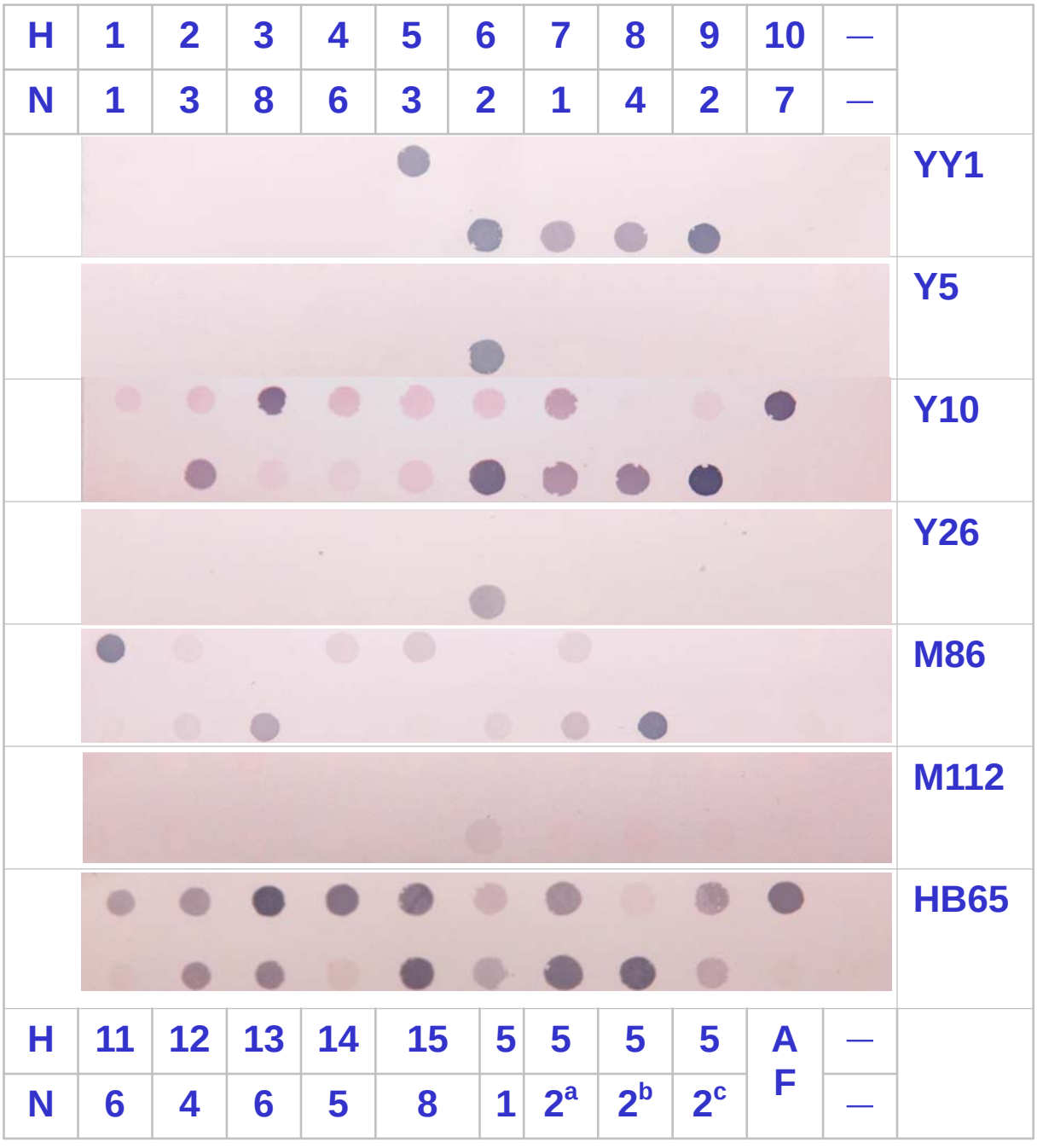


Fig. 6. Fluorescent Assay of mAbs against A/Dk/CHN/E319-2/2003 virus. The monoclonal antibodies were harvested from the culture of hybridoma cells. The anti-mouse IgG labeled with FITC was used to detect the specific monoclonal antibodies from hybridoma cells. The MDCK cells were infected with 200 $TCID_{50}$ of the virus for 16 hrs and were fixed with 10% formalin.



a, H5N2 (Chen)
b, H5N2 (Den)
c, H5N2 (Duck)
All specimen were diluted ten fold

Fig. 7. Dot blot analysis to determine the interaction between viruses and monoclonal antibodies. The avian influenza viruses from H1 to H15 were fixed on the nitrocellulose membrane and incubated with the monoclonal antibodies from hybridoma cells to determine the interaction between viruses and antibodies.