PROCEEDINGS OF THE JOINT ELEVENTH ANNUAL MEETINGS OF THE NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES OF COUNTRIES OF THE EUROPEAN UNION

HELD AT:

AVIAN VIROLOGY AND AGROCHEMICAL RESEARCH CENTRE, UCCLE, BRUSSELS, BELGIUM

28th September to 29th September 2005

Edited by Dennis J. Alexander

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PROGRAMME FOR WEDNESDAY 28 SEPTEMBER 2005

Annual meeting of the National Laboratories for avian influenza (AI)

9:15 − 9:30	Welcome	
9:30 − 9:50	Country reports on AI based on questionnaires	D. Alexander
9:50 − 10:20.	Theory and practice of prophylactic vaccination against H5 and H7 subtype AI in Italy and the implications for other EU member states	I. Capua
10:20 − 10:40	*Coffee*	
10:40 − 11:00	Surveys for AI in poultry and wild birds Original contributions on AI	R. Manvell
11:00 – 11.30	Vaccine efficacy against Asian H5N1 in chickens, ducks and geese	D. Swayne
11:30 – 11.50	Progressive truncation of the Non-structural 1 gene of H7N1 Avian influenza viruses following extensive circulation in poultry.	W. Dundon
11:50 – 12:20	Unusual Characteristics of HPAI of North American Lineage	D. Senne
12:20 – 12:50	HPAI in SE Asia	A. Chaisingh
12:50 − 14:00	*Lunch*	
14:00 -14:15	Working with H5N1 and other AI viruses that infect humans	J. Banks
14:20 – 14:50	Changing pathobiology of H5N1 viruses for chickens and ducks	D. Swayne
14:50 – 15.10	AIV Monitoring of targeted free-range mule ducks in France	V. Jestin
15:10 – 15:30	Transmission of avian influenza in vaccinated and unvaccinated pheasants and ducks	J. van der Goot
15:30 − 15:50	*Coffee*	
15.50 − 16:20	Evaluation of the potential use of a M2e-specific ELISA for DIVA testing	B. Lambrecht
16:20 − 16:40	Isolation and characterisation of highly pathogenic H5N1 virus from Thai eagles smuggled into Europe	T. van den Berg
16.40 – 17:00	Bracing Real Time RT-PCR's Achilles heel: applying controls to a diagnostic settings	S. van Borm
17:00 – 17.30	The EU Diagnostic Manual for AI	J. Banks
17.30 - 17.45	The AI situation in Kazakhstan	G. Cattoli
17.45 – 18.00	Discussion, laboratory matters, recommendations etc	

PROGRAMME FOR THURSDAY 29 SEPTEMBER 2005

Annual meeting of the National Laboratories for Newcastle disease (ND)

9:30 − 9:50	Country reports on ND based on questionnaires	D. Alexander
9:50 − 10:10	Report from the European Commission	R. Freigofas
10:10 − 10:40	Coffee	
10.40 – 11.00	Technical Report from the EU Reference Laboratory for 2004	D. Alexander
11.00 – 11.30	Use of Real Time RT-PCR in Outbreaks and in Surveillance for Newcastle Disease	D. Senne
11.30 – 11.50	The ND outbreak in pheasants in GB	R. Manvell
11.50 − 12.10	Events around one outbreak of ND in pheasants in France in July 2005	J-P Picault
12.10 – 12.30	In ovo vaccination with third generation La Sota escape mutant	S. Marché
12.30 − 13.40	*Lunch*	
13:40 − 14:00	ND situation worldwide excluding EU and USA	R. Manvell
14:00 − 14:20	Interlaboratory comparative tests	D. Alexander
14:20 − 14:35	Work plan of the Community Reference Laboratory for 2005	R. Freigofas
14:35 − 15.00	Discussion, laboratory matters, recommendations etc and close	

Annual Meeting of the National Laboratories for Avian Influenza

COUNTRY REPORTS ON AVIAN INFLUENZA FOR 2004 BASED ON RESPONSES TO THE QUESTIONNAIRE

Dennis J. Alexander and Ruth J. Manvell

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INTRODUCTION

Continuing the format adopted at the 7th Meeting the information for this report was taken from answers supplied by National laboratories to the following questionnaire:

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AVIAN INFLUENZA

1. How many samples from which species of bird/type of poultry have been processed that would have resulted in the isolation of avian influenza viruses in eggs and in cell culture?

Example response:

broilers	*200 cloacal swabs*	*in eggs*
	60 tissue samples	*in eggs*
turkeys	*100 cloacal swabs*	*in eggs*
	140 tissue samples	*in eggs*
	140 tissue samples	*in cell cultures*

2. State the number of influenza viruses isolated, their subtype, and the type of bird from which they were isolated.

Example response:

meat turkeys	*3 x H6N2*
	2 x H9N2
waterfowl	*2 x H4N6, 1 x H5N2*

3. For all influenza viruses isolated state type of poultry or species of bird and IVPI. For H5 and H7 isolates give amino acid sequence at the HA0 cleavage site and conclusion.

Example response:

Bird	***subtype***	***IVPI***	***HA0 cleavage site***	***conclusion***
Turkeys	*H9N2*	*0.00*	*nd*	*LPAI*
feral duck	*H5N2*	*0.00*	*PQRETR*GLF*	*LPAI*

4. Was any active surveillance for avian influenza carried out? If so give details of birds sampled, number of samples and results.

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RESULTS

A total of 33 questionnaires was sent to different laboratories in 30 countries. Responses were received for 19/25 EU countries [21/27 laboratories]: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece x 2, Ireland, Italy, Latvia, Netherlands, Poland, Slovakia, Slovenia, Sweden, UK Great Britain, UK Northern Ireland and from 3/5 [3/6 laboratories] non-EU countries: Croatia, Norway and Switzerland. The samples tested and the results for avian influenza are summarised in the following pages.

VIRUS ISOLATION REPORTS BY COUNTRY

AUSTRIA

Samples tested
None

BELGIUM

Samples tested

Type of bird	Sample	Method	Number
Poultry (chickens and turkey)	Tissue Cloacal swabs	eggs eggs	95 10
Poultry (others)	Tissue	eggs	2
Psittacine	Tissue	eggs	53
Duck and geese	Cloacal swabs	eggs	41
pigeon	Tissue	eggs	33
Pet birds	Tissue Tissue	eggs Cell culture	204 612
Quarantine birds	Cloacal swabs	eggs	735
Wild birds	Tissue	eggs	7

Influenza virus isolated
Illegally imported eagle: 1x H5N1

Virus characterization

Bird	Subtype	IVPI	HA0 cleavage site	conclusion
eagle	H5N1	2.94	KRRKKR*GLF	HPAI

CYPRUS

Samples tested by inoculation into eggs

Type of bird	Sample	Number
Chicken broilers	tissue	2
Chicken layers	tissue	4
Falcons	tissue	5
Flamingos	tissue	21
Hawks	tissue	3
Partridges	tissue	7
Pet birds, (parrots, canaries, finches)	tissue	33
Pigeons	tissue	17
Thrushes	tissue	5
Various free living birds	tissue	8

Influenza viruses isolated
None.

CZECH REPUBLIC

Samples tested by inoculation into eggs:

Birds	Sample	Number	Result
chickens	tissues	6	negative
ducks	tissues	2	negative
exotic birds	faeces	30	negative
	tissues	23	negative

Influenza viruses isolated
None

DENMARK

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
chickens and hens	tissue	660
psittacine and other caged birds	tissue	3549
ducks and geese	tissue	44
game birds	tissue	112
turkeys and ostriches	tissue	60
pigeons	tissue	30
wild birds	faeces	3000*
wild ducks	cloacal swabs	65

*in pools of 5

Influenza virus isolated

Wild birds [All isolates were from wild ducks]
2 x H2N3, 1 x H3N2, 1 x H3N?, 2 x H5N2 (LPAI), 1 x H6N2, 2 x H8N1, 1 x H8N4, 3 isolates with subtyping still pending - not H5/H7 (19.7% of the samples were positive by RT-PCR for influenza A)

Characterisation of AIV isolates

Bird	subtype	IVPI	HA0 cleavage site	conclusion
Feral ducks	H5N2	nd	PQKETR*GLF	LPAI

ESTONIA

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	*cloacal swabs*	1
	tissue samples	2

Influenza viruses isolated
None

FINLAND

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
Layers	tissue samples	22
	faecal samples	7
Turkeys	tissue samples	18
	faecal samples	2
Broilers	tissue samples	9
Water and shorebirds	tissue samples	12
	cloacal swabs	309
Other wild birds	tissue samples	4
	cloacal swabs	87
Partridges	tissue samples	8
Pigeons	tissue samples	4

Influenza viruses isolated
None

FRANCE

Samples tested by inoculation into eggs:

Type of bird	Sample	*Number*
Exotics birds	cloacal swabs	5
	tracheal swabs	5
	pooled tissues	4
Chickens/layers	cloacal swabs	200
	tracheal swabs	150
	pooled tissues	11
Ducks	cloacal swabs	222
	tracheal swabs	6
	pooled tissues	7
Pigeons	cloacal swabs	1
	tracheal swabs	1
	pooled tissues	30
Swallow	pooled tissues	4
Turkeys	cloacal swabs	170
	tracheal swabs	75
	pooled tissues	3

Influenza viruses isolated

turkey breeders: 3 x H6N2
meat turkeys: 2 x H6N2

Characterisation of AIV isolates

Bird	Subtype	IVPI	HA0 cleavage site	Conclusion
turkeys breeders	H6N2	0.00	3 isolates, and 2 cleavage sites determined: PQVETR*GLF PQIETR*GLF	LPAI
meat turkeys	H6N2	0.00	2 isolates with the same cleavage site : PQIETR*GLF	LPAI

GERMANY

Samples tested

Type of bird	Sample	Method	Number
broilers	cloacal swabs	eggs	200
	tissue samples	eggs	60
turkeys	cloacal swabs	eggs	100
	tissue samples	eggs	140
	tissue samples	cell culture	140

Influenza viruses isolated

meat turkeys 3 x H6N2, 2 x H9N2
waterfowl 2 x H4N6, 1 x H5N2

Characterisation of AIV isolates

Bird	subtype	IVPI	HA0 cleavage site	conclusion
turkeys	H9N2	0.00	nd	LPAI
feral duck	H5N2	0.00	PQRETR*GLF	LPAI

GREECE - THESSALONIKI

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
Broilers	cloacal swabs	95
	tissue samples	40
Broiler breeders	cloacal swabs	33
Layers	cloacal swabs	87
	tissue samples	22
Meat turkeys	cloacal swabs	82
	tissue samples	27

Influenza viruses isolated
None

IRELAND

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
Broiler Breeders	cloacal swabs	20
	tracheal swabs	20
Broilers	tissue samples	16
*Chickens	tissue samples	29
Pheasants	tissue samples	18
Ducks	tissue samples	1
Pigeons	tissue samples	14
*Exotic	tissue samples	1
Geese	tissue samples	3
Turkeys	tissue samples	6
Wild	cloacal swabs	228
Other	tissue samples	8

Influenza viruses isolated
None

ITALY

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	pools of cloacal swabs tissue samples	7 40
turkeys	pools of cloacal swabs pools of tracheal swabs	24 18
domestic duck	pools of cloacal swabs pools of tracheal swabs	4 9
ostriches	pools of cloacal swabs	87
quail	pools of cloacal swabs pools of faeces	8 1
pigeons	tissue samples	7
parrots	pools of faeces	6
geese	pools of cloacal swabs pools of faeces	3 2
Samples from wild birds:		
mallard *(Anas platyrhynchos)*	cloacal swabs	236
teal *(Anas crecca)*	cloacal swabs	2
pintail *(Anas acuta)*	cloacal swabs	2
gadwall *(Anas strepera)*	cloacal swabs	2
shoveller *(Anas clypeata)*	cloacal swabs	2
tufted duck *(Aythya fuligula)*	cloacal swabs	9
coot *(Fulica atra)*	cloacal swabs	8
dunlin *(Calidris alpina)*	cloacal swabs	17
black-headed gull *(Larus ridibunot doneus)*	cloacal swabs pools of tracheal swabs	22 10
cormorant *(Phalacrocorax carbo)*	cloacal swabs	23
Cetti's warbler *(Cettia cetti)*	cloacal swabs	1
lapwing *(Vanellus vanellus)*	cloacal swabs	3
moorhen *(Gallinula chloropus)*	cloacal swabs	2

Influenza viruses isolated

From intensively reared birds
turkeys 11 x H7N3
quail 1 x H7N3

From backyard flocks
ducks 2 x H1N1, 1 x H1N2, 1 x H4N2, 1 x H5N3, 1 x H4N6, 1 x H7N7, 3 x H10N4
geese 1 x H1N1, 1 x H3N8, 1 x H4N2, 1 x H7N7, 1 x H9N8, 1 x H11N2, 1 x H11N9

From wild birds
mallard *(Anas platyrhynchos)* 1 x H4N6, 3 x H7N7, 1 x H7N4

Characterisation of AIV isolates

Bird	subtype	IVPI	HA0 cleavage site	conclusion
turkey	H7N3	0.00	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	***LPAI***
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
turkey	H7N3	Not done	PEIPKGR*GLF	LPAI
quail	H7N3	Not done	PEIPKGR*GLF	LPAI
duck	H1N1	Not done	Not done	-
duck	H1N1	Not done	Not done	-
duck	H4N6	Not done	Not done	-
duck	H10N4	Not done	Not done	-
duck	H10N4	Not done	Not done	-
duck	H10N4	Not done	Not done	-
duck	H5N3	0.00	PQRDTR*GLF	LPAI
duck	H1N2	Not done	Not done	-
duck	H7N7	Not done	Not done	LPAI
duck	H4N2	Not done	Not done	-
goose	H1N1	Not done	Not done	-
goose	H3N8	Not done	Not done	-
goose	H4N2	Not done	Not done	-
goose	H11N9	Not done	Not done	-
goose	H11N2	Not done	Not done	-
goose	H9N8	Not done	Not done	-
goose	H7N7	Not done	PEIPKGR*GLF	LPAI
wild mallard	H4N6	Not done	Not done	-
wild mallard	H7N7	Not done	PEIPKGR*GLF	LPAI
wild mallard	H7N7	Not done	PEIPKGR*GLF	LPAI
wild mallard	H7N7	Not done	PEIPKGR*GLF	LPAI
wild mallard	H7N4	Not done	PEIPKGR*GLF	LPAI

LATVIA

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
Broilers	tissue samples	40
Layers	tissue samples	245
Quail	tissue samples	6

Influenza viruses isolated
None

NETHERLANDS

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
chickens	trachea swabs lung/trachaea samples	40 12
turkeys	trachea swabs	40
ducks	110 cloaca swabs faeces sample	110 1
"poultry"	lung/trachea samples tracheal swabs	20 279
miscellaneous	miscellaneous samples	57
unknown	swabs	3
Exotic birds in quarantine		
psittacines	faeces samples litter samples cloacal swabs	12 12 12
other birds	faeces samples litter samples cloacal swabs	30 206 607

Influenza viruses isolated
Domestic duck 1 x H11N?

Infection detected by RT- PCR

Bird	subtype	IVPI	HA0 cleavage site	conclusion
duck	H5	ND*	GLRNVPQKETR*GLF	LPAI

* No virus was isolated. 1 pool was positive in M- and H5-RT-PCR.

POLAND

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	pooled tissue samples	1
broiler breeders	pooled tissue samples	1
turkeys	pooled tissue samples	3
ostriches	pooled tissue samples	2
pigeons	pooled tissue samples	1
black grouse	pooled tissue samples	4
sparrows	faeces samples	6
storks	cloacal swabs	16

Influenza viruses isolated
None

SLOVAKIA

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	tracheal swabs cloacal swabs tissue samples	4 2 2
layers	cloacal swabs tissue samples	66 3
turkeys	cloacal swabs	34
ducks and geese	cloacal swabs	123
pigeons	cloacal swabs	26
wild birds	cloacal swabs tissue samples	117 4
others	cloacal swabs faeces samples	196 3

Influenza viruses isolated
None

SLOVENIA

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	tissue samples	5
pigeons	tissue samples	2
ducks	tissue samples	1
grebes	tissue samples	1

Influenza viruses isolated
None

Surveillance in wild birds

Cloacal swabs or samples from carcases tested by isolation in eggs and RT-PCR

Species of bird	Number of samples
waterfowl and pheasants	
ducks	39
geese	13

storks	3
herons	1
swans	2
grebes	1
pheasants	25
other free-living birds*	30
Total number	114

**Sylvia attricapila, Sylvia borin, Panus major, Tardus merula, Erithacus rubecula, Acrocephalus schoenobaenus*

Influenza viruses isolated or detected
None

SWEDEN

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	tissue samples	6
imported layer breeders	tissue samples	8
breeders	tissue samples	3
layers	cloacal swabs	25
	tissue samples	10
pigeons	tissue samples	8
wild birds	tissue samples	15
zoo birds	tissue samples	16

Influenza viruses isolated
None

UK GREAT BRITAIN

Samples tested

Type of bird	Sample	Method	Number
chickens	tissues	eggs	166
		cell culture	170
	faeces	eggs	2
		cell culture	2
	swabs	eggs	25
		cell culture	23
turkeys	tissues	eggs	41
		cell culture	58
	faeces	eggs	1
	swabs	eggs	182
pet birds	tissues	eggs	15
		cell culture	15

	faeces	eggs	276
	swabs	eggs	677
game birds	tissues	eggs	45
		cell culture	34
	swabs	eggs	3
		cell culture	3
pigeons	tissues	eggs	86
		cell culture	84
	faeces	eggs	8
	swabs	eggs	337
		cell culture	27
waterfowl	tissues	eggs	74
		cell culture	112
	swabs	eggs	2
		cell culture	3
other	tissues	eggs	20
		cells	15

Influenza viruses isolated
None

UK NORTHERN IRELAND

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
chickens	cloacal swabs	30
	faeces	113
	tissues	42
pigeons	cloacal swabs	23
	tracheal swabs	22
	tissue samples	45
turkeys	cloacal swabs	30

Influenza viruses isolated
None

CROATIA

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	tissue samples	39
broiler breeders	tissue samples	21
layers	tissue samples	4
backyard chicken	tissue samples	3
pigeons	tissue samples	14

	cloacal swabs	20
doves	tissue samples	7
ostriches	tissue samples	3
pheasant	tissue sample	1
swan	tissue sample	1
blackbird	tissue sample	1
vulture	tissue samples	12
buzzard	tissue samples	2

Influenza viruses isolated
None

NORWAY

Samples tested

Bird	Specimen	Detection method	Number
Wild pigeon	cloacal swab	Inoc. in eggs	1
Wild duck	cloacal swabs	Inoc. in eggs	3
Wild duck	cloacal swabs	RT-PCR	63

Influenza viruses isolated or detected
None

SWITZERLAND.

Samples tested by inoculation into eggs:

Type of bird	Sample	Number
broilers	tissue samples	31
laying hens	tissue samples	6
duck	tissue samples	1
duck	tissue samples	1

Influenza viruses isolated
None

DISCUSSION

Responses
The questionnaire for 2004 reversed the trend of more countries responding each year seen over recent years. The responses for the last 4 years compared to the number of countries invited to complete the questionnaire have been:
2000 19/29; 2001 22/29; 2002 25/30; 2003 28/30, but for 2004 this was 22/30.

Samples tested

The overall isolation attempts for avian influenza are summarised in Tables 1 and 2 for egg inoculations and Table 3 for cell culture inoculations. The overall total of 18,441 was considerably lower than totals of 45,866 in 2004 and 35,374 for 2002, but is more than twice the total of 8,498 in 2001.

Table 1 Summary of virus isolation attempts in eggs from tissue samples by countries responding to the questionnaire

Bird	Number countries	Number
chickens	20	1544
turkeys	5	247
ducks & geese	7	63
game birds etc	8	287
ostriches	1	3
pigeons	14	340
cage, zoo & Q	10	4050
wild birds	10	121
TOTAL		**6,655**

Table 2 Summary of virus isolation attempts in eggs from cloacal swabs, tracheal swabs and faecal samples by countries responding to the questionnaire

Bird	Number countries	Number
chickens	9	937
turkeys	8	666
ducks & geese	5	516
game birds etc	1	9
ostriches	1	87
pigeons	5	507
cage, zoo & Q	5	3516
wild birds	9	4379
others	2	498
TOTAL		**11,115**

Table 3 Summary of virus isolation attempts from all samples* in cell cultures by countries responding to the questionnaire

Type of bird	Number countries reporting attempts	Number
chickens	1	195
turkeys	2	198
ducks & geese	1	115
game birds	1	37
pigeons	1	111
cage, zoo, pet, quarantine etc	1	15
TOTAL		**671**

* tissues/tracheal swabs/cloacal swabs/faeces

Viruses isolated

Of the 24 laboratories responding 18 reported no isolations of AI viruses.

HPAI

The only isolations of HPAI in the EU in 2004 were the H5N1 viruses obtained from the mountain hawk eagles smuggled from Thailand and detected at Brussels Airport in October 2004 [see these proceedings].

LPAI H5 and H7 subtypes

The H5 and H7 LPAI viruses isolated in the EU during 2004 are summarised in Table 4.

Table 4 Summary of H5 or H7 subtype LPAI viruses isolated.

Subtype	Country	Bird	Number	HA0 cleavage site
H5N2	Denmark Germany	feral ducks	2 1	PQKETR*GLF PQRETR*GLF
H5N3	Italy	domestic duck	1	PQRDTR*GLF
H5N?	Netherlands	domestic duck	1	PQKETR*GLF
H7N3	Italy	meat turkeys quail	11 1	PEIPKGR*GLF PEIPKGR*GLF
H7N4	Italy	feral mallard	1	PEIPKGR*GLF
H7N7	Italy	domestic duck domestic goose feral mallards	1 1 3	PEIPKGR*GLF PEIPKGR*GLF PEIPKGR*GLF

Other LPAI viruses

A total of 38 LPAI influenza viruses of subtypes other than H5 or H7 was isolated from 5 countries (Table 3). Thirteen of these isolates were obtained from wild ducks and a further 15 from domestic ducks or geese. Isolates of H6N2 viruses were obtained from turkeys in France and Germany and two

H9N2 viruses from meat turkeys in Germany. There were no isolates of these viruses from chickens.

Table 5 Summary of other LPAI viruses isolated by countries responding to the questionnaire

Type of bird	Subtype	No. of isolates	No. Countries
turkeys	H6N2	8	2
	H9N2	2	1
commercial ducks	H1N1	2	1
	H1N2	1	1
	H4N2	1	1
	H4N6	1	1
	H10N4	3	1
	H11N?	1	1
commercial geese	H1N1	1	1
	H3N8	1	1
	H4N2	1	1
	H9N8	1	1
	H11N2	1	1
	H11N9	1	1
wild ducks	H2N3	2	1
	H3N2	1	1
	H3N?	1	1
	H4N6	1	1
	H6N2	1	1
	H8N1	2	1
	H8N4	2	1
	untyped	3	1

Vaccination for AI: from theory to practice

Ilaria Capua & Stefano Marangon

Reference Laboratory for Newcastle Disease and Avian Influenza,
Istituto Zooprofilattico Sperimentale delle Venezie
Legnaro –Padova, Italy

AI challenge in Italy

- Wetlands and resting sites for migratory waterfowl in close proximity of densely populated poultry areas (DPPA)
- Significant numbers of highly susceptible species (turkeys) in the DPPA
- Multiple introductions from the wild host (1997-2004) resulting in most cases in major epidemics

Surveillance activities

- Between November 2003 and March 2005 surveillance on migratory and free range domestic waterfowl in NE Italy has yielded:
  - 1 H5 virus (H5N3)
  - 9 H7 viruses (H7N7, H7N4)
  - Other subtypes H10, H4
- This indicates that the DPPA's located in North Eastern Italy are at high risk of introduction.

1997-2004 Avian influenza

- 1997-1998 HPAI: H5N2 8 outbreaks, mainly backyard flocks, prompt eradication
- 1999-2001 LPAI-HPAI: H7N1 devastating epidemic, @ cost 500 million euros (110 million euros direct compensation costs), 17 million birds involved
- 2002-2003: H7N3 LPAI 388 outbreaks
- 2004 H7N3 LPAI : 28 outbreaks
- 2005 H5N2 LPAI: 15 outbreaks

Total direct costs 170 million euros

AI challenge in Italy

- This situation requires intervention in order to limit the economic losses to the public and private sectors
  - Long term strategy: dependant on the applicability of structural and organisational changes in the intensive poultry rearing system
  - Short term strategy: "holding strategy" to limit the financial losses in the interim period

Long-term strategy

Densely populated poultry areas

- Financial support to farmers:
  - to encourage a re-addressing of their activity to a different type of farming (other than turkey)
  - to refurbish poultry-houses improving bio-security standards
- Specific provisions aimed to a gradual removal of turkey farms from urban areas
- Specific measures to facilitate early retirement of poultry farmers
- Implementation of a traceability system of live poultry and poultry products
- Education and training of poultry farmers

Short-medium term control strategy

- Direct control measures (biosecurity, surveillance and restriction) coupled with vaccination to:
  - Reduce transmission dynamics
  - Increase resistance of birds to field challenge
  - Reduce the shedding levels in case of infection

Vaccination for AI

- "Political issue" following the re-emergence of H7N1 LPAI, after 13 000 000 birds had been stamped out for H7N1 HPAI in 1999-2000
- EU Conditions
  - No GMO vaccine
  - Framework of a vaccination programme under official control
  - Necessary to establish whether the virus was circulating in the vaccinated population
  - A tool to support eradication
  - Restrictions on trade (live birds and products)

Development of "DIVA" vaccination strategy against H7N1

- "DIVA" vaccine (Differentiating Infected from Vaccinated Animals)
- Vaccination with inactivated oil emulsion containing (A/ck/Pakistan/95/H7N3)
- Development of a diagnostic test to differentiate anti-N1 from anti-N3 antibodies
- Antibodies to N1 as a marker of field infection

Vaccination against AI (2000)

- Very limited field experience
- Establish the efficacy of the "DIVA" system using heterologous vaccination in the field
- Develop and apply monitoring programmes
- Be transparent with Commission and other Member States
- Gain credibility

1st Italian vaccination campaign

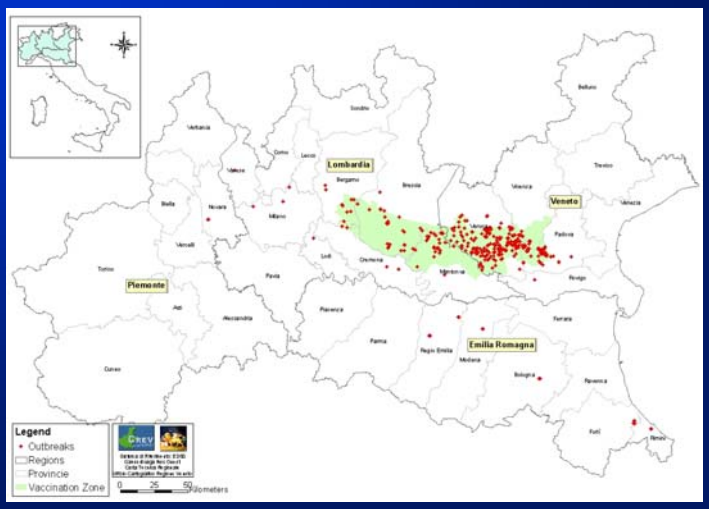
15 November 2000- 1 March 2002

- H7N3 vaccine to control H7N1
- H7N1 virus was eradicated (last outbreak stamped out on 26th March 2001)
- Trade restrictions on fresh meat were lifted in December 2001
- In July 2002 there were no seropositive (vaccinated) slaughterbirds present in the area, and the domestic poultry population was not immune

H7N3 (2002)

- Index case October 2002
- Low Pathogenicity
- Novel introduction from the wild host

H7N3 LPAI EPIDEMIC IN ITALY
Distribution of affected poultry farms (10/10/02 – 10/10/03)



H7N3 EPIDEMIC IN ITALY (2002-2003)
ERADICATION STRATEGY

- Bio-security measures
- Restriction policies to restocking
- Movement restrictions
- Monitoring: prompt identification of LPAI outbreaks
- Controlled marketing and stamping out measures on affected premises
- "DIVA" vaccination (vaccine availability)
- No heterologous vaccine immediately available
  - Only one product (A/ck/It/H7N1/99) was in preparation
  - Only basic safety tests required
  - 1st Batch availability on 31.12.2002

2nd vaccination campaign in Italy: "DIVA" strategy against H7N3

- Inactivated oil emulsion vaccine

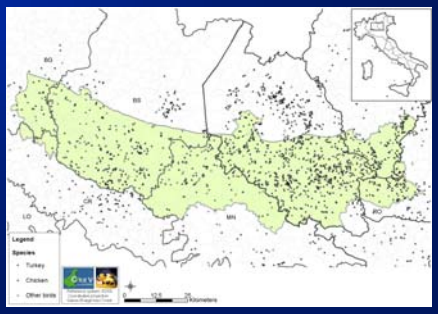
- strain A/ck/IT/99-H7N1 from 31 December 2002 (heterologous vaccine with discriminatory test)

- Only birds at high risk of infection: meat-type turkeys, layers, capons and cockerels

Vaccination area

@ 1,550 poultry farms

More than 45 million bird places



Efficacy of vaccination programme

Field evidence

➢**Due to the delay in the implementation of the vaccination campaign AI spread significantly**

➢NO outbreaks of H7N3 AI virus infection were ever detected in vaccinated layer or other vaccinated chicken flocks, but only in turkeys (80/380)

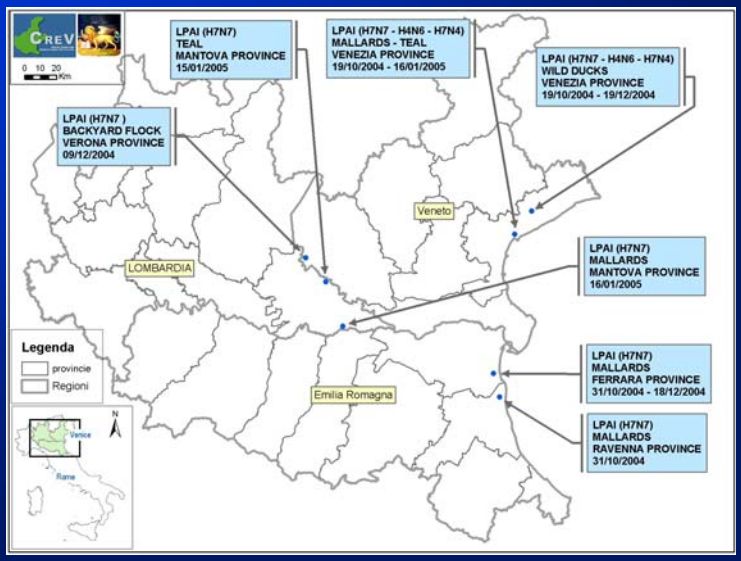
➢ Following the beginning of the vaccination campaign there was evidence of minor spread of infection in the vaccination area (only 5 outbreaks were identified in non-vaccinated poultry flocks located in the vaccination area)

➢ High and long- lasting immune response (titres and duration) in vaccinated chickens (layers)

Eradication of H7N3

- In September 2003 H7N3 AI was eradicated
- The DIVA strategy using heterologous vaccination (H7N1) was successful
- Monitoring results indicated that the virus was no longer circulating in the vaccinated population
- Lifting of trade restrictions was extended to commercial eggs and on fresh meat from vaccinated poultry (Commission Decision 2004/159/EC)

ITALY: AI monitoring on wild waterfowl – 31.10.2004/16.01.2005



Given the high risk is it wiser to chase or to attempt to prevent an epidemic?

MEASURES TO PREVENT AND CONTROL LPAI VIRUS INTRODUCTIONS

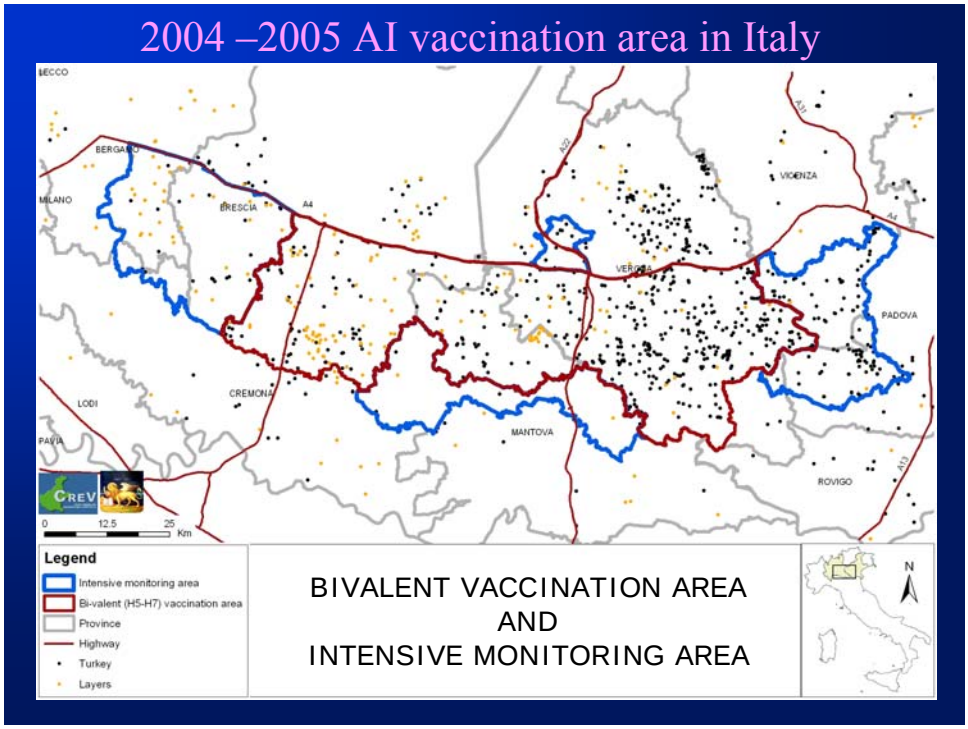
- Bio-security measures
- Monitoring and surveillance, early warning systems
- Prompt detection of any AI virus introduction
- **Pilot bivalent vaccination with H5–H7 virus subtypes**

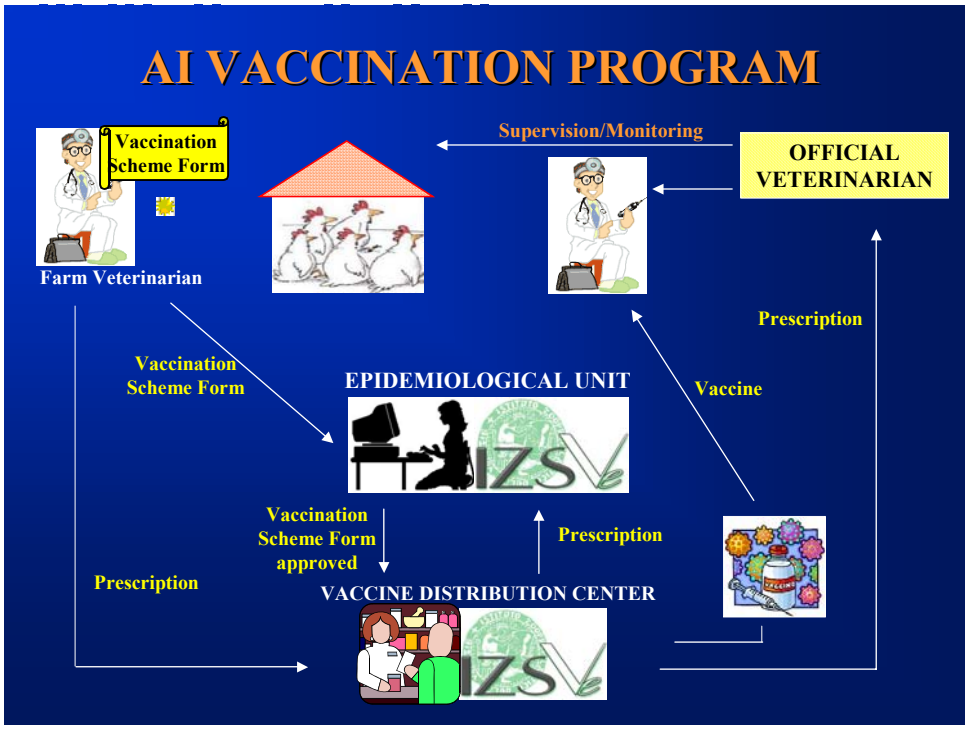
Prophylactic Vaccination

- In July 2004 Italy proposed and obtained the authorisation by the EU Commission to implement a prophylactic vaccination campaign (Decision 2004/666/EC)
- This is based on a bivalent (H5N9 and H7N1) vaccination programme based on DIVA
- Only in the area at high risk

Prophylactic Vaccination

- Rationale: to give the population at risk a minimal level of immunity that can be boosted if a novel strain is introduced
- An immune population will be less susceptible to field challenge and will shed less virus thus generating less secondary outbreaks


2004 –2005 AI vaccination area in Italy
LECCO
BERGAMO
MILANO
BRESCIA
A4
VICENZA
VERONA
PADOVA
CREMONA
LODI
PAVIA
MANTOVA
ROVIGO
A13
CReV
0 12.5 25 Km
Legend
Intensive monitoring area
Bi-valent (H5-H7) vaccination area
Province
Highway
Turkey
Layers
BIVALENT VACCINATION AREA
AND
INTENSIVE MONITORING AREA
N


AI VACCINATION PROGRAM
Vaccination Scheme Form
Farm Veterinarian
Supervision/Monitoring
OFFICIAL VETERINARIAN
Prescription
Vaccination Scheme Form
EPIDEMIOLOGICAL UNIT
Vaccine
Vaccination Scheme Form approved
Prescription
Prescription
VACCINE DISTRIBUTION CENTER
IZSVe

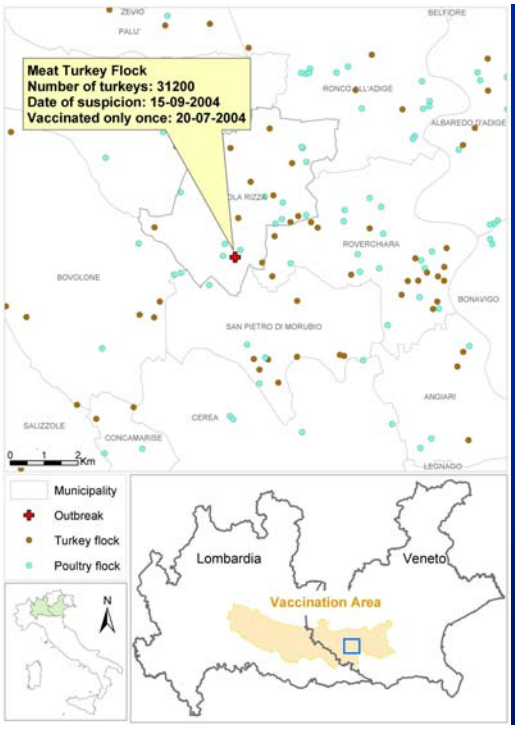
VACCINATION PROGRAMME

"DIVA" vaccine

(**D**ifferentiating **I**nfected from **V**accinated **A**nimals)

Monitoring measures

- Vaccinated flocks
  - * sentinel birds **(1% of birds not vaccinated and properly identified)**
  - * vaccinated birds (**discriminatory test N1-N3 and N2-N9)**
- Unvaccinated flocks
- Vaccine efficacy



15 September 2004

- **LPAI (H7) seropositive meat turkey flock**
- **Vaccinated only once**
- **About 11 months after the depopulation of the last LPAI outbreak**
  - **H7N3 subtype**
  - **Low Pathogenicity**
  - **Cleavage site sequence ..PEIPKGR*GLF..**

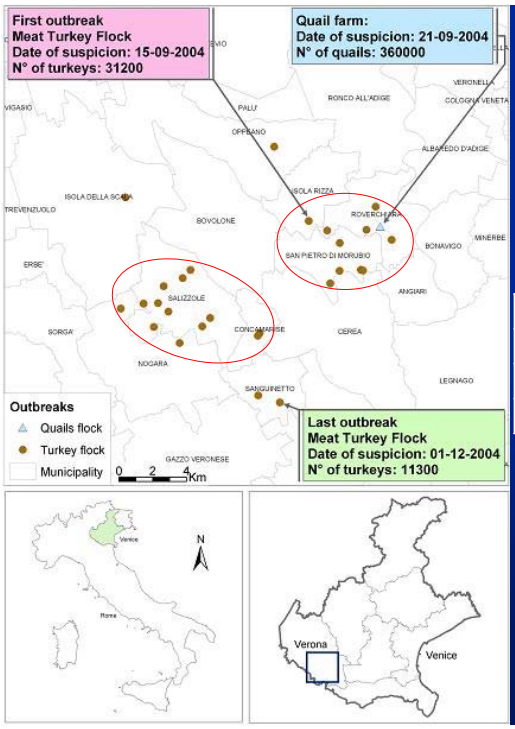
LOW PATHOGENIC AVIAN INFLUENZA (H7N3)

Origin (where did it come from?)

- **Genetic data available:**
  - **Presence of stalk deletion in the NA molecule and of potential AGS (149) in the HA molecule indicates a certain degree of adaptation to the domestic host**
  - **Based on the sequencing data, the HA is genetically related to the previous H7N3 Italian epidemic strain with a nucleotide homology up to 99.3 %**

LOW PATHOGENIC AVIAN INFLUENZA (H7N3)

- **No evidence of virus circulation from September 2003 to August 2004 in the monitored poultry population (vaccinated and not vaccinated)**
- **The monitoring program was able to detect virus circulation in the vaccinated turkey population**
- **Data suggest that this virus can be considered a re-emergence of the 2002-2003 H7N3 LPAI epidemic strain, possibly present in a reservoir (quail?)**



LPAI (H7N3) EPIDEMIC IN ITALY

Geographical distribution of affected farms

(15/09/2004 – 10/12/2004)

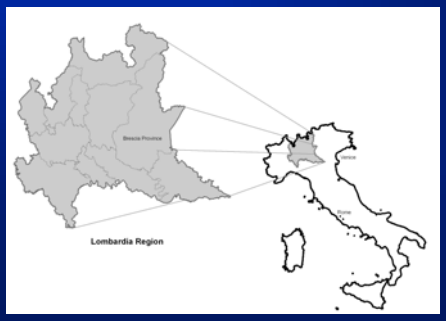
Type and category	No. of Farms	No. of Birds
Meat turkeys	27	384,000
Quails	1	360,000
TOTAL	28	744,000

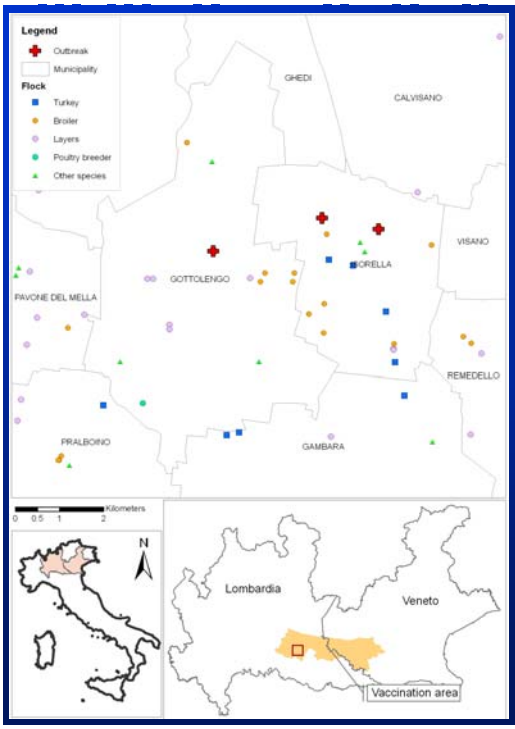
Last outbreak depopulated on 10/12/2004

AVIAN INFLUENZA IN ITALY 2005

LOW PATHOGENIC AVIAN INFLUENZA (H5N2) EPIDEMIC

LOMBARDY REGION





LOW PATHOGENIC AVIAN INFLUENZA (H5N2) EPIDEMIC LOMBARDY REGION ITALY

11 April 2005

- **3 LPAI (H5) seropositive meat turkey flocks**
- **Total n° of birds: 43,400**
- **Brescia province**
- **All infected flocks vaccinated twice**
- **All flocks resulted negative to the monitoring tests carried out in the second half of March**

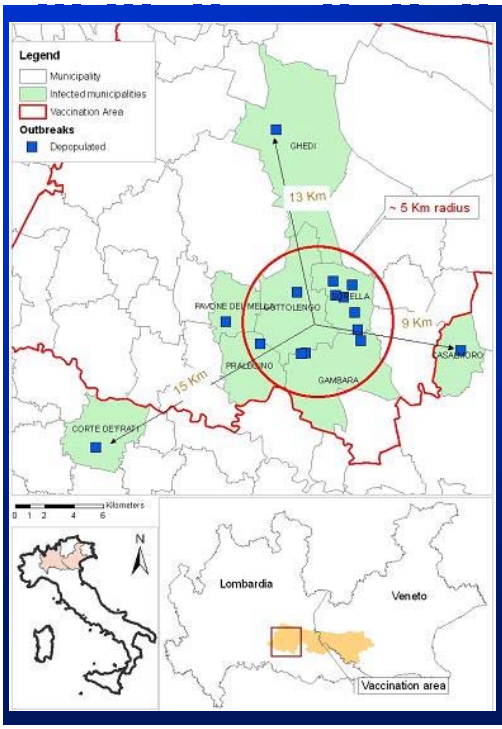
LOW PATHOGENIC AVIAN INFLUENZA (H5N2)
LPAI virus strain isolated on April 14

- H5N2 subtype (A/tk/Italy/295/05)
- Low Pathogenicity (IVPI=0)
- Cleavage site sequence ….. PQRETRGLF …

Correlation with:

- A/duck/Primorie/2633/01(H5N3) (97.5%)
- A/chicken/Italy/8/98(H5N2) (93.8%)
- A/duck/Italy/1349/04(H5N3) (93%)

From the molecular analysis:
Poor adaptation to the domestic host, probably due to the recent introduction from the wild bird reservoir



LOW PATHOGENIC
AVIAN INFLUENZA (H5N2)
LOMBARDY REGION – ITALY
(11.04.2005 – 15.05.2005)

Provinces	Municipalities	No. of Farms	No. of Birds
Brescia	Gambara	3	44,000
	Ghedi	1	22,800
	Gottolengo	1	15,000
	Isorella	6	110,500
	Pralboino	1	13,800
	Pavone Mella	1	14,600
Cremona	Corte dei Frati(*)	1	13,300
Mantova	Casalmoro(*) (**)	1	31,000
Total		**15**	**265,000**
Outbreaks depopulated		**15**	**265,000**

(*) Municipalities in which farms are not vaccinated with H7/H5 vaccine
(**) Last outbreak detected on 10.05.2005

LAST OUTBREAK DEPOPULATED
ON 15.05.2005

AI epidemics of the H5/H7 subtype since 1999

- H7N1 (HP/LP) (1999-2000) – in absence of vaccination @ 700
- H7N1(LP) (2001) with vaccination @ 20
- H7N3 (LP) (2002-2003) emergency vaccination with delayed implementation @ 390
- H7N3 (LP) (2004) in vaccinated population @ 30
- H5N2 (LP) (2005) in vaccinated population @15

Conditions for the successful application of a vaccination programme

1. Have a "system" that can manage it. This includes : data collection and processing, management of field outbreaks, vaccine distribution, laboratory diagnosis
2. Upgrade biosecurity
3. Have a high quality vaccine, immediately available
4. Have a vaccination programme, approved by relevant authorities

Vaccination for AI with reference to trade

- New OIE regulations allow trade of commodities originating from vaccinated birds provided that the latter are also proven to be not field exposed
- In order to trade in commodities originating from vaccinated birds enhanced surveillance must be carried out

Summary of events

1999-2000	2000- 2001	2002- 2003	2004 - 2005
HPAI H7N1 epidemic	LPAI H7N1 epidemic	LPAI H7N3 epidemic	Prophylactic vaccination
↓ stamping out	↓ stamping out and vaccination	↓ stamping out and vaccination	↓ absence of virus circulation
↓ eradication	↓ eradication	↓ eradication	↓ No restrictions on meat and eggs
	↓ Lifting of restrictions on meat	↓ Lifting of restrictions on meat and eggs	

Summary

- The Italian experience has shown that a "DIVA" vaccination strategy against AI using inactivated heterologous seed and companion anti-neuraminidase diagnostic test can be used both for emergency and prophylactic purposes limiting financial losses generated by major epidemics
- In order to mantain trade, vaccination must be coupled with monitoring and aim at eradication
- Transparency on results builds credibility to trade partners

Acknowledgements

- The success of this project was achieved thanks to the joint effort of public and private bodies that opened possibilities through funding and extensive collaboration, in particular:
  - Italian Ministry of Health
  - EU Commission (DG SANCO)
  - EU Commission (DG Research)
  - OIE
  - Staff of the Istituto Zooprofilattico delle Venezie

Surveys for avian influenza in poultry and wild birds in member states 2004

Ian Brown, L. Jordan, R.J. Manvell and A.J. Cook

Community Reference Laboratory

Veterinary Laboratories Agency, UK

Background

- Control of highly pathogenic avian influenza (AI) (Directive 92/40/EEC)
- Surveillance not forseen in directive
- Low pathogenic strains not covered by directive may circulate and acquire virulence
- Severe economic losses may be alleviated by intervention strategies

Adopted OIE AI definition change

For the purpose of diagnostic procedures for the confirmation and differential diagnosis of avian influenza:

"Avian influenza' means an infection of birds caused by any influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype."

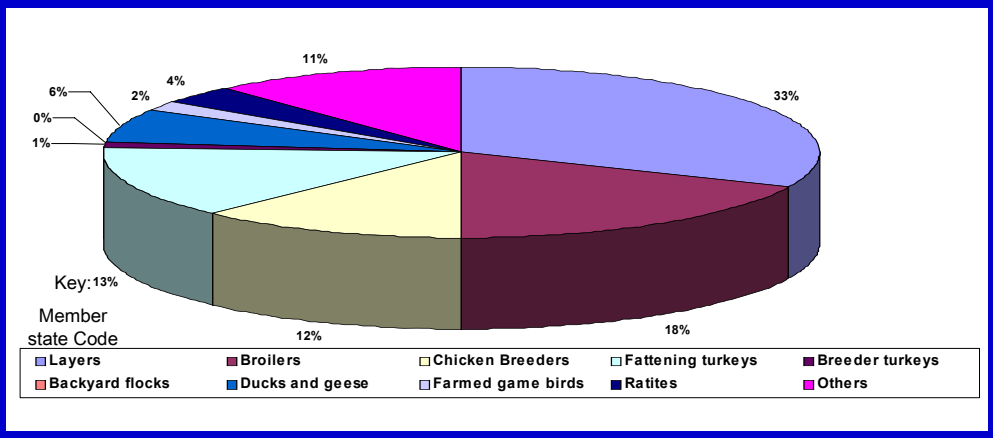
Programme objectives

- To investigate the prevalence of infections with influenza A viruses of H5 and H7 subtypes in different species of poultry in the EU
- To contribute to a cost–benefit study in relation to eradication of all H5 and H7 subtypes from poultry as a result of the change in definition of avian influenza
- To take the preliminary steps towards the connection and integration of human and veterinary networks for influenza surveillance

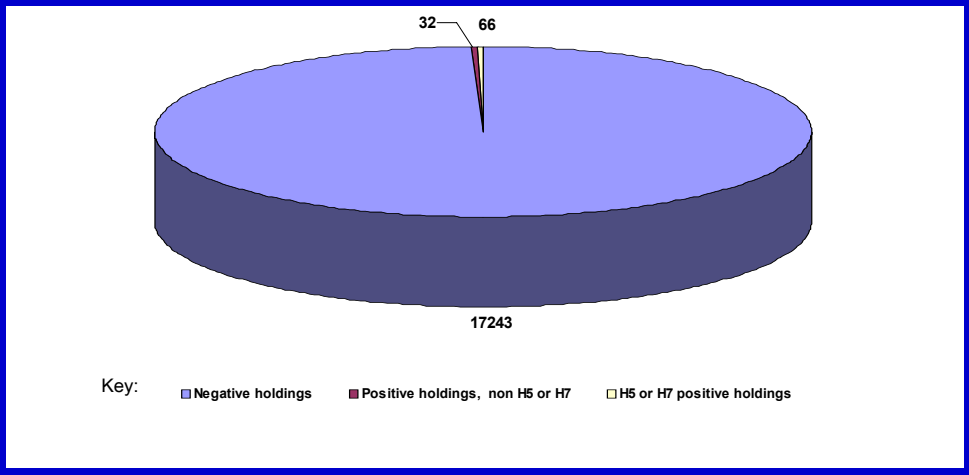
General structure of programme

- Refinements to 2003 programme
  - Increased sampling for turkeys at holding level
  - Chicken broilers not recommended
  - Serology for ducks & geese
- All categories of poultry
- Statistical based programme
- Sampling
  - adapted according to host
- Laboratory tests
- Wild bird surveillance optional

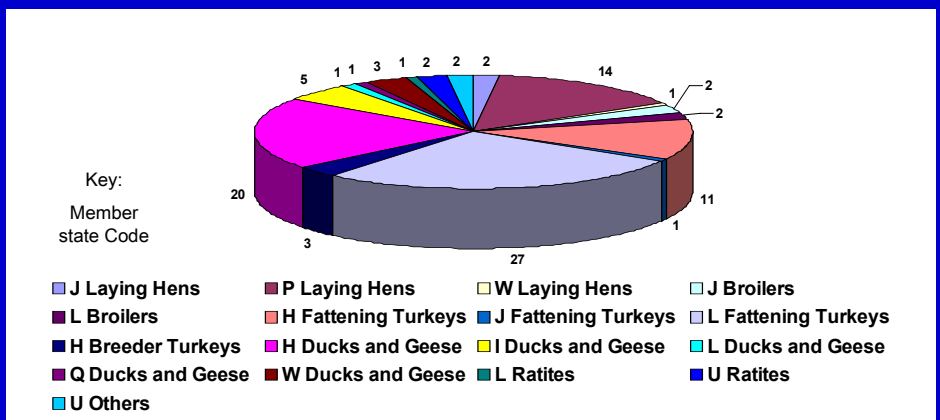
Percentage of categories sampled by holdings



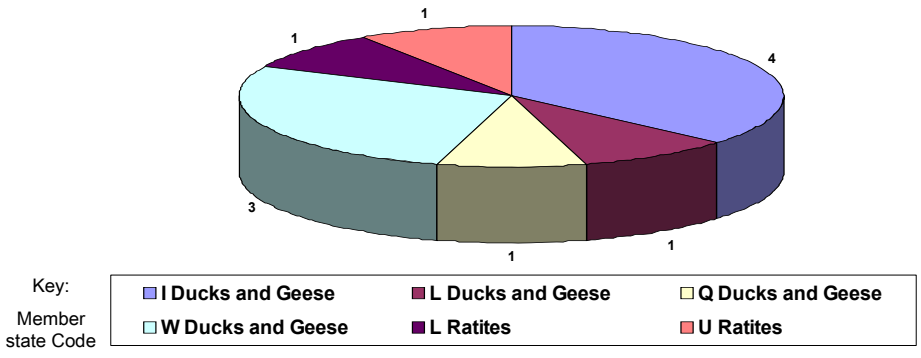
Total holdings tested/positive



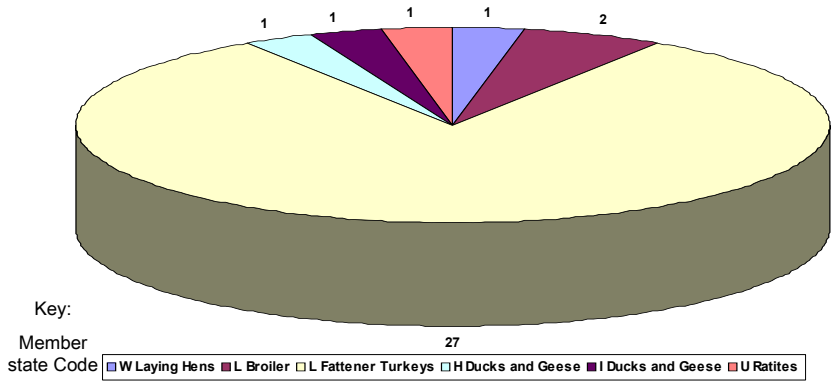
Total holdings positive by category and MS



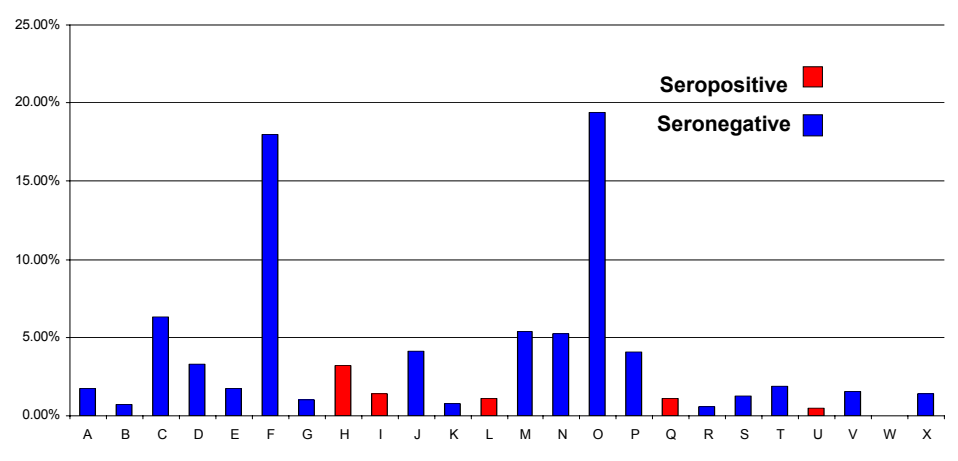
H5 positive holdings in Member States



H7 positive holdings in Member States



Histogram of upper limits for prevalence in poultry of H5/H7 Influenza A virus at 95% confidence level



AI survey in wild birds

- Diversity of species
  - waterfowl, shorebirds & other free-living birds
- Virus detection using faecal material
  - test sample pools from same host species
- Results received from 16 MS's
- 7482 samples examined
- 214 (2.9%) influenza A viruses
  - 15 x H5
  - 7 x H7

Avian influenza viruses isolated in Europe in 2003/4

Other subtypes

In wild birds:

Ducks and geese H1N1, H2N3, H3N2, H3N8, H4N6, H6N1, H6N2, H6N5, H6N6, H6N8, H9N9, H10N4, H10N5, H10N6, H10N7, H11N9

Other birds H9N9, H10N4, H13N6 H16N3

Avian influenza viruses isolated in Europe 2003/4

H5 & H7 subtypes

LPAI in wild ducks

- H5N2/3 in Germany
- H5N2 in Denmark
- H7N1 in Germany
- H7N3/7 in Germany
- H7N4/7 in Italy

HPAI in other birds

- H5N1 in smuggled Eagles in Belgium

Integration of human and animal influenza surveillance networks

- Preliminary steps
- Formal interaction AI CRL and EISS
  - Attendance at annual meetings
- Workshop in June 2005
  - Assessing areas for collaboration
  - Improved and rapid data sharing

Enhanced and improved surveillance in wild birds

- New initiative through apparent increased threat for introduction
- Further funds approved for enhanced programme
  - Better targeted to species/locations
  - Start September 2005
- Initiatives for more integrated EU wide programme

Conclusions

- Prevalence of H7 and H5 viruses was reported in several MS's since active surveillance began
  - Low prevalence in ducks and/or geese and/or ratites
  - Spread to other hosts in clearly defined outbreaks
- Some of MS's with positive holdings had concurrent outbreak with H7 in the wider/within region at the time of the survey
- Apparent (?) increase in isolation of H5/H7 from wild ducks
- Generally the point prevalence study indicated low prevalence with estimates of limits for most MS's with major production in the range 0 to 6%.
- Comparison to 2003: continued circulation of H5/H7; apparent increased detection?
- **Surveillance in high risk areas/hosts important for improved knowledge**

Future

- Repeat of 2004 programme with modifications based on risk and results from last years programme
- Results submitted no later than 31/3/06 but earlier if possible
- Increased sampling at holding level
  - Ducks, Geese, Quail

Issues

- Information consistency

AI Survey Data Collection & Reporting
The Current System

- Freeform System
  - Member States collate laboratory results at a national level
  - Standardised word documents are provided for the collection of data
    - Data not always received in standard format
  - Completed data is passed to the VLA via the EU
  - Data is entered onto an Excel spreadsheet database by staff at the VLA
  - Report is generated from the data
  - Completed report is passed to the EU Commission for review

Problems with the current system

- Data not in a consistent format
  - Standard templates disregarded
  - Data in languages other than English
  - Terms used can be ambiguous
- Delays in the reporting of data
  - Data reported late
  - Slow response to queries regarding discrepancies in the data
- Data must be interpreted at VLA in order to make it compliant with the standards in place
- Data must be entered manually onto the spreadsheet
  - Duplication of effort
  - Transcription errors can occur

New System Proposed

- Web System
  - Member States collate laboratory results at a national level
  - Member States enter summarised data onto web site
    - Can only be viewed by source member state and commission only
    - Web site secured to allow only authorised access, not public
  - Tabulation of data performed automatically
  - Commission and Member States able review reports on website
    - In accordance with EU protocols

Advantages of the new system

- Data can be validated at time of entry
- Data is entered in a single, consistent format
- MS can review their own data following entry
- Delays in the reporting of data can be avoided
  - Direct entry of data with no intermediate steps
  - Increased efficiency of data entry
  - Reports generated more efficiently
  - Queries should be reduced due to data validation

Summary

- Estimated development time for this system 3-6 months
- Any other questions should be directed to:
  - Vincent Adcock
  - E-mail: v.adcock@vla.defra.gsi.gov.uk
    - (English only please)

Thank you for submitting results promptly

We look forward to receiving viruses isolated

Vaccine Efficacy Against Asian H5N1 Highly Pathogenic Avian Influenza in Chickens, Ducks and Geese

David E. Swayne

Southeast Poultry Research Laboratory
Agricultural Research Service
U.S. Department of Agriculture
Athens, Georgia

EU Ref 2005

Disease Control Basics

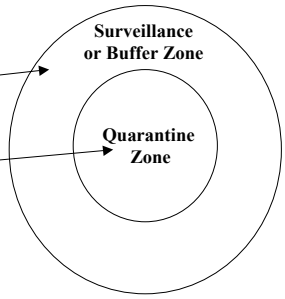
- **Strategies for dealing with poultry disease are developed to achieve one of 3 goals or outcomes:**
  - *Prevention*: preventing introduction
  - *Management (Control)*: reducing losses by minimizing negative economic impact through management practices
  - *Eradication*: total elimination
- **These goals are achieved through various strategies developed using universal components:**
  - Biosecurity (exclusion and inclusion) including quarantine
  - Diagnostics and surveillance
  - Elimination of AI virus infected poultry

EU Ref 2005

Vaccination - AI

1. Vaccination as a Tool During Eradication

- **"Ring" vaccination: difficult - compartment**
- **"Suppressor" vaccination**
  - **Repopulation**
  - **In face of outbreak?**



2. Vaccination as a Tool for Management

- **Decrease clinical disease and reduce economic losses**
- **Use in high risk areas – ex H1N1 in turkey breeders**

EU Ref 2005

Properly Used AI Vaccines

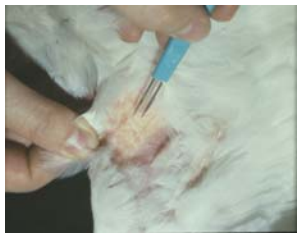
Protection – Positive Aspects

- **Increase resistance to AI virus infection**
- **Prevent clinical signs and death**
- **Reduced shedding of field virus when infected**
- **Prevent contact transmission**
- **Provide long protection from single vaccination**
- **Protect against low or high exposure dose of field virus**
- **Protect against a changing virus, but vaccine strains have finite lifespan**

EU Ref 2005

Avian Influenza Vaccines: Poultry

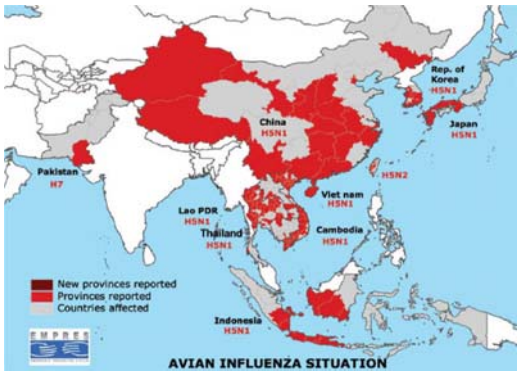
- Vaccination not routine in most of the world
- No single vaccine for AI viruses
- Anti-hemagglutinin antibodies are protective, but anti-neuraminidase also protective, less effective
- Types of Vaccines
  - Inactivated whole AI virus (C,E)
  - Recombinant live virus vectors: Fowl Pox (C), Adenovirus (E)VEE (E), ALV (E), Vaccinia (E), ILT (E), NDV (E)
  - Subunit AI proteins (E) - HA, NA: Baculovirus, Yeast, Bacterial, Plant
  - Naked DNA vaccines (E)



EU Ref 2005

Asian H5N1 HPAI Epizootics

- 24 Epizootics of HPAI since 1959
- 1996-2005: 11 Asian countries affected by H5N1
- Total dead or culled: 100-200m
- Endemic in village poultry and domestic ducks
- 107 human cases – 54 fatalities (6/15/05)



EU Ref 2005

- Vaccines as tool in a control strategy:
  - Protection of poultry against H5N1 viruses in Asia
  - Prevention of bird-to-human transmission – prevent human infections
  - Protection in USA should the H5N1 Asian viruses be introduced

Avian Influenza Vaccines in Asia

- **Inactivated vaccine strains:**
  - **A/turkey/England/73 (H5N2) LPAIV**
  - **A/chicken/Mexico/94 (H5N2) LPAIV**
  - **A/chicken/Indonesia/03 (H5N1) HPAIV**
  - **Infectious clone: H5 & N1 genes of A/goose/Guangdong/96, 6 internal genes PR8**
  - **A/turkey/Wisconsin/68 (H5N9) LPAIV**
- **Recombinant fowlpox with cDNA inserts of AI viral genes**
  - **H5 gene - A/turkey/Ireland/83**
  - **H5 & N1 genes - A/goose/Guangdong/96**

EU Ref 2005

Inactivated AI Vaccines Protection Against H5N1 in Chickens

Chickens vaccinated SQ 3 wks with inactivated whole AIV vaccine and IN challenged 3 wks later with high dose ($10^{6.0}$ EID_{50} of HPAIV A/chicken/Indonesia/7/2003 [H5N1])

- **1994 North American vaccine virus (Mexico/94)**
- **1986 Eurasian vaccine virus (Pottsdam/86)**

Group	Vaccine	Morbidity (3-4+)*	Mortality (MDT)**	Virus Isolation, 2 DPC (Log_{10} EID_{50} titer/ml)	
				oral	cloacal
1	Nobilis Hepatitis + ND Inac (Control)	$10/10^{A}$	$10/10^{A}$ (2.2)	$10/10^{A}$ (6.16 [a])	$10/10^{A}$ (5.82 [a])
2	Nobilis I.A. Inactivated H5N2 (Mexican Strain)	$0/10^{B}$	$0/10^{B}$	$5/10^{B}$ (1.23 [b])	$3/10^{B}$ (1.00 [b])
3	Nobilis Influenza, H5N2 (European Strain)	$1/10^{B}$	$1/10^{B}$ (2.0)	$6/10^{AB}$ (1.78 [b])	$3/10^{B}$ (1.53 [b])

EU Ref 2005

Recombinant Fowlpox Vaccine Protection Against H5N1 in chickens

Chickens vaccinated SQ 1d with fowlpox-AIV-H5 recombinant* or inactivated whole AIV vaccine and IN challenged at 3 wks with low challenge dose ($10^{3.3}$ EID_{50} of HPAIV A/chicken/South Korea/2003 [H5N1])**

Vaccine Group	Morbidity	Mortality (Mean Death Time in days)	Virus Isolation, 2 days Post-challenge (Log_{10} EID_{50} titer/ml)	
			Oral swab	Cloacal swab
rFP-H5 (10^3 $TCID_{50}$)*	$0/12^{A}$	$0/12^{A}$	**$3/5^{A,B}$ (1.67)**	**$0/5^{A}$ (<0.97)**
rFP-H5 (10^4 $TCID_{50}$)*	$1/12^{A}$	$1/12^{A}$ (6.0)	**$0/5^{A}$ (<0.97)**	**$0/5^{A}$ (<0.97)**
Diluent	$9/10^{B}$	$9/10^{B}$ (4.7)	**$4/5^{B}$ (3.06)**	**$4/5^{B}$ (1.98)**
TW/68 Oil Emusified (2-3 µg HA protein)**	$0/12^{A}$	$0/12^{A}$	**$0/5^{A}$ (<0.97)**	**$0/5^{A}$ (<0.97)**

***H5 gene of A/turkey/Ireland/83**
**** A/turkey/Wisconsin/68 (H5N9)**

EU Ref 2005

Recombinant Fowlpox Vaccine Protection Against H5N1 in chickens: Low to High Challenge Doses

Chickens vaccinated SQ 1d with fowlpox-AIV-H5 recombinant* and IN challenged at 3 wks with various challenge dose ($10^{0.5\text{-}8.0}$ EID_{50} of HPAIV A/chicken/South Korea/2003 [H5N1])

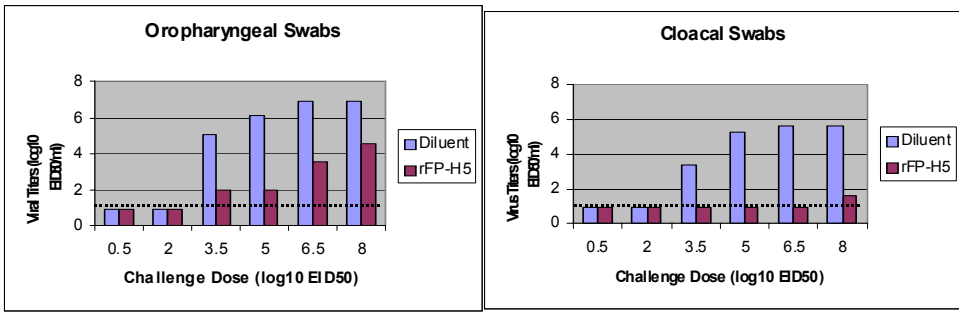
Vaccine	Challenge Dose	Morbidity	Mortality (MDT)
rFP-H5	0.5 logs	0/10	0/10
	2.0 logs	0/10	0/10
	3.5 logs	0/10	0/10
	5.0 logs	0/10	0/10
	6.5 logs	0/10	0/10
	8.0 logs	2/10	2/10 (4.5)
Diluent/Adjuvant	0.5 logs	0/10	0/10
	2.0 logs	0/10	0/10
	3.5 logs	8/10	8/10 (2.75)
	5.0 logs	10/10	10/10 (2.4)
	6.5 logs	10/10	10/10 (2.0)
	8.0 logs	10/10	10/10 (2.0)

***H5 gene of A/turkey/Ireland/83**

EU Ref 2005

Recombinant Fowlpox Vaccine Protection Against H5N1 in chickens: Low to High Challenge Doses

Chickens vaccinated SQ 1d with fowlpox-AIV-H5 recombinant* and IN challenged at 3 wks with various challenge dose ($10^{0.5-8.0}$ EID_{50} of HPAIV A/chicken/South Korea/2003 [H5N1])



***H5 gene of A/turkey/Ireland/83**

EU Ref 2005

Recombinant Adenovirus Vaccine Protection Against H5N1 in chickens

Chickens vaccinated SQ 3 wks with d-Adenovirus-AIV-H5 recombinant* and IN challenged at 6 wks with 10^6 EID_{50} of HPAIV A/Vietnam/1203/2004 [H5N1])

Vaccine Group	Morbidity	Mortality (Mean Death Time in days)	Virus Isolation, 2 days Post-challenge (Log10 EID50 titer/ml)	
			Oral swab	Cloacal swab
d-Adenovirus Vector	10/10	10/10 (1.8)	10/10 (6.96)	10/10 (6.26)
d-Adenovirus-AIV-H5	0/10	0/10	9/10 (3.84)	0/10 (=0.9)

***H5 gene of A/Vietnam/1203/04, cooperative project with A. Gambotto – University of Pittsburgh**

EU Ref 2005

Inactivated Vaccine Protection Against Asian H5N1 in Geese

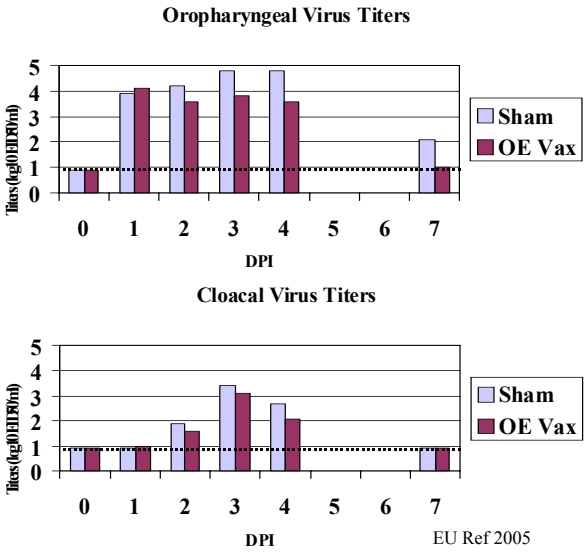
Chinese Geese vaccinated SQ 1wk with inactivated whole AIV (A/ck/HGO/28159-232/95, A/tk/Eng/N28/73, A/ck/Indonesia/7/03) of recombinant Fowlpox-H5 AI vaccine and IN challenged at 4-wks-old with $10^{6.0}$ EID_{50} of HPAIV (A/chicken/Indonesia/7/2003 [H5N1])

Group	Morbidity	Mortality
Sham	11/12	10/12
A/ck/HGO/28159-232/95	0/6	0/6
A/tk/Eng/N28/73	2/6	2/6
A/ck/Indonesia/7/03	0/6	0/6
A/tk/WI/68	0/6	0/6
Fowlpox-H5 AI	2/6	1/6

EU Ref 2005

Inactivated Vaccine Protection Against Asian H5N1 in Geese

White Chinese Geese vaccinated subcutaneously (SQ) 1wk with inactivated whole AI vaccine (A/turkey/Wisconsin/68 [H5N9]) and IN challenged at 4-wks-old with $10^{6.0}$ EID_{50} of HPAIV (A/chicken/Indonesia/7/2004 [H5N1])



EU Ref 2005

Inactivated Vaccine Protection Against Asian H5N1 in Ducks

Pekin ducks vaccinated SQ 1wk with inactivated whole AIV vaccine (A/ck/HGO/28159-232/95, A/tk/Eng/N28/73, A/ck/Indonesia/7/03) and IN challenged at 4-wks-old with $10^{6.0}$ EID_{50} of HPAIV (A/chicken/Indonesia/7/2004 [H5N1])

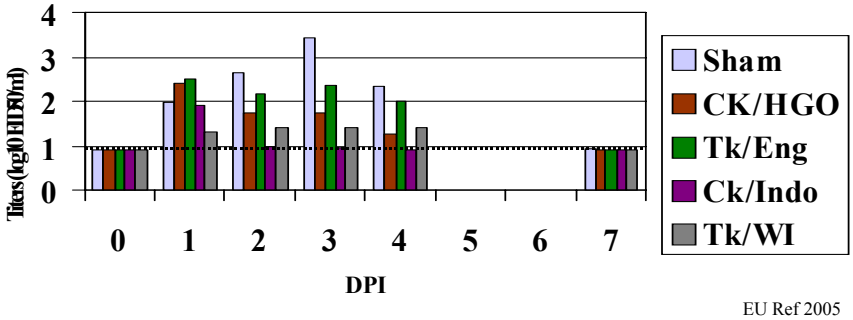
Group	Morbidity	Mortality
Sham	0/6	0/6
A/ck/HGO/28159-232/95	0/6	0/6
A/tk/Eng/N28/73	0/6	0/6
A/tk/WI/68	0/6	0/6
A/ck/Indonesia/7/03	0/6	0/6

EU Ref 2005

Inactivated Vaccine Protection Against Asian H5N1 in Ducks

Pekin ducks vaccinated SQ 1wk with inactivated whole AIV vaccine (A/ck/HGO/28159-232/95, A/tk/Eng/N28/73, A/ck/Indonesia/7/03, A/tk/WI/68) and IN challenged at 4-wks-old with $10^{6.0}$ EID_{50} of HPAIV (A/chicken/Indonesia/7/2004 [H5N1])

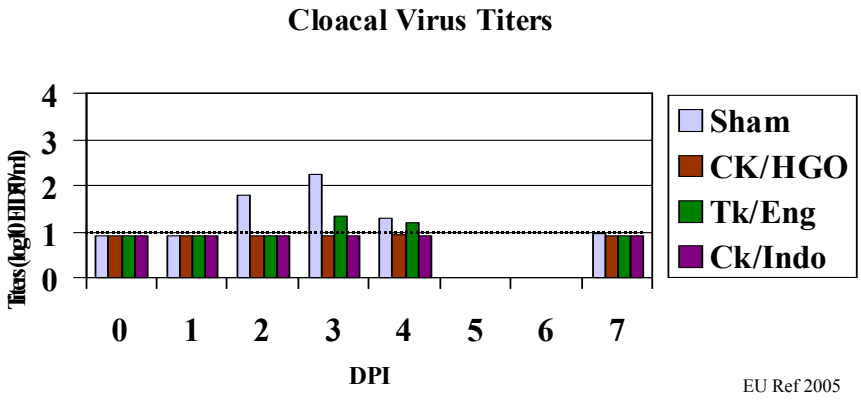
Oropharyngeal Virus Titers



EU Ref 2005

Inactivated Vaccine Protection Against Asian H5N1 in Ducks

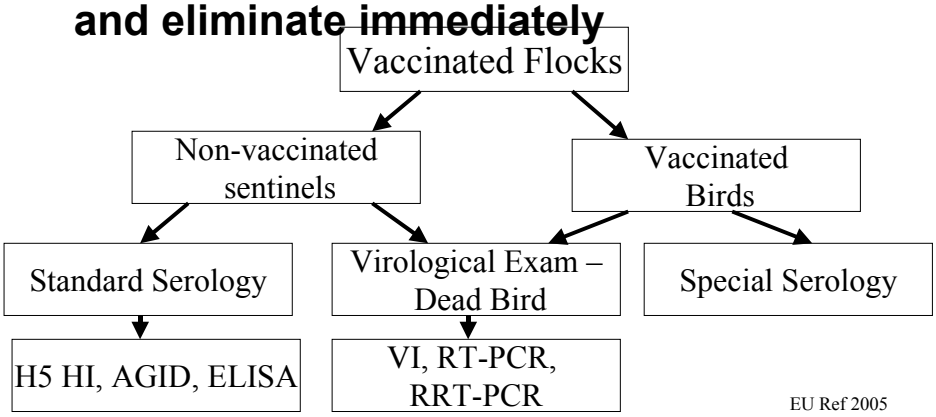
Pekin ducks vaccinated SQ 1wk with inactivated whole AIV vaccine (A/ck/HGO/28159-232/95, A/tk/Eng/N28/73, A/ck/Indonesia/7/03) and IN challenged at 4-wks-old with $10^{6.0}$ EID_{50} of HPAIV (A/chicken/Indonesia/7/2004 [H5N1])



EU Ref 2005

Surveillance is essential in vaccinated flocks

- **Must be able to distinguish infected from non-infected flocks in vaccinated populations - detect "silent" infections and eliminate immediately**



EU Ref 2005

Interference of AI Vaccination with Surveillance

	Serological Test				
			Homo.	Hetero.	
	NP/M (AGP/ELISA)	HA (HI)	NA (NI)	NA (NI)	NS
AI Field Virus	X	X	X	-	X
Homologous NA inactivated AIV vaccine	X	X	X	-	-
Heterologous NA inactivated AIV vaccine	X	X	-	X	-
Recombinant Fowlpox, subunit HA & DNA HA vaccines	-	X	-	-	-
Unvaccinated sentinels	-	-	-	-	-

EU Ref 2005

DIVA - AI Vaccine vs Infected

Chickens vaccinated SQ 1d with fowlpox-AIV-H5 recombinant* or inactivated whole AIV vaccine and IN challenged at 3 wks with low challenge dose ($10^{3.3}$ EID_{50} of HPAIV A/chicken/South Korea/2003 [H5N1]).**

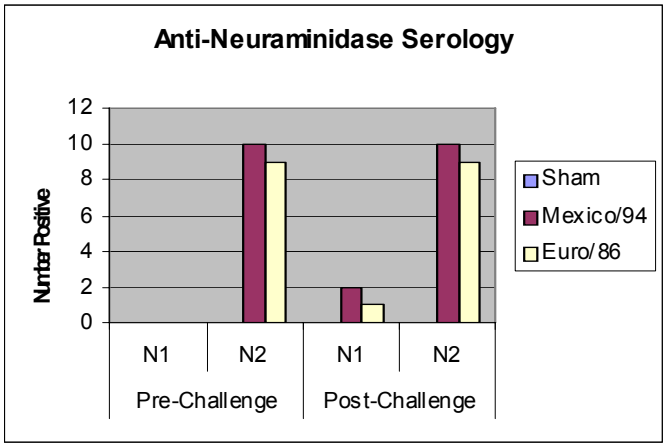
	3 weeks Post-Vaccination		14 days Post-Challenge	
	HI Serology, # positive/total (GMT)		HI Serology, # positive/total (GMT)	
Vaccine Group	TK/IRE/83	AGID	TK/IRE/83	AGID
Fowlpox recombinant (10^3 $TCID_{50}$)	$12/12^{A}$ $(446)^{ab}$	0/12	$12/12^{B}$ $(1261)^{a}$	1/12
Fowlpox recombinant (10^4 $TCID_{50}$)	$12/12^{A}$ $(478)^{a}$	0/12	$11/11^{A}$ $(1552)^{a}$	2/11
Diluent	$0/10^{B}$ $(NA)^{c}$	0/10	$0/1^{A}$	0/1
TW/68 Oil Emusified (2-3 µg hemagglutinin protein)	$12/12^{A}$ $(119)^{bc}$	12/12	$12/12^{A}$ $(478)^{a}$	12/12

- **Surveillance is very important – need to use homologous HI antigen**

EU Ref 2005

DIVA - AI Vaccine vs Infected

Chickens vaccinated SQ 3 wks with inactivated whole AIV vaccine and IN
Challenged 3 wks later with high dose (106.0 EID50 of HPAIV
A/chicken/Indonesia/7/2003 [H5N1])
1994 North American vaccine virus (Mexico/94)
1986 Eurasian vaccine virus (Pottsdam/86)



EU Ref 2005

DIVA Using Inactivated AI Vaccine: NS1 ELISA with Diluted Sera

Vaccine/Live	NS1 ELISA	AGID
Sham (3)	0/20	0/20
Live Virus (H1N1) – IN Tk	5/5	5/5
Live Virus (H7N2) – Field Ck	19/25	19/25
VX-commercial 2-3X (H1N1) – Field Tk	0/20	20/20
VX-commercial 2-3X (H7N2) – exp. Ck	0/5	5/5
VX-commercial & live – exp. tk	20/20	20/20

EU Ref 2005

Conclusions

- **Vaccines can be used to prevent, manage or eradicate AI in poultry**
- **In H5N1 Asian epizootic, four inactivated AI vaccines have been primary used, but live fowl pox recombinants has begun usage in Vietnam**
- **AI vaccines provide protection by preventing clinical signs and death, and reducing respiratory and intestinal virus replication in chicken and geese**
- **AI vaccines provide protection by reducing respiratory and intestinal virus replication in ducks**
- **Protection in ducks and geese is less than in chickens**
- **More research on adjuvants for inactivated vacciens to improve immune response in ducks and geese is needed**

EU Ref 2005

Thank You For You Attention!

EU Ref 2005

PROGRESSIVE TRUNCATION OF THE NON STRUCTURAL 1 GENE OF H7N1 AVIAN INFLUENZA VIRUSES FOLLOWING EXTENSIVE CIRCULATION IN POULTRY

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~~To determine whether the truncation could be the result of antibody selection pressure due to the immunogenicty of the carboxy terminal of the NS1 protein, a peptide spanning residues 219 aa to 230 aa was synthesized and tested in an indirect ELISA against sera obtained from turkeys experimentally infected with a virus strain known to have a full length NS1 protein. The peptide proved to be immunogenic suggesting that the observed carboxy-terminal truncation of the NS1 protein might be the result of antibody selection pressure.~~

1. Introduction

Avian influenza (AI) virus poses significant threats to both animal and human health. It is estimated that since 2000 approximately 200 million birds have died or have been culled worldwide as a result of AI. Since 1997, AI viruses belonging to the H5 or H7 subtype have crossed the species barrier and caused fatal disease in humans in several Asian countries and in The Netherlands. This event represents a serious threat in terms of the loss of human lives and a biological opportunity for the generation of a new human pandemic virus (For reviews see Horimoto and Kawaoka, 2001; Capua and Alexander, 2004).

The control of AI infections in poultry appears to be crucial in order to reduce the pandemic risk, as actively circulating virus in domestic poultry populations represents the main source of infectious virus for humans. International organisations have issued a series of recommendations aimed at bringing the ongoing Asian H5N1 epidemic under control (FAO\OIE, 2004; OIE\FAO 2005). In addition to direct control measures based on biosecurity, restriction policies and stamping out the appropriate use of vaccines is encouraged to maximise eradication efforts. It is known that vaccination prevents clinical disease, increases resistance to infection and reduces virus shedding levels, but does not prevent infection if birds are challenged with a sufficiently high dose of virus (Swayne and Suarez, 2000). For this reason, vaccinated birds may still become infected and shed virus into the environment without displaying any clinical signs, and therefore represent a means of spreading infection. In order to achieve the goal of eradication, so-called “DIVA” vaccination strategies, enabling the Differentiation of Infected

from Vaccinated Animals must be implemented. These systems coupled with an appropriate monitoring system, enable the detection of field exposure in vaccinated flocks and through this, infected flocks may be properly managed.

Several "DIVA" systems have been developed to date although they have some limitations in the field (Capua et al., 2002; Cattoli et al., 2003; Lee et al., 2004; Pasick, 2004). A promising system, based on the detection of antibodies against a specific antigen, the Non-Structural 1 (NS1) protein of AI has been deemed a good candidate (EU-SCAHAW 2003; Tumpey et al., 2005). The NS1 protein is synthesized in large amounts in infected cells but is not incorporated into the mature virions, and for this reason it could represent the ideal candidate to elicit a specific immune response only in the presence of active viral replication. It is a multifunctional protein, normally consisting of 230 aa residues, that has been implicated in the inhibition of host antiviral defences that are mediated by interferons. This is achieved through binding double stranded RNA which is a potent inducer of interferon. In addition, NS1 inhibits the posttranscriptional processing of cellular pre-mRNAs by binding and inhibiting the function of two cellular proteins that are required for the 3'-end processing of cellular pre-mRNA, namely the 30kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII) (for review see Krug et al.,2003).

One of the criteria for the success of DIVA is that the candidate antigen should be highly conserved thereby eliciting a similar immune response in the host regardless of the challenging viral strain. To determine whether this was the case for the NS1 protein, the *ns1* gene from a collection of AI isolates obtained between 1999 and 2003 in Northern Italy was analysed by sequencing.

The occurrence of four epidemic waves of H7N1 (LPAI and HPAI) between 1999 and 2001 (Capua and Marangon, 2000; Capua and Alexander, 2004), and the subsequent H7N3 epidemic between 2002 and 2003 (Capua and Alexander, 2004), represents a unique opportunity to examine AI isolates in a longitudinal manner, and evaluate the effects of extensive circulation in poultry including the effects on the antigenicity of viral proteins.

2. Materials and methods

2.1 Viruses

Viruses were obtained from the repository of the International Reference Laboratory in Padova, Italy and were typed using standard methods (CEC, 1992). They were isolated throughout the 1999-2001 H7N1 and 2002-2003 H7N3 avian influenza epidemics that occurred in Italy. The viruses used in this study, which were passaged in Specific pathogen free (SPF) eggs not more than twice, are listed in Table 1.

2.2 Gene amplification and sequencing

Viral RNA was extracted using the High Pure RNA isolation kit (Roche). The *ns1* gene was amplified by a one-step RT-PCR protocol using primers NS1F: 5′ gtgacaaaaacataatggattccaac 3′ and NS1R 5′ tcattaaataagctgaaacgagaaag 3′. The amplicon was sequenced directly using

the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) using primers NS1F, NS1R and an internal primer PNSR: 5′ cacaattgcaccatcttct 3′.

2.3 Sequence analysis

The nucleotide and deduced amino acid sequences obtained were analysed by ClustalW available at www.embl.de and MEGA (Kumar et al., 2004). The immunogenicity of the NS1 protein was analysed using the physico-chemical profiles program (Parker et al., 1986) available at http://npsa-pbil.ibcp.fr. Sequences have been deposited with Genbank under accession numbers DQ090025 to DQ090064.

2.4 Passage in eggs

The LPAI strains of subtype H7N1 A/Turkey/Italy/977/99 and A/Chicken/Italy/1082/99 were inoculated into 10-day-old embryonated chicken and 12-day-old embryonated turkey eggs by the allantoic route at a EID_{50} of $10^{5.7}$ and 10^{5} respectively. Allantoic fluid collected from each egg after chilling 48 hr post-inoculation was tested for hemagglutinating activity. The allantoic fluid was then diluted 1:10,000 in sterile phosphate-buffered saline solution (PBS) and inoculated into a new embryonated egg. This process was repeated 19 times.

3. Results

3.1 Sequence analysis

In order to determine the variability of the NS1 protein among Italian isolates, the complete *ns1* gene from 40 isolates were amplified by RT-PCR and sequenced. The sequences were compared phylogenetically and as previously reported the viruses separated into two subtypes A and B (Table 2) (Treanor et al., 1989; Ludwig et al., 1991). For *ns1* genes of influenza A viruses obtained from different species it is known that subtype A includes viruses from humans, horses, pigs and birds while subtype B represents those from birds only. In this study all of the H7N3 were located in the subtype A clade of the tree while H7N1 subtypes located in the subtype B clade.

Of the 40 isolates 16 had a full-length NS1 protein of 230 aa, 6 had a truncated protein of 220 aa and 18 had an intermediate carboxy-terminal truncation resulting in a protein of 224 aa (Table 2). All of the HPAI isolates had the intermediate truncation. Closer examination of the nucleotide sequence revealed that the carboxy-terminal truncations in the H7N1 isolates were due to a single nucleotide change at the respective positions. For the 220 aa truncated protein a C to A transversion was observed at nucleotide position 663 bp while for the 224 aa protein a C to T transversion was observed at nucleotide position 673 bp resulting in a TAA and a TGA stop codon respectively. No truncation was observed in the *ns1* gene of the H7N3 viruses.

3.2 Passage in eggs

It was imperative to determine that the carboxy-terminal truncations observations were not due to laboratory manipulation. For this reason, viruses A/ty/Italy/977/99 and A/ck/Italy/1082/99, both of which have complete NS1 proteins, were passaged 20 times in SPF chicken and turkey embryonated eggs. The gene was amplified and sequenced at passages 3, 10 and 20. No truncation was observed at any of the passages or either of the embryo species tested confirming that the carboxy-terminal truncation was not due to *in vitro* manipulation of the virus

4. Discussion

The NS1 protein has previously been described as a remarkably conserved protein amongst type A influenza viruses (Ludwig et al., 1991; Suarez and Perdue, 1998; Tumpey et al., 2005) and it is for this reason that it has been deemed a good candidate protein for used in the DIVA strategy. Indeed, a paper by Birch-Machin et al. (1997) reported that antibodies to NS1 could be detected in serum samples of ponies experimentally infected with equine influenza virus, but not in animals vaccinated with whole inactivated virus. Likewise a study by Ozaki et al. (2001) identified antibodies to the NS1 exclusively in the sera of mice infected with equine influenza viruses and not in those mice immunized with inactivated virus. These data indicated that the NS1 protein could be used for serological diagnosis to distinguish horses infected with equine influenza viruses from those immunized with inactivated vaccines. Similarly, recent work on a limited number of avian samples has indicated that antibodies to the NS1 protein could possibly be used as part of a DIVA strategy as seroconversion to antibodies against the NS1 protein was achieved in chickens and turkeys experimentally infected with different subtypes of influenza A virus. A similar reaction was not detected in birds inoculated with inactivated vaccines (Tumpey et al., 2005).

The present study, however, has shown that in the case of extensive viral circulation in poultry, the NS1 protein is not as conserved as originally believed. Variability in NS1 sequence length has been previously reported in influenza A viruses isolated from birds, pigs, horses and humans (Suarez and Perdue, 1998). In the afore mentioned study the NS1 protein of 3 out of 65 avian influenza isolates was truncated. Two of these isolates had a predicted NS1 protein of 217 aa while one had a truncated protein of 124 aa. A more recent report by Guan et al., (1999) identified a similar 13 aa truncation NS1 protein in five out of fourteen avian influenza isolates analysed. In addition, there has been a recent Genbank submission of 19 sequences of influenza virus isolates of the H9N2 subtype from Southern China all of which have the 13 aa carboxy-terminal truncation of (Genbank accession numbers AY664736 to AY664754). Although these data confirm that truncation in the NS1 protein occurs in nature and is a noted phenomenon there has been little or no discussion as to the significance of these truncations.

The 10 amino acid truncation in the NS1 protein demonstrated in this present work has also been recently reported by two other groups (MacRae et al., 2005; Gorvorkova et al, 2005). Interestingly MacRae et al., (2005) have identified the same truncation in one strain of influenza A (H3N8) isolated from

horses in the United Kingdom while Govrorkova et al, (2005) have identified the truncation in a Vietnamese influenza A isolate (A/Vietnam/1203/04) isolated from a human and shown to cause severe disease in a ferret model. MacRae et al., (2005) have suggested that because the 10 amino truncation removes the putative PAB(II) binding site from the NS1 protein the truncation may explain the increased pathogenicity observed in their isolate. From the data presented in this work~~, this, ,~~ however, it is not possible to determine whether the truncations have an effect on the pathogenicity of the isolates investigated.~~s unlikely given that the strains possessing the 10 amino acid truncation are less pathogenic that those with just the 6 amino truncation. One would expect the isolate with the longer truncation to be, if not more pathogenic, at least equal in it pathogenicity to the isolate with the 6 amino acid truncation.~~

The longitudinal approach of the present investigation has shown that the truncation is a progressive occurrence during the epidemic. Indeed, all the LPAI viruses circulating at the beginning of the H7N1 epidemic had full-length NS1, while the protein was progressively more truncated in the LPAI viruses that were circulating later in the epidemic. Furthermore, all of the HPAI viruses and only them, had the intermediate truncation. Whether this truncation may be correlated with the increased virulence of these strains is presently unknown and cannot be concluded from this study.

~~The demonstrated immunogenicty of the carboxy terminal provides one possible explanation for the truncations observed in this study. It has been proposed by other researchers that selection by antibodies or other immune mechanisms may play a major role in the rapid evolution of viruses in nature (Rojas et al., 1992; Schiappacassi, 1995, Price et al, 2000). Populations of RNA viruses exists as quasispecies due to the error rate of the RNA polymerase which replicates the RNA genomes (Holland, 1993). If a virus is under selective pressure from the host immune system, mutations that confer an increase in fitness can be selected for and can eventually predominate in the viral population. Therefore the truncations observed in this work may be due to selective pressure on the *ns1* gene following extensive circulation in poultry although the effects of this selective pressure on viral efficiency remains unclear. In the presence of widespread antibodies to NS1, isolates with a truncation of the *ns*1 gene may supplant isolates with the full length *ns*1 in viral population dynamics.~~

The data obtained from this investigation should be taken into account when validating a diagnostic test based on the detection of antibodies to the NS1 protein of AI. Although the truncations were only seen in the H7N1 isolates and not the H7N3 isolates, the progressive truncation of the gene, resulting in a modified antigenicity, is most probably not an isolated event that just occurred during the Italian H7N1 epidemic. It is possible that a similar progressive truncation occurs with other AI subtypes following extensive circulation in poultry, including the ongoing H5N1 Asian epidemic. Further investigations on this aspect should be carried out on viruses occurring in endemic situations.

Provided they occur in the *ns*1 gene after prolonged circulation~~following endemicity~~, these modifications could be used as molecular markers in epidemiological studies. An example of this would be the possibility of

establishing whether the re-emergence of AI viruses that have occurred in certain situations worldwide (e.g. Mexico, Italy, Pakistan~~, Asia~~) can be correlated with the re-introduction of isolates that have been previously identified either at an early or late stage in an epidemic. These data would be useful in better understanding the transmission dynamics and the role of reservoirs in the eco-epidemiology of the AI.

Acknowledgements

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Table 1. Influenza viruses used in this study.

Virus	Subtype	Pathotype	Virus	Subtype	Pathotype
A/Turkey/Italy/977/99	H7N1	LP	A/Turkey/Italy/1713/00	H7N1	HP
A/Chicken/Italy/1081/99	H7N1	LP	A/Quail/Italy/1764/00	H7N1	HP
A/Chicken/Italy/1082/99	H7N1	LP	A/Pheasant/Italy/1847/00	H7N1	HP
A/Turkey/Italy/1086/99	H7N1	LP	A/Duck/Italy/1848/00	H7N1	HP
A/Chicken/Italy/1279/99	H7N1	LP	A/Turkey/Italy/2023/00	H7N1	HP
A/Turkey/Italy/1744/99	H7N1	LP	A/Turkey/Italy/2330/00	H7N1	HP
A/Turkey/Italy/2716/99	H7N1	LP	A/Ostrich/Italy/2332/00	H7N1	HP
A/Turkey/Italy/2732/99	H7N1	LP	A/Turkey/Italy/4426/00	H7N1	LP
A/Turkey/Italy/3675/99	H7N1	LP	A/Turkey/Italy/7002/00	H7N1	LP
A/Turkey/Italy/4580/99	H7N1	HP	A/Turkey/Italy/223/01	H7N1	LP
A/Chicken/Italy/5074/99	H7N1	HP	A/Turkey/Italy/284/01	H7N1	LP
A/Chicken/Italy/78/00	H7N1	HP	A/Chicken/Italy/322/01	H7N1	LP
A/Turkey/Italy/421/00	H7N1	HP	A/Turkey/Italy/1351/01	H7N1	LP
A/Chicken/Italy/522/00	H7N1	HP	A/Turkey/Italy/8000/02	H7N3	LP
A/Duck/Italy/551/00	H7N1	HP	A/Turkey/Italy/8534/02	H7N3	LP
A/Turkey/Italy/577/00	H7N1	HP	A/Turkey/Italy/8535/02	H7N3	LP
A/Chicken/Italy/662/00	H7N1	HP	A/Turkey/Italy/2962/03	H7N3	LP
A/Chicken/Italy/910/00	H7N1	HP	A/ Turkey/Italy/3620/03	H7N3	LP
A/Chicken/Italy/914/00	H7N1	HP	A/Quail/Italy/4610/03	H7N3	LP
A/Ostrich/Italy/984/00	H7N1	HP	A/Chicken/Italy/4616/03	H7N3	LP

Table 2. ClustalW analysis of the carboxy terminal of the NS1 protein. The sequence of the peptide NS1$^{219-230}$ is underlined.

Isolate	Subtype	Pathotype	Allele	Carboxy-terminal sequence
A/Turkey/Italy/977/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Chicken/Italy/1081/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Chicken/Italy/1082/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Turkey/Italy/1086/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Chicken/Italy/1279/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Turkey/Italy/1744/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Turkey/Italy/2716/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRYMARRVESEV- 230
A/Turkey/Italy/2732/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRHMARRVEPEV- 230
A/Turkey/Italy/3675/99	H7N1	LP	B	FAWGIRDENGGPPLPPKQKRHMARRVEPEV- 230
A/Turkey/Italy/4580/99	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/5074/99	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/78/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/421/00	H7N1	HP	B	FAWGIHDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/522/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Duck/Italy/551/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/577/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/662/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/910/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Chicken/Italy/914/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Ostrich/Italy/984/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/1713/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Quail/Italy/1764/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Pheasant/Italy/1847/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Duck/Italy/1848/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/2023/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/2330/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Ostrich/Italy/2332/00	H7N1	HP	B	FAWGIRDENGGPPLPPKQKRYMAR------- 224
A/Turkey/Italy/4426/00	H7N1	LP	B	FAWGIRDENGGPPLPPKQKR---------- 220
A/Turkey/Italy/7002/00	H7N1	LP	B	FAWGIRDENGGPPLPPKQKR---------- 220
A/Turkey/Italy/223/01	H7N1	LP	B	FAWGIRDENGGPPLPPKQKR---------- 220
A/Turkey/Italy/284/01	H7N1	LP	B	FAWGIHDENGGPPLPPKQKR---------- 220
A/Chicken/Italy/322/01	H7N1	LP	B	FAWGIRDENGGPPLPPKQKR---------- 220
A/Turkey/Italy/1351/01	H7N1	LP	B	FAWGIRDENGGPPLPPKQKR---------- 220
A/Turkey/Italy/8000/02	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKRKMARTIEPEV- 230
A/Turkey/Italy/8534/02	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKRKMARTIESEV- 230
A/Turkey/Italy/8535/02	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKRKMARTIESEV- 230
A/Turkey/Italy/2962/03	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKWKMARTIESEV- 230
A/Turkey/Italy/3620/03	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKWKMARTIESEV- 230
A/Quail/Italy/4610/03	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKRKMARTIESEV- 230
A/Chicken/Italy/4616/03	H7N3	LP	A	FAWRSSNEDGRPPLPPKQKRKMARTIESEV- 230

Unusual Characteristics of Highly Pathogenic Avian Influenza Viruses of North American Lineage

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Presentation Outline

- Recent changes in reporting
- Emergence of HPAI
- Outbreaks – Chile, USA, Canada
- Virus characteristics
- Conclusions

USDA-APHIS

Recent Changes in Requirements for International Reporting

- May 2005
- Adopted new code chapter on HPAI
- All infections of H5 and H7 (LPAI and HPAI) are now Notifiable
  - ✓Isolation of H5 or H7 virus
  - ✓Detection of H5 or H7 specific RNA
  - ✓Detection of H5 or H7 antibodies (not due to vaccination or non specific)

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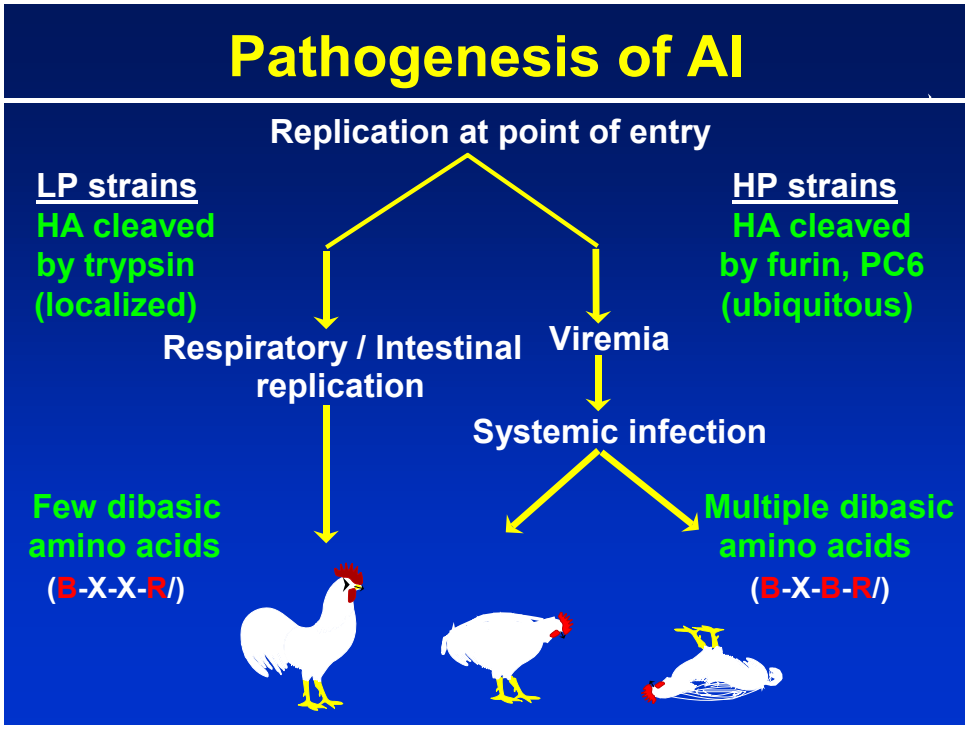
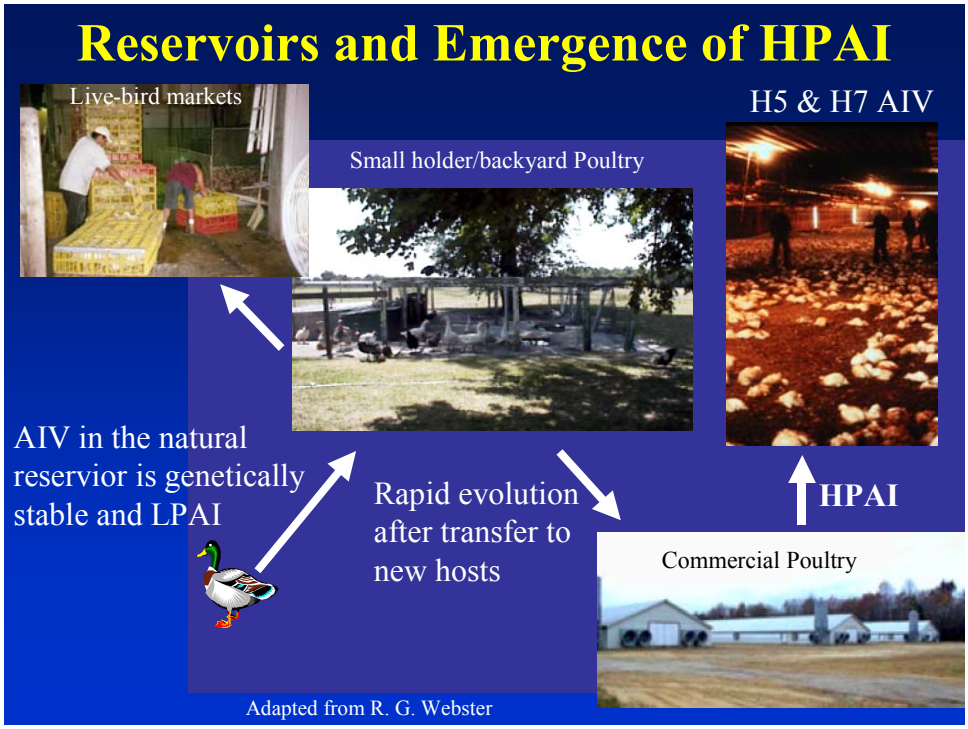
Highly Pathogenic Avian Influenza

Current USDA/OIE definition:

1. Any influenza virus that kills 6, 7, or 8 of 8 chickens (75% mortality) or IVPI of >1.2
2. Any H5 or H7 subtype that does not meet the criteria in item 1, but has an amino acid sequence at the cleavage site of the hemagglutinin that is compatible with other HPAI viruses


Pathogenesis of AI
Replication at point of entry
LP strains
HA cleaved
by trypsin
(localized)
HP strains
HA cleaved
by furin, PC6
(ubiquitous)
Respiratory / Intestinal
replication
Viremia
Systemic infection
Few dibasic
amino acids
(B-X-X-R/)
Multiple dibasic
amino acids
(B-X-B-R/)


Reservoirs and Emergence of HPAI
Live-bird markets
H5 & H7 AIV
Small holder/backyard Poultry
AIV in the natural
reservior is genetically
stable and LPAI
Rapid evolution
after transfer to
new hosts
HPAI
Commercial Poultry
Adapted from R. G. Webster

Examples of Emergence of HPAI from LPAI Precursors (Mutation)

- 1983 – United States (H5N2)
  - ✓ 6 months (loss of carbohydrate)
- 1994 – Mexico (H5N2)
  - ✓ 14 months (insertion of dibasic amino acids)
- 2002 – Chile (H7N3)
  - ✓ 4 weeks (recombination with NP gene)
- 2004 – United States (H5N2)
  - ✓ 2 years? (substitution)
- 2004 – Canada (H7N3)
  - ✓ 11 days (recombination with M gene)

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HPAI – Santiago, Chile (2002)

- May 2002 – H7N3 LPAI
  - ✓ Broiler breeder flock – >540,000
  - ✓ Egg drop, slight increase in mortality
  - ✓ IVPI = 0.0
  - ✓ PEKPKTR/GLF
- June 2002 – H7N3 HPAI
  - ✓ Sharp increase in mortality
  - ✓ IVPI = 2.8 – 3.0
  - ✓ **P E K P K T C S P L S R C R K T R / G L F**
  - ✓ **P E K P K T C S P L S R C R E T R / G L F**

30 nt insertion (from nucleoprotein gene)

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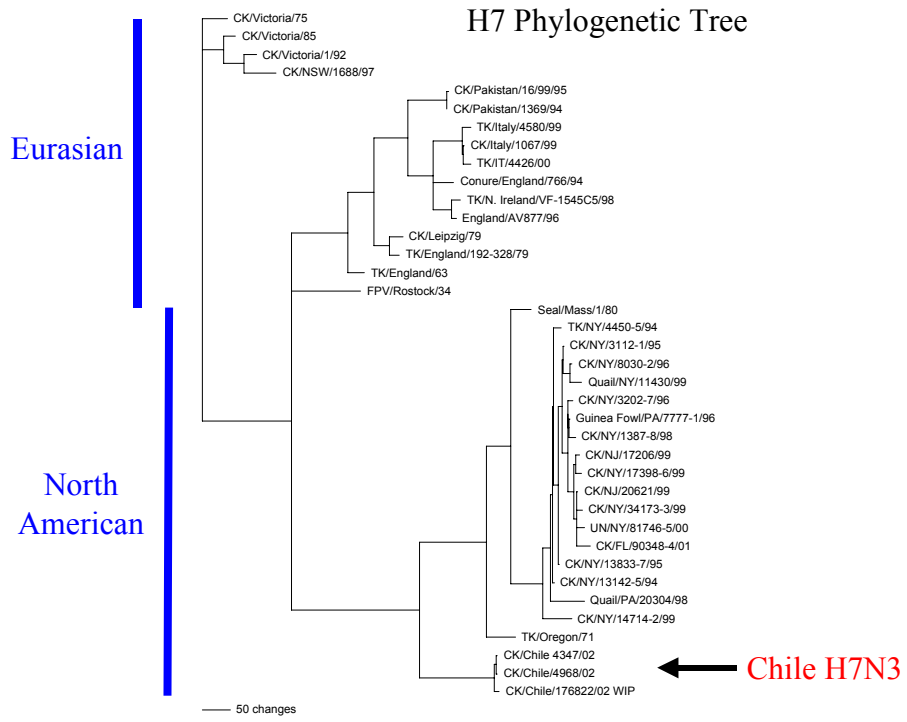

H5N2 – Gonzales, Texas (2004)

Phantom HPAI

February 23, 2004

- First HPAI outbreak in U.S. in 20 years
- Broilers produced for Houston LBM – > 6,600
- Respiratory signs (mycoplasma suspected)

Texas HP H5N2 (2004): *Virus Characteristics*

- Amino acid sequence compatible with HPAI
- **H5N2 – PQRKKR/GLF**
- **H5N1 – PQRKKR/GLF** (A/Ck/Scotland/59)
- IVPI = 0.0
- Closely related to A/CK/TX/02 (H5N3)
  - ✓98% sequence homology (HA gene)
  - ✓Two nucleotide changes near cleavage site:

(TX/02) **PQREKR/GLF** → (TX/04) **PQRKKR/GLF** (?)

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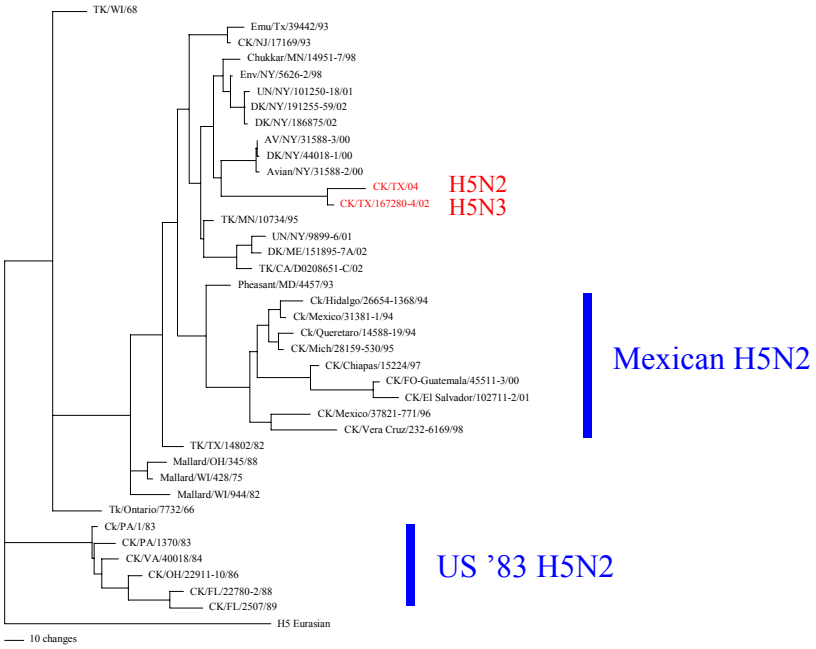
H5 Phylogenetic Tree



HPAI in B.C., Canada
Frazer Valley (2004)

- Feb 19 – LPAI H7N3 in 52-week-old broiler breeders
- March 8 – HPAI H7N3 in 24-week-old broiler breeders on same farm
- First report of HP since 1966
- Disease spread to 42 farms
- Approximately 19 million birds destroyed

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Virus Characteristics
HPAI H7N3, Canada (2004)

- LP H7N3
  - ✓IVPI = 0.0
  - ✓**PENPKTR / GLF**
- HP H7N3
  - ✓IVPI = 2.96
  - ✓**PENPKQAYRKRMTR / GLF**

21 nucleotide insert (from matrix gene)

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Summary

- All H5 and H7 infections (LPAI and HPAI) are now Notifiable to OIE
- Three HPAI outbreaks in the Americas since 2002 with unusual characteristics…
  - ✓Two – viruses (H7N3) met the virulence but not the molecular criterion (Chile, Canada)
  - ✓One – virus (H5N2) met the molecular but not the virulence criterion (USA)

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Conclusions

- AI surveillance is important!
- Non-homologous recombination is a novel mechanism for increased virulence in H7 AIV
- H5 and H7 AI viruses are unpredictable!!
- The outbreaks of HPAI in Chile, the USA and Canada since 2002 provide further support for treating all H5 and H7 infections in domestic poultry as potentially highly pathogenic and subject to international reporting

USDA-APHIS

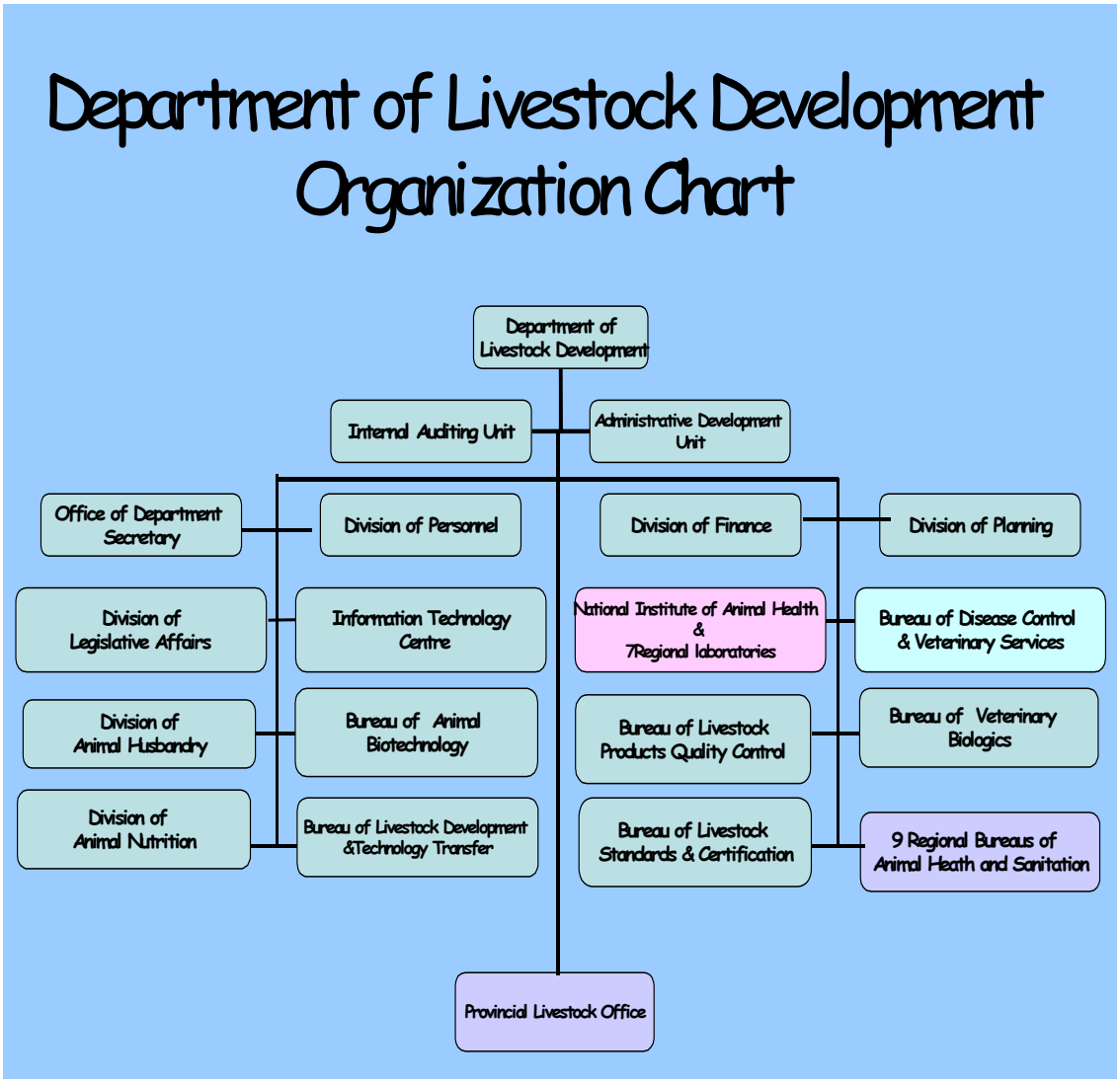
HPAI in SE Asia : Thailand

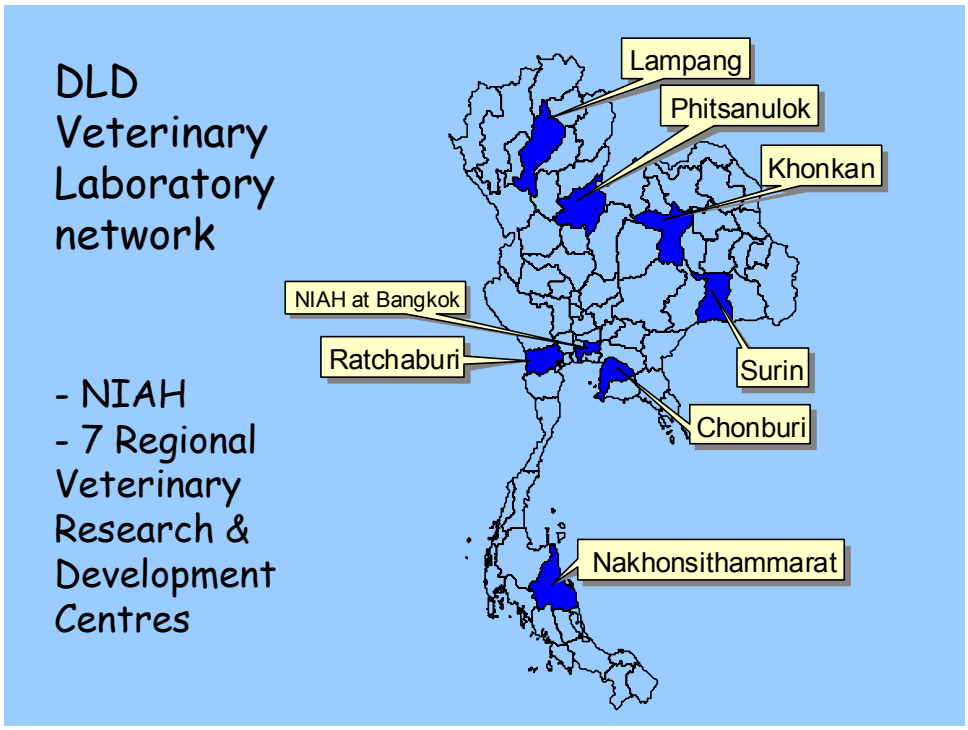
Department of Livestock Development

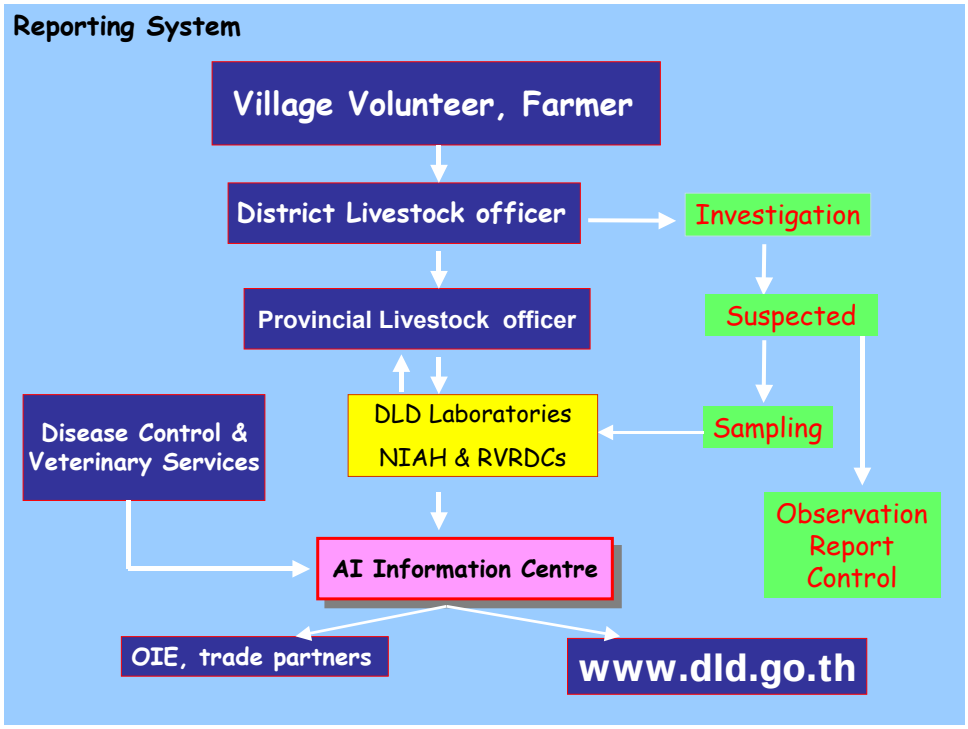
Arunee Chaisingh
National Institute of Animal Health
Bangkok, Thailand

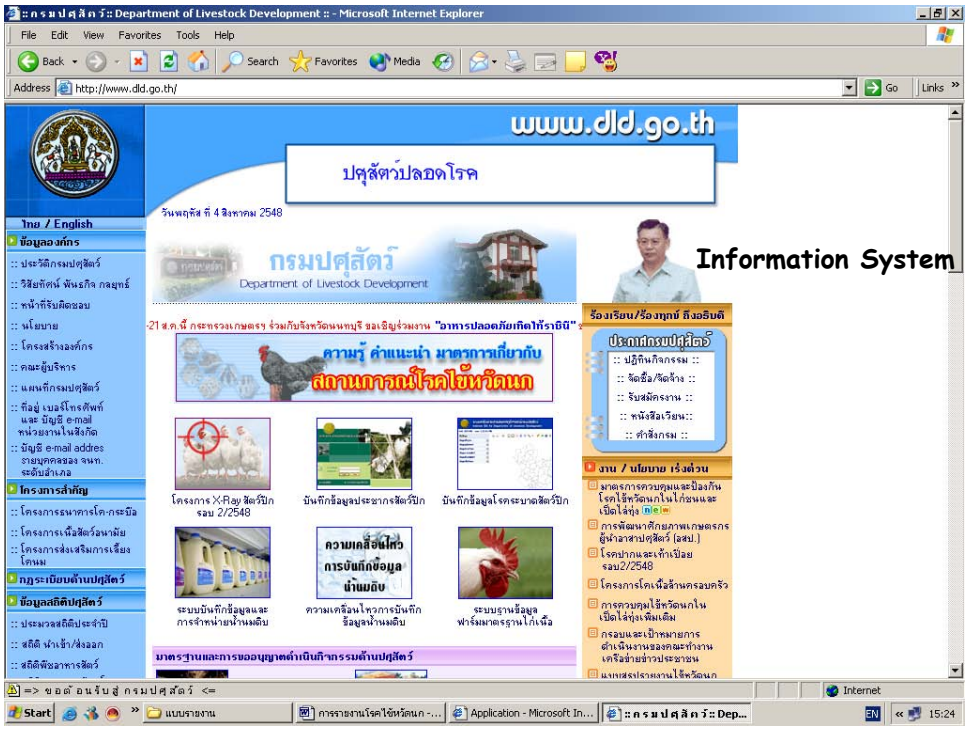
Outline

- HPAI Information Network System
- HPAI Disease Status
- HPAI Disease Control Measures
- HPAI Disease Surveillance


Department of Livestock Development Organization Chart
Department of Livestock Development
Internal Auditing Unit
Administrative Development Unit
Office of Department Secretary
Division of Personnel
Division of Finance
Division of Planning
Division of Legislative Affairs
Information Technology Centre
National Institute of Animal Health & 7Regional laboratories
Bureau of Disease Control & Veterinary Services
Division of Animal Husbandry
Bureau of Animal Biotechnology
Bureau of Livestock Products Quality Control
Bureau of Veterinary Biologics
Division of Animal Nutrition
Bureau of Livestock Development &Technology Transfer
Bureau of Livestock Standards & Certification
9 Regional Bureaus of Animal Heath and Sanitation
Provincial Livestock Office


DLD
Veterinary
Laboratory
network
- NIAH
- 7 Regional
Veterinary
Research &
Development
Centres
Lampang
Phitsanulok
Khonkan
NIAH at Bangkok
Ratchaburi
Surin
Chonburi
Nakhonsithammarat


Reporting System
Village Volunteer, Farmer
District Livestock officer
Investigation
Provincial Livestock officer
Suspected
Disease Control &
Veterinary Services
DLD Laboratories
NIAH & RVRDCs
Sampling
Observation
Report
Control
AI Information Centre
OIE, trade partners
www.dld.go.th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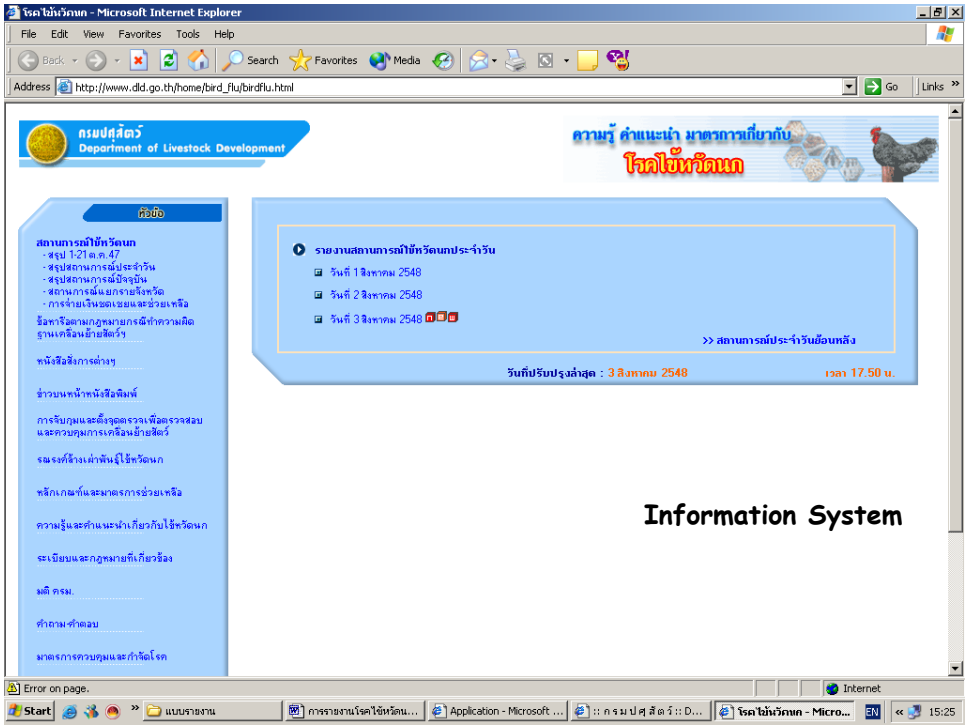
Information System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กรมปศุสัตว์
Department of Livestock Development
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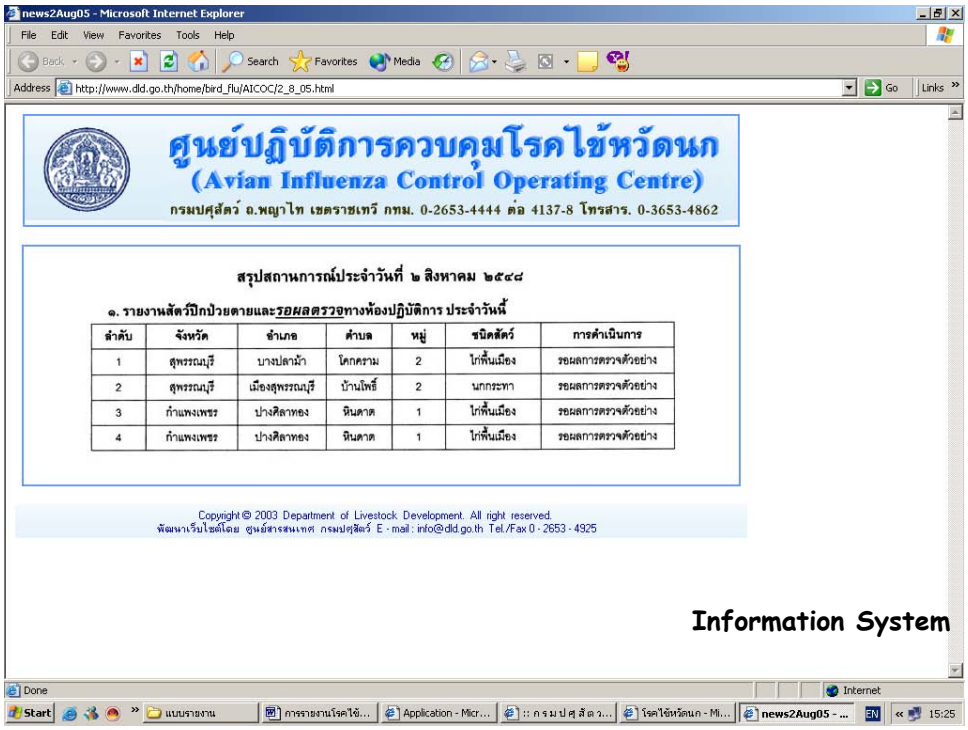
news2Aug05 - Microsoft Internet Explorer
http://www.dld.go.th/home/bird_flu/AICOC/2_8_05.html
ศูนย์ปฏิบัติการควบคุมโรคไข้หวัดนก
(Avian Influenza Control Operating Centre)
กรมปศุสัตว์ ถ.พญาไท เขตราชเทวี กทม. 0-2653-4444 ต่อ 4137-8 โทรสาร. 0-3653-4862
สรุปสถานการณ์ประจำวันที่ ๒ สิงหาคม ๒๕๔๘
๑. รายงานสัตว์ปีกป่วยตายและรอผลตรวจทางห้องปฏิบัติการ ประจำวันนี้
ลำดับ | จังหวัด | อำเภอ | ตำบล | หมู่ | ชนิดสัตว์ | การดำเนินการ
1 | สุพรรณบุรี | บางปลาม้า | โคกคราม | 2 | ไก่พื้นเมือง | รอผลการตรวจตัวอย่าง
2 | สุพรรณบุรี | เมืองสุพรรณบุรี | บ้านโพธิ์ | 2 | นกกระทา | รอผลการตรวจตัวอย่าง
3 | กำแพงเพชร | ปางศิลาทอง | หินดาด | 1 | ไก่พื้นเมือง | รอผลการตรวจตัวอย่าง
4 | กำแพงเพชร | ปางศิลาทอง | หินดาด | 1 | ไก่พื้นเมือง | รอผลการตรวจตัวอย่าง
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พัฒนาเว็บไซต์โดย ศูนย์สารสนเทศ กรมปศุสัตว์ E - mail : info@dld.go.th Tel./Fax 0 - 2653 - 4925
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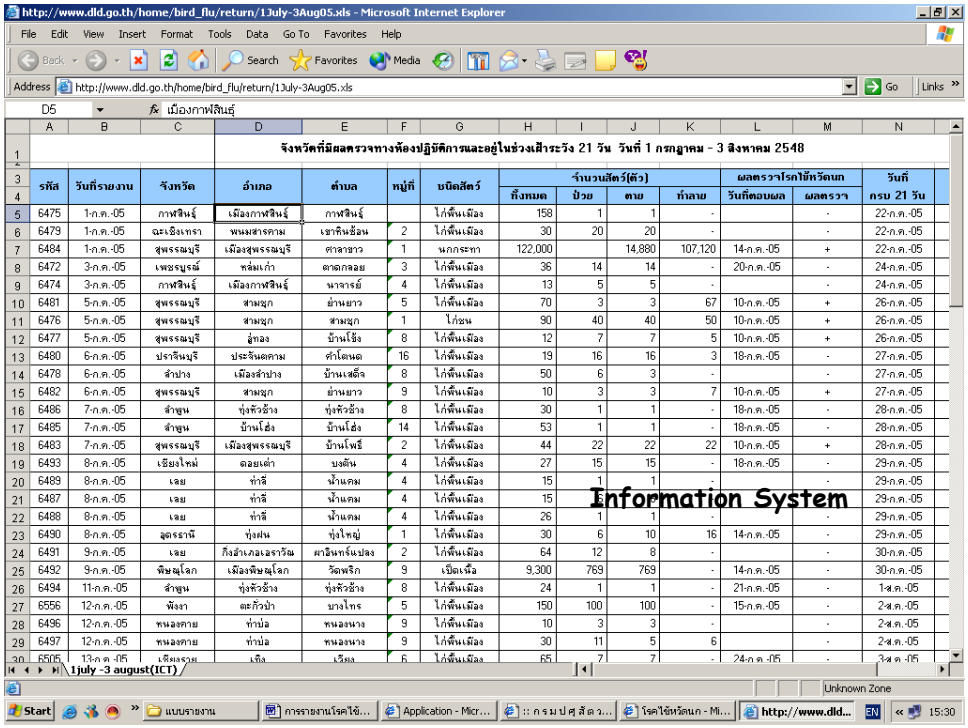
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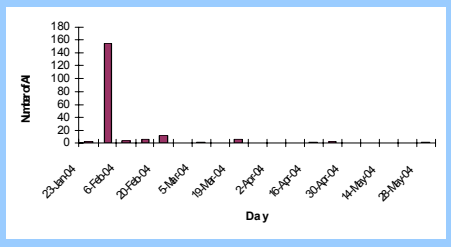
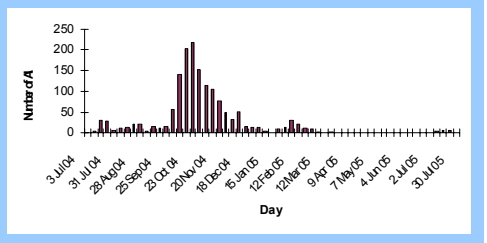
จังหวัดที่มีผลตรวจทางห้องปฏิบัติการและอยู่ในช่วงเฝ้าระวัง 21 วัน วันที่ 1 กรกฎาคม - 3 สิงหาคม 2548
Information System

HPAI Disease Status

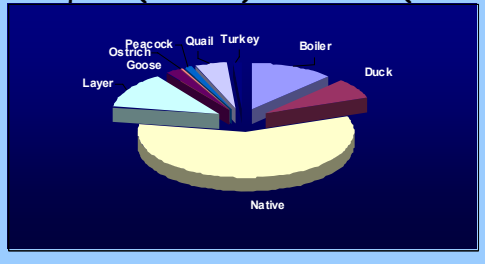
- First wave (23rd January 04 – 24th May 04)
- Second wave (3rd July 04 – 12th April 05)
- Third wave (1st July 05 – 22th September 05)

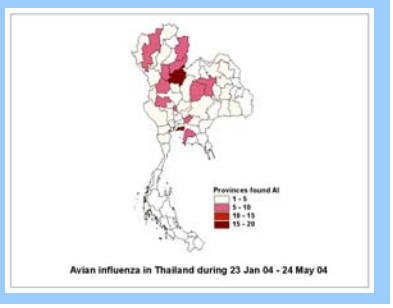




HPAI : 1st wave (23rd Jan – 24th May 04)

- 190 cases in 89 districts, 42 provinces
- Affected animals
  - native chicken (63.68%)
  - boiler (11.58%)
  - layer (10.53%)
  - duck (6.32%)
  - quail (4.74%) & others (3.15%)





HPAI : 1st wave (23rd Jan – 24th May 04)

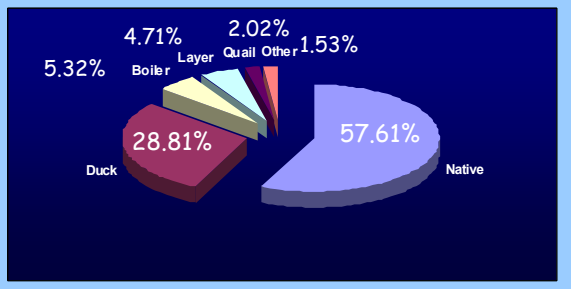
- Destroyed ~ 30 Million birds
- Culling poultry
  - 5 Km. around HPAI cases
    23 Jan – 10 Feb 2004
  - 1 Km. around HPAI cases
    11 – 29 Feb 2004
  - Affected HPAI cases
    From 1 Mar 2004 - Present

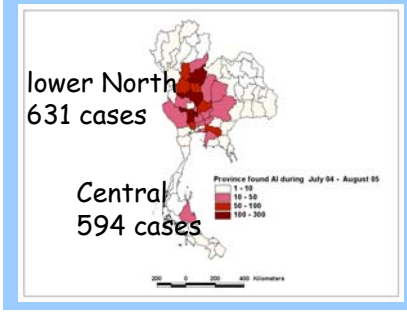
HPAI : 1st wave (23rd Jan – 24th May 04)

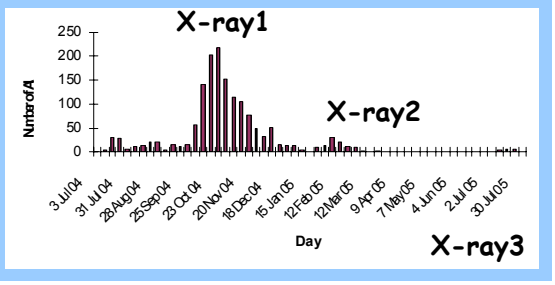
- Government's spent 2,999.2 million Baht (60 M euro) compensation (either in cash according to an official rate, or in terms of breeding stock)
- Programmes for rehabilitation cost 2,824 million Baht (56 M euro) to affected poultry farmers (low interest rate or soft loan, longer debt payment & interest suspension period etc)

HPAI: 2nd wave (3rdJuly 04 – 12thApril 05)

- 1,539 cases in 264 districts, 51 provinces

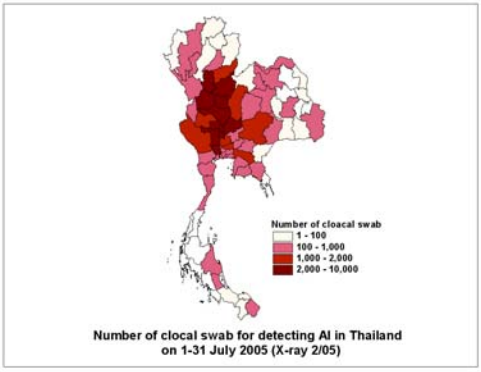




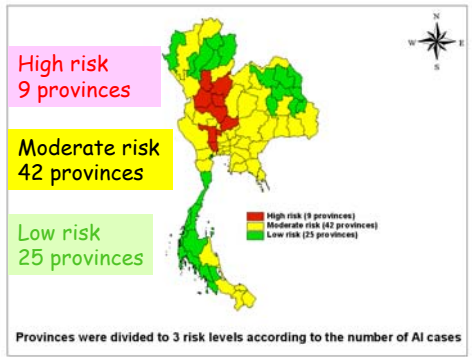


Third wave (1st July – 22th Sep 05)

- 37 cases in 13 districts, 5 provinces



Number of clocal swab for detecting AI in Thailand on 1-31 July 2005 (X-ray 2/05)



Provinces were divided to 3 risk levels according to the number of AI cases

Affected Poultry

native chicken (81.1%)
quail (8.1%)
boiler (5.4%)
layer& duck (2.1% each)

HPAI Disease Control Measures

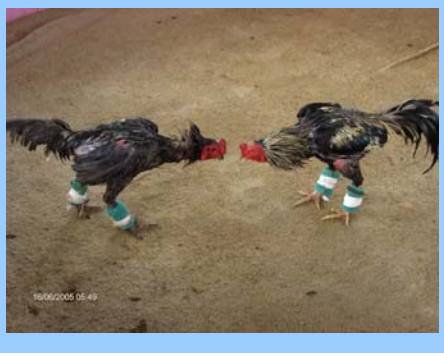
- HPAI is a national notifiable disease under Animal Epidemic Act B.E.2499 (A.C.1956)
- Establishment of HPAI Disease Control Committee & Operating Centre (National to Provincial levels)
- Depopulation

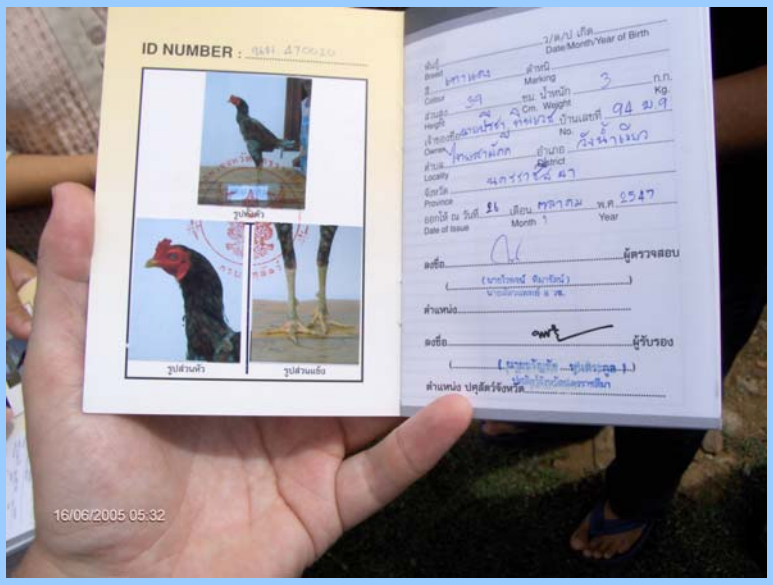
HPAI Disease Control Measures

- Compensation 75%, by Laws
- Disinfection & Disposal (carcasses, products & wastes)

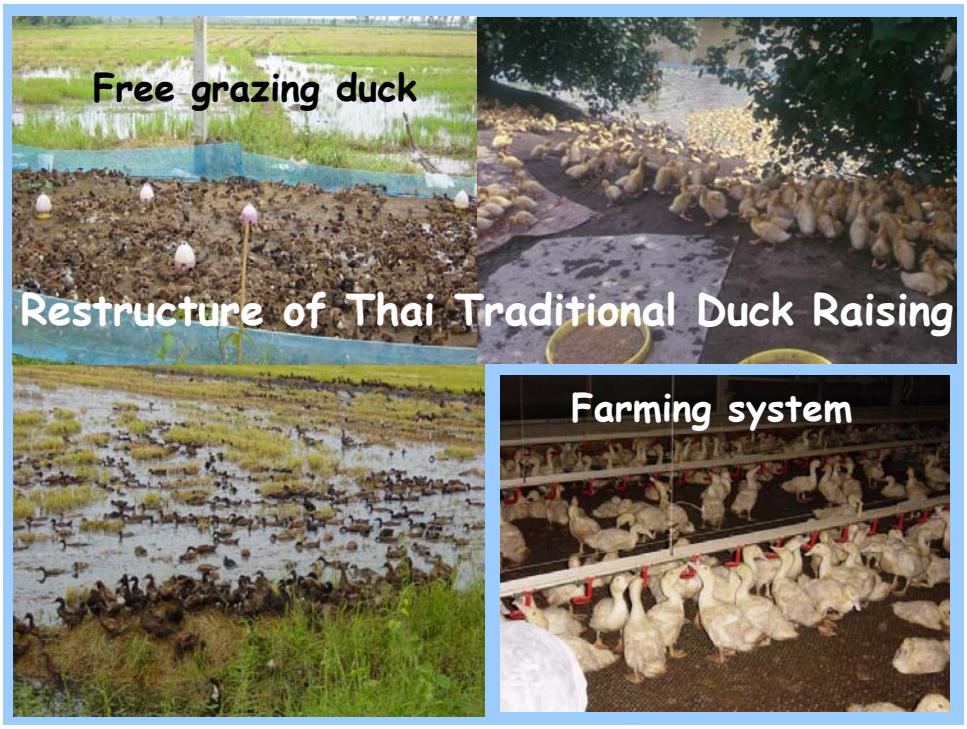
HPAI Disease Control Measures

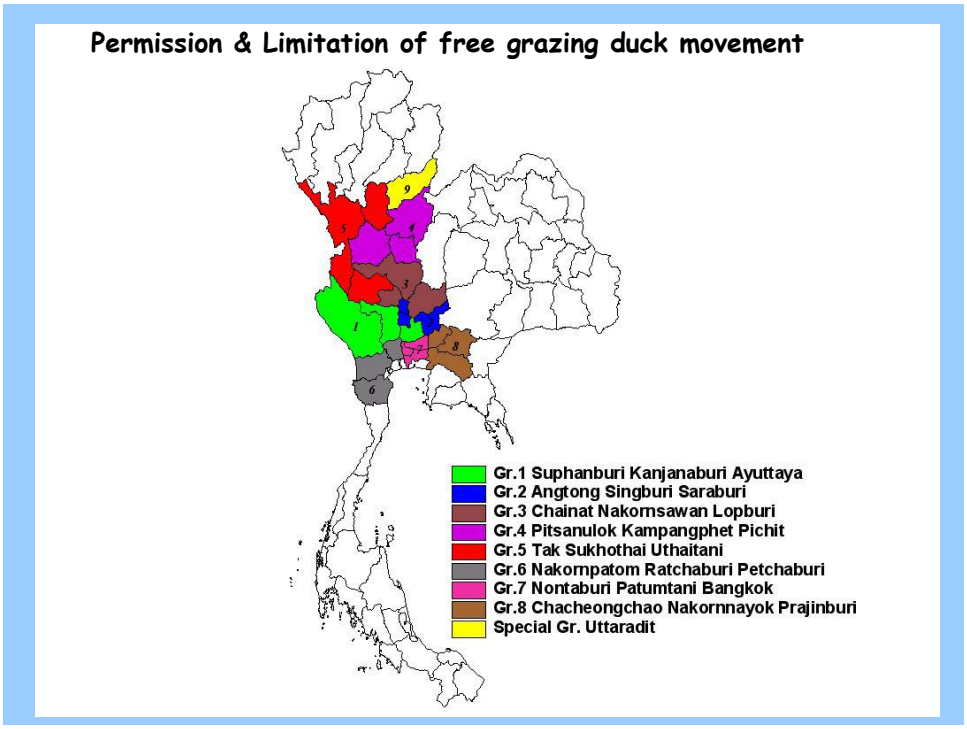
- Quarantine & Movement control
  - around affected areas
  - within zone and between zone
    esp.free grazing ducks & fighting cocks





Fighting cock passport


Free grazing duck
Restructure of Thai Traditional Duck Raising
Farming system


Permission & Limitation of free grazing duck movement
Gr.1 Suphanburi Kanjanaburi Ayuttaya
Gr.2 Angtong Singburi Saraburi
Gr.3 Chainat Nakornsawan Lopburi
Gr.4 Pitsanulok Kampangphet Pichit
Gr.5 Tak Sukhothai Uthaitani
Gr.6 Nakornpatom Ratchaburi Petchaburi
Gr.7 Nontaburi Patumtani Bangkok
Gr.8 Chacheongchao Nakornnayok Prajinburi
Special Gr. Uttaradit

HPAI Disease Control Measures

- Poultry zoning or Regionalization
- Investigation, Surveillance & Monitoring
- Intensive Farm Biosecurity

32 quarantine stations between each zones

HPAI Disease Control Measures

- National Cooperation
- International Coordination
- Public awareness & education on HPAI
- Human Health Emphasis : Government policy
- No AI Vaccination in poultry (Thailand Policy)

Disease surveillance

Clinical surveillance

- 175,215 officials & 892,072 volunteers
- poultry death >10% within a day
- sudden death, resp., diarrhea, nervous signs
- in duck : cataract, swelling head

Disease surveillance

Active surveillance : VI

1. Chicken safety (Jan - Mar 04)
2. Nation wide surveillance : x-ray 1 (Oct 04)
3. Nation wide surveillance : x-ray 2 (Feb 05)
4. Nation wide surveillance : x-ray 3 (Jul 05)

Next Plan : VI & HI

1. Before the end of 2005
2. Early of 2006

Disease surveillance

- Strengthening lab capability & biosafety (BSL-2 ⇨ BSL-3)
  - Testing method
    - : VI (egg/cell culture)
    - : Real time PCR (H5, N1, M)
    - : PCR/Gene sequencing

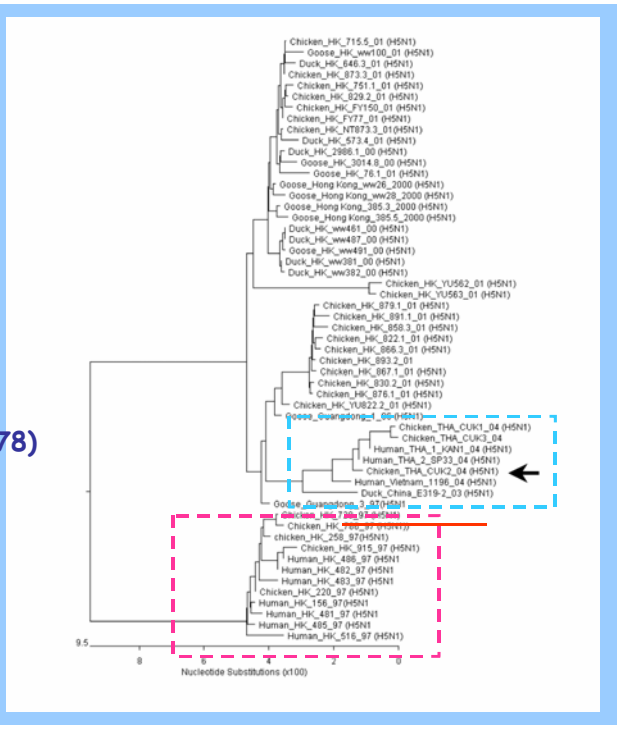
Hemagglutinin (HA) gene

...predicting the catastrophe

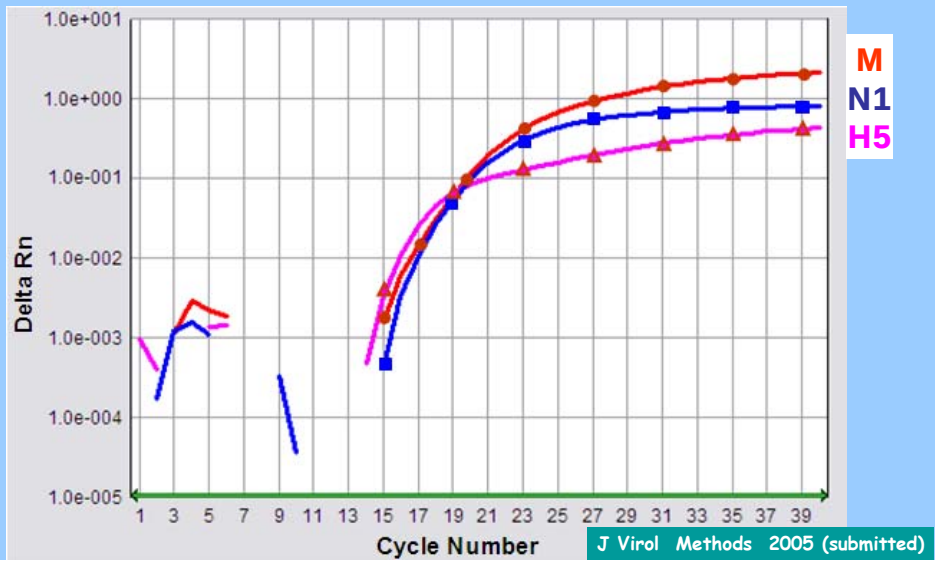
R E _ _ _ _ T R ← Mexico 94
R E R R R K K R ← Vietnam/04, Human_Thai/04
R E R R R K K R ← Chicken/Thailand
R E R K R K K R ← Pheasant/Thailand
R E K R R K K R ← Wild bird/Thailand

NA gene

20 aa deletion (59-78)

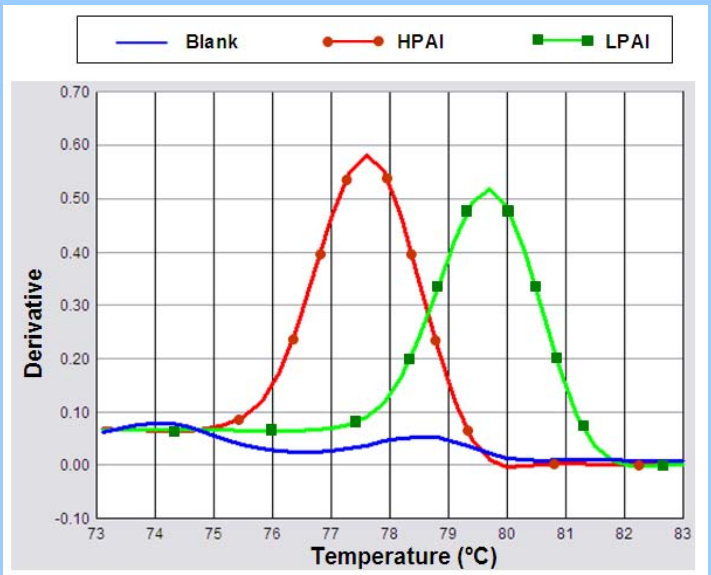


Multiplex real-time PCR for H5N1 influenza A virus detection

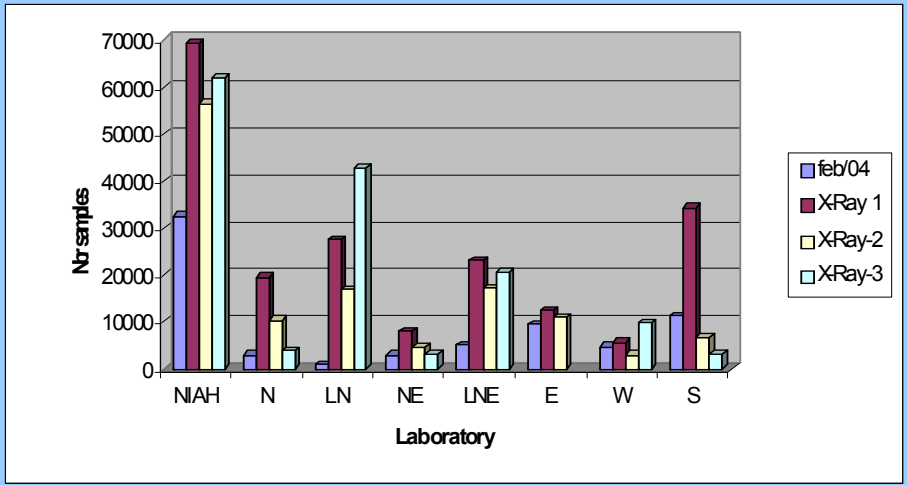


J Virol Methods 2005 (submitted)

Discrimination between HPAI & LPAI by melting curve analysis



- Increase testing capacity



Number of tests performed during nation-wide surveillance

HPAI : Asian Collaboration

- Laboratory & Epidemiological network under FAO projects
- Japan & Thailand collaboration : "Animal Disease Control in Thailand & Neighboring Countries"
- Bilateral cooperation between Thailand and Vietnam, Cambodia, Laos, Malaysia.....
- ASEAN : HPAI Task Force

Thank you

Working with H5N1 and other AI viruses that infect humans

Jill Banks & Ian Brown
CRL,VLA Weybridge, UK

- Until recent years direct infection of humans with AI viruses had not been considered an important zoonosis.
  - Only three reported instances up to 1996
  - Conjunctivitis
  - Volunteer experiments showed that only transitory infections occurred in humans infected with some viruses of avian origin
- In last eight years AI infections in humans have increased dramatically, with four different lineages of viruses from three subtypes being isolated – H7N7 (x2), H9N2, H5N1.

Implications for persons working with avian influenza

- WHO has issued laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus
  - Many of the recommendations are applicable to work in veterinary laboratories
  - WHO recommends that BSL3 precautions are adopted for work with H5 viruses
- OIE is currently reviewing its standards for laboratory biosafety

- Many countries Health and Safety executives also issue guidelines:

  For the UK the Advisory Committee on Dangerous Pathogens recommends that:
  - Laboratories knowingly handling influenza A virus subtypes H5N1 and H7N7, in addition to the containment requirements of Defra must protect workers from potential exposure.
  - CL3 (BSL3) is appropriate. The use of close-fronted microbiological safety cabinets should be considered (ie Class III cabinets or Class I/III in Class III mode)
  - The appropriate containment must be selected after performing a risk assessment
    - Risks presented by the strain of virus
    - The type of work that will be carried out

Laboratory risk assessment

- All procedures (apart from those where inactivated virus is used) involving avian influenza subtypes H5, H7 and H9 are handled at all times within the class I/ III biological safety cabinet operating in a class I mode.
- Viruses strains where human infections have been demonstrated are handled in biological safety cabinets in class III mode or where it is impractical to do so personal protective equipment e.g. positive pressure hoods should be worn.
- Personnel who are pregnant, have acute respiratory symptoms of a 'flu like'syndrome will not work with AIV

General recommendations

- Good laboratory technique is fundamental to laboratory safety
- Standard precautions
  - Barrier protection (e.g. gowns, gloves)
  - Eyes should be protected
  - All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets
  - Biological safety cabinets should be used for all manipulations that may cause splashes, droplets, or aerosols of infectious materials
  - Should avoid the use of hyperdermic syringes
- When a procedure cannot be conducted within a biological safety cabinet, appropriate combinations of personal protective equipment must be used

Health Surveillance Protocols

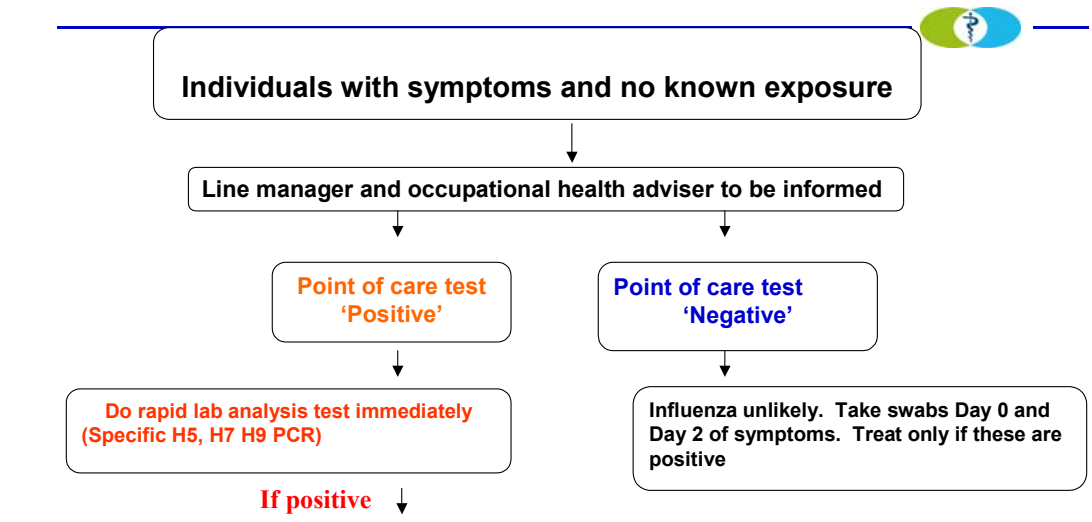
- A procedure for health monitoring of staff working with AI viruses that have infected humans should be in place e.g.H5, H7 and H9 AI subtypes.
  - Staff vaccinated against 'human' influenza, screened for contraindications to treatment with Oseltamivir (Tamiflu)
- Procedures in the event of an accidental exposure
  - Breaches in containment and accidents must be reported
    E.g. needle stick injury, power failure whilst handling virus in biological safety cabinet, spillages outside the cabinet.
  - Staff member will be withdrawn from work and isolated at home for 7 days
  - Prophylactic treatment will be commenced – Tamiflu

- Exposed individuals will take throat and nose swabs, and conjunctival swabs if appropriate.
- Post exposure blood samples will be collected on day 0 and day 14.
- The staff members GP will take any necessary action with respect to other family members that may be at risk

- The swabs will be tested for the presence of influenza virus and any virus subtyped.
- Blood samples will be tested for AI antibodies.

Procedures in the event of a staff member developing symptoms in absence of known exposure

- This will apply if a staff member, in the absence of a known exposure, develops symptoms compatible with influenza



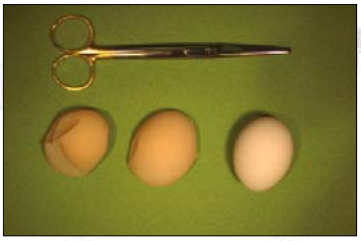
1. Immediately commence treatment with Tamiflu
2. Recommend prophylaxis for co-workers and close contacts
3. Follow the post exposure protocol with regards to swab testing and subsequent management of the individual
4. If subsequent tests indicate human rather than avian strain, individual may discontinue treatment

Changing Pathobiology of HPAI Viruses for Chickens and Ducks

David E. Swayne and Mary Pantin-Jackwood
USDA/Agricultural Research Service
Southeast Poultry Research Laboratory
Athens, Georgia

Pathogenicity of AIV

- **Pathogenicity: ability of an AI virus strain to produce lesions, disease and/or death**
  - **<u>Low pathogenicity</u>:**
    - **Subclinical, drops feed and water consumption, respiratory problems, egg production drops, ruptured egg yolks in coelomic cavity, rarely renal disease with visceral urate deposition**
    - **Multiple poultry species – galliformes, anseriformes, columbiformes, etc.**

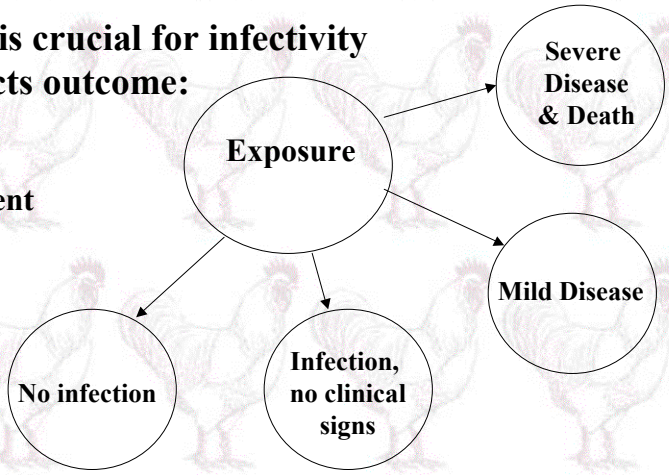


Introduction

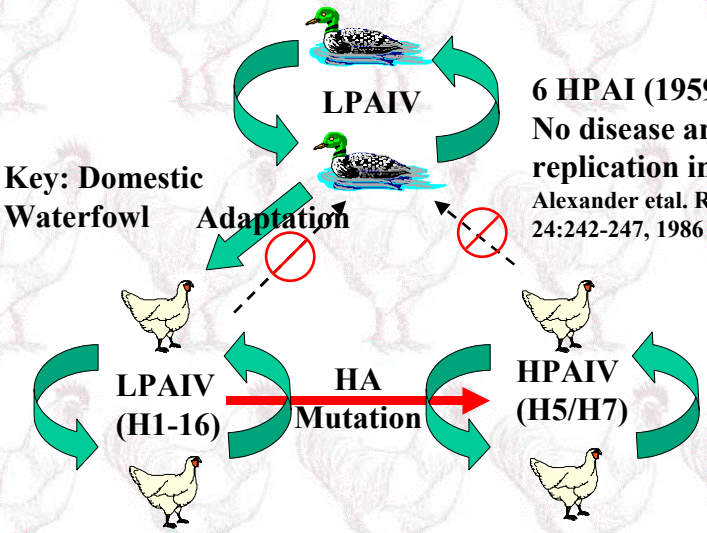
- **<u>High pathogenicity</u>:**
  - **Multi-organ systemic disease – hemorrhages, edema, necrosis, inflammation**
  - **Defined in chickens by high lethality;**
  - **Mechanism – enzyme for cleavage of hemagglutinin**

Introduction

- **Critical Factors:**
  - **Infectivity (ability to replicate in a host)–**
    - **Pathogenicity directly associated with quantity of virus replication**
  - **Adaptation is crucial for infectivity**
  - **Triad impacts outcome:**
    - **Virus**
    - **Host**
    - **Environment**



Ecology and Epidemiology



Pathobiology of A/CK/HK/220/97 (H5N1): Prototype Asian H5N1 AIV

4 groups based on morbidity, mortality, pathology and virus replication

- **Natural Route Exposure – IN**
- **Standard dose: 10^6 EID50 virus**
- **Young birds when available**

Av Dis 47:956-967, 2003

Species	Morbidity[B] (DPI)	Mortality[B] (DPI)
WL Chickens	8/8[C] (1.5-2.0)	8/8[C] (1.5-2.0)
WR Chickens	8/8[C](1.5-2.0)	8/8[C](1.5-2.0)
J. Quail	8/8[C](1.5-2.5)	8/8[C](1.5-2.5)
B. Quail	8/8[C](1.5-3.5)	8/8[C](2.0-3.5)
Turkeys	6/6[C](1.5-2.5)	6/6[C](2.0-2.5)
Guineafowl	8/8[C](2.0-5.0)	8/8[C](2.0-5.0)
Pheasants	8/8[C](2.0-4.0)	8/8[C](2.5-4.0)
Partridges	8/8[C](3.0-6.5)	6/8[C](4.0-6.5)
Z. finches	7/7[D](3-5)	7/7[D](3-5)
Geese	5/11(4-10)	0/11
Emus	1/2(8-14)	0/2
H. finches	7/9[D](4-13)	4/7[E](6-13)
Budgerigars	7/8[D](5-9)	6/8[D](5-9)
H. sparrows	3/7(4-7)	0/7
Ducks	0/9	0/9
Gulls	0/8	0/8
Starlings	0/4	0/4
Pigeons	0/10	0/10
Rats	0/6	0/6
Rabbits	0/6	0/6

Pathobiology of A/CK/HK/220/97 (H5N1)

Species	Morbidity[A]	Mortality[B]	Gross Lesions[C]	Histological Lesions[D]	Viral Antigen[E]	Virus Reisolation[F]
WL Chickens	+++	+++	+++	+++	+++	+++
WR Chickens	+++	+++	+++	+++	+++	+++
J. Quail	+++	+++	+++	+++	+++	+++
B. Quail	+++	+++	+++	+++	+++	+++
Turkeys	+++	+++	+++	+++	+++	+++
Guineafowl	+++	+++	+++	+++	+++	+++
Pheasants	+++	+++	+++	+++	+++	+++
Partridges	+++	+++	++	++	++	+++
Z. finches	+++	+++	+	++	+++	+++
Geese	++	-	+	++	++	++
Emus	++	-	+	++	++	++
H. finches	+++	++	+	++	+++	++
Budgerigars	+++	++	-	++	++	++
H. sparrows	+	-	-	+	+	+/-
Ducks	-	-	-	+	+/-	+
Gulls	-	-	-	+/-	-	+/-
Starlings	-	-	-	-	-	+/-
Pigeons	-	-	-	-	-	-
Rats	-	-	-	-	-	-
Rabbits	-	-	-	-	-	-

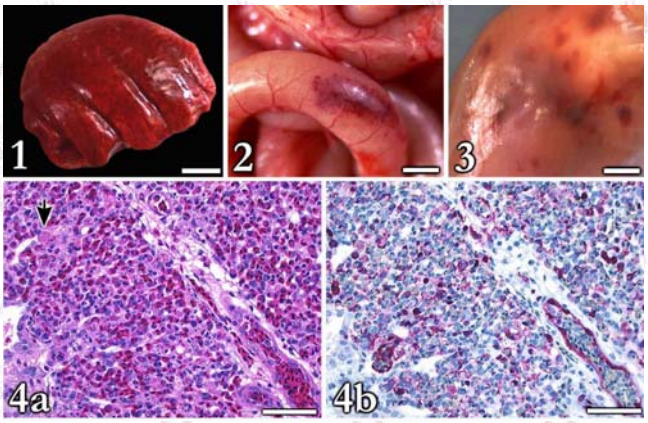
Av Dis 47:956-967, 2003

Pathobiology in Group 1 (Galliformes & Zebra Finches): A/CK/HK/220/97

Pathobiological Changes in Visceral Organs: Exudative, Necrotic, Hemorrhagic, Suppurative

Virus location:

- **Group A: vascular endothelium, phagocytic leucocytes**
- **Group B: parenchymal cells (cardiac myocytes, adrenal corticotrophic cells, pancreatic acini, neurons & glia of brain)**



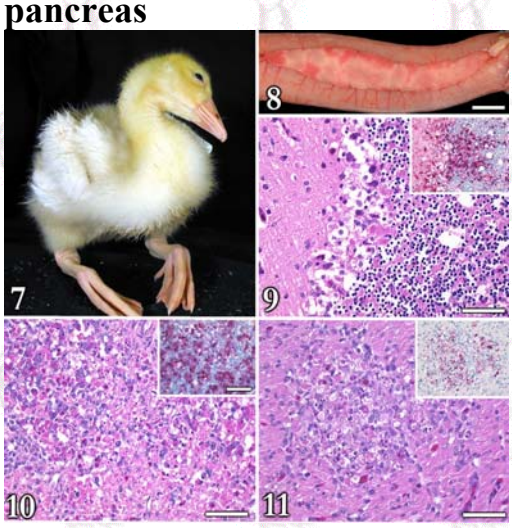
Av Dis 47:956-967, 2003

Pathobiology in Group 2 (geese, emus, house finches & budgerigars): A/CK/HK/220/97

Pathobiological Changes: predilection for nervous system, but less so for heart and pancreas

Features:
- **Morbidity delayed**
- **Mortality: 0-75%**
- **Neurological signs**
- **Brain: most severe lesions & greatest antigen content**
- **Other organs: heart & pancreas**

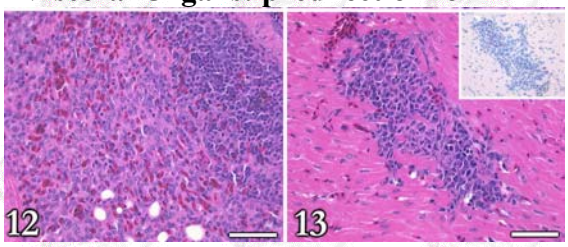
Av Dis 47:956-967, 2003



• Pathobiology in Group 3 (ducks, house sparrows & gulls): A/CK/HK/220/97

• Pathobiological Changes in Visceral Organs: predilection for respiratory system

Features:
- **Morbidity minimal**
- **Mortality: 0%**
- **Mild respiratory lesions predominate (D,G): pneumonia & air sacculitis**
- **Myocarditis & orchitis (H.S.)**
- **Minimal virus detection & low titers**



• Pathobiology in Group 4 (pigeons, starlings, rats & rabbits): A/CK/HK/220/97

• Pathobiological Changes in Visceral Organs: None

Features: Morbidity: None, Mortality: None, Single virus isolation from starlings

Av Dis 47:956-967, 2003

Change in Asian H5N1 since 1997 Chicken IN- Lethality and Replication

Avian-Origin Isolates

Virus	Age	MDT	Virus Titers (EID50/ml or gm)			
			Oral	Cloacal	Brain	Heart
Ck/HK/220/97	4	1.5	4.7	4.2	6	
Gs Env./HK/437-6/99	4	5.5	1.7	-		
Dk/Anyang/AVL-1/01	4	2.9	3.1	6.7		
Gs/Vietnam/113/01	4	2.6				
Gs/Vietnam/324/01	4	2.4				
Ck/Korea/ES/03	3-6	2	6.2	6	7.2	9.3
Ck/Indonesia/7/03	4-6	2.1	6.4	5.6	7.6	
Crow/Thailand/1C/04	4	1.8	6.8	4	6.9	9

- All cause 100% mortality
- MDT longer than for IV group and varied with strain

Change in Asian H5N1 since 1997 Chicken IN-Infectivity & Lethality

Human Isolates

Virus	MDT	Virus Titers (EID50/ml or gm)			
		Oral	Cloacal	Brain	Heart
HK/156/97	2.4	6	3.7		
HK/491/97	1.6	6.4	5.8	6.3	9.8
HK/213/03	2.1	7.4	4.5	7.4	10
Vietnam/1203/04	1.5	6.3	5.9	7.7	10

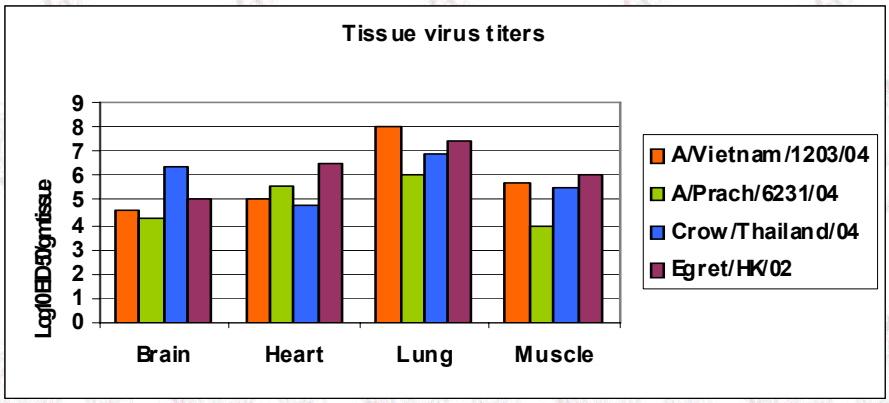
- All cause 100% mortality
- MDT longer than for IV group and varied with strain

Asian H5N1 (1997-2005) Virus in Ducks (IN) – Lethality and Replication

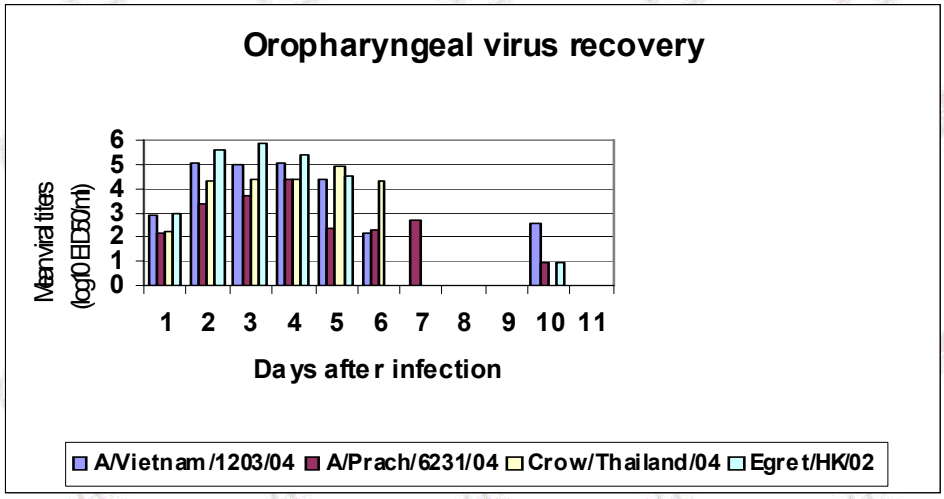
Virus	Mortality dead/ inoculated	MDT	Oral (mean titer)[A] 3DPI	Cloacal (mean titer) 3DPI	Brain (mean titer)
A/Whooper Swan/Mongolia/244/05	7/8	4.3	?	?	?
A/Crow/Thailand/04	8/8	4.5	4.4	1.98	6.4
A/Egret/HK/757.2/02	7/8	4.1	5.8	2.36	5.0
A/Vietnam/1203/04	7/8	4.2	5.0	2.0	4.6
A/Prachinburi/6231/04	3/8	6.3	3.7	1.36	4.3
A/Ck/Korea/ES/03	2/8	4	1.62	1.55	
A/Gs/Vietnam/113/01	0/8	-	1.8	<1.6	1.5
A/Ck/HK/317.5/01	0/8	-	1.92	2.45	
A/Dk/Anyang/ALV1/01	0/8	-	2.5	1.3	2.1
A/Env/HK/437-6/99	0/8	-	2.05	2.57	0.0
A/Ck/HK/220/97	0/8	-	1.97	1.22	

[A] Mean titer reported as $\log_{10}EID_{50}$

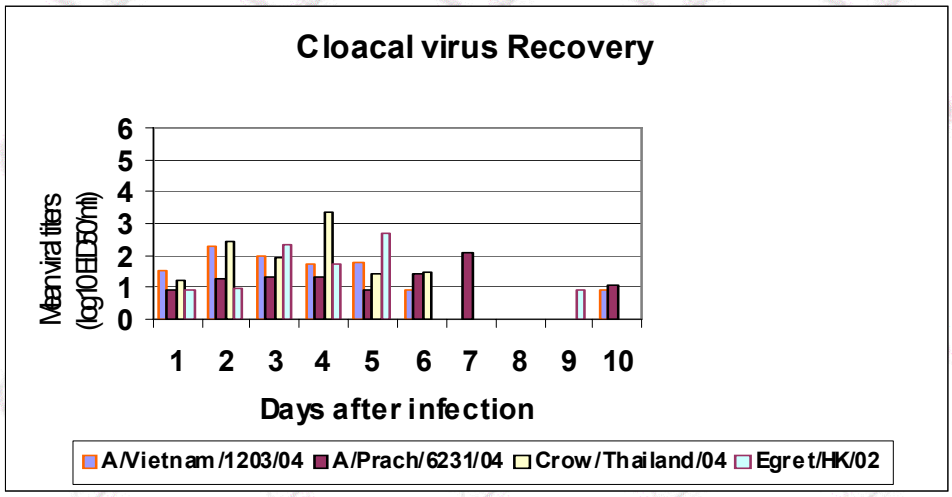
New Asian H5N1 (2002-2004) Virus in Ducks (IN) – Tissue Replication



New Asian H5N1 (2002-2004) Virus in Ducks (IN) – Oropharyngeal Replication



New Asian H5N1 (2002-2004) Virus in Ducks (IN) – Cloacal Replication



Change in Asian H5N1 since 1997 Duck IN-Lethality & Replication

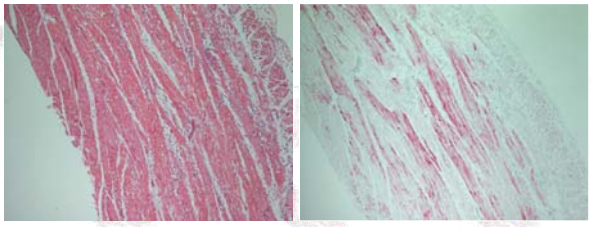
A/Ck/Indonesia/7/03, IN, Pekin Ducks

Age	% Deaths	Virus Titers (EID50/ml or gm)			
		Oral	Cloacal	Brain	Heart
2	50 (7)	3/4 (2.45)	0/4	4/4 (3.7)	4/4 (4.6)
4	0	3/4 (2.97)	2/6 (1.23)		
4	0				

- Age difference for mortality rates
- Predominate respiratory & not GI replication
- Moderate replication titers in brain and heart

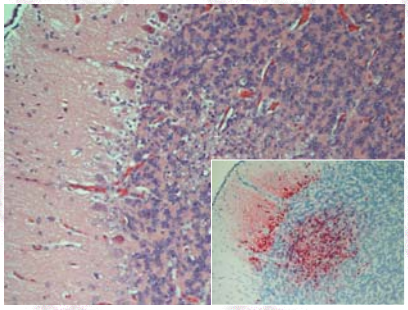
Lesions in IN-inoculated Ducks

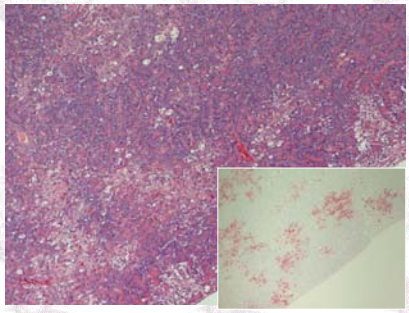
- 1997-2001 HK H5N1 viruses: no lesion >>> mild respiratory [Group 3 & 4]
- Dk Meat/Anyang/01: respiratory (rhinitis, sinusitis, laryngitis, tracheitis, pneumonia) > skeletal muscle > encephalitis (Group 3)
- GS/VN/113/01 & Human/HK/213/03: respiratory (Air sacs and lungs) >> myocardial (Group 3)
- Ck/S. Korea/ES/03: sinusitis, rhinitis, air sacculitis>>> myocardial cell necrosis [Group 3]
- Ck/Indonesia/7/03, A/Prachinburi/6231/04 : autonomic ganglioneuritis, encephalitis, myocarditis, sinusitis [Group 2]



Lesions in IN-inoculated Ducks

•Egret/HK/757.2/02, Gs/HK/739.2/02, A/Vietnam/1203/04, A/Crow/Thailand/04: systemic lesions - nonsuppurative meningoencephalitis, autonomic neuritis, pancreatic necrosis, hepatic necrosis, myocardial necrosis & myocarditis (10-80%+), adrenal gland necrosis, skeletal muscle degeneration and necrosis, respiratory tract inflammation [Group 1]





Lesions in IN-inoculated Pigeons & Crows

Virus	Species	Morb.	Mort.	Sero +	VI+
A/crow/Thailand/1C/04	Pigeon	1/6	1/6	2/5	2/5
A/Pigeon/Thailand/1B/04	Pigeon	0/6	0/6	2/6	3/6
A/crow/Thailand/1C/04	Crows	2/2	2/2	2/2	2/2

Crows and dead pigeon – severe encephalitis and high titers of virus in brain

Conclusions

1. **Two pathogenicity categories based on experimental chicken studies; i.e LP and HP**
2. **In chickens, pathobiology of HPAI viruses varies with isolates**
3. **Similar pathogenicity in other galliformes**
4. **Generally ducks resistant to HPAI viral infections or produce minor respiratory lesions**
5. **Since 2001, H5N1 HPAI viruses have shown great variations in virulence for domestic ducks changing from respiratory only to lesions in a few internal organs to severe systemic infection and lesions**

Contributors

- **SEPRL - Laura Perkins, Chang Wan Lee, David Suarez, Mike Perdue, Terry Tumpey, Joan Beck**
- **CDC – Terry Tumpey, Jackie Katz, Nancy Cox, Alexander Klimov, Yumi Matsoaka, Doan Nguyen**
- **NIH – Kanta Subbarao**
- **HK Department of Fisheries, Agriculture & Conservation – Les Sims, Trevor Ellis, Howard Wong**
- **Thailand Dept Livestock Development – Drs. Chantanee, Arunee and Sudarat**
- **S. Korea, National Veterinary Research and Quarantine Service – Drs. Mo, Kim and Kwon**

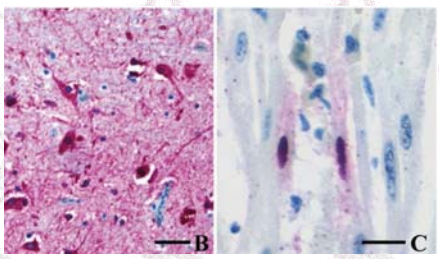
Mongolia Results

- **July-August 2005, survey live apparently healthy wild water birds on nine lakes in central Mongolia**
- **Wild bird mortality on Erhel Lake (site 7)**
  - **41 dead birds of nine species**
  - **6500 live apparently healthy birds of 39 species**
  - **Additional 15 species but no accurate counts**
  - **Whooper Swans (*Cygnus cygnus*), Swan Geese (*Anser cygnoides*) and Velvet Scoter Duck (*Melanitta fusca*) mortality rates greater than 6%**

EU Ref 2005

Mongolia Results

- **H5N1 influenza A virus was identified by RRT-PCR & VI from a dead Whooper Swan**
  - **AI viral antigen in brain (Fig. 1), autonomic nerves of intestine, heart muscle, pancreatic glandular epithelium, and inflammatory cells in the pneumonic lung**
  - **HPAI – 8/8 chickens IV test died in less 27 hrs**
  - **Lethal neurological disease in IN test of ducks (7/8), and killed contact ducks**
  - **Fluid accumulation in pericardial sac of the experimental ducks and the Whooper Swans**



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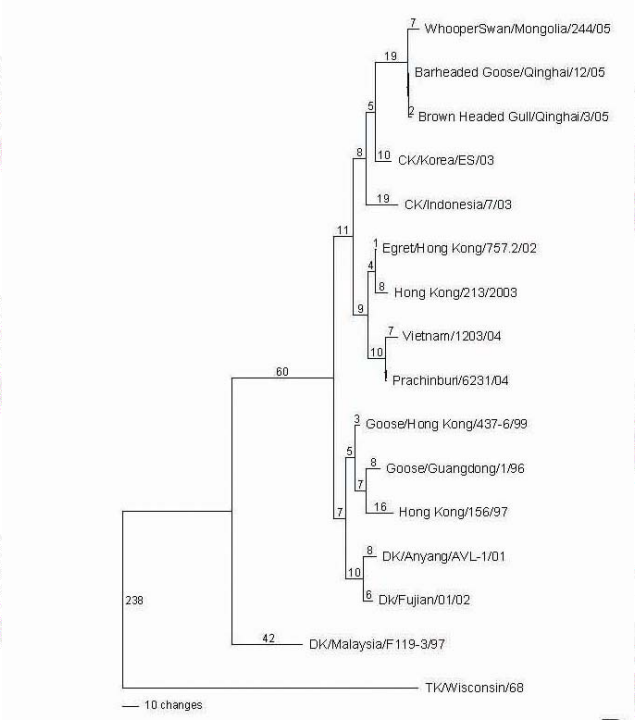
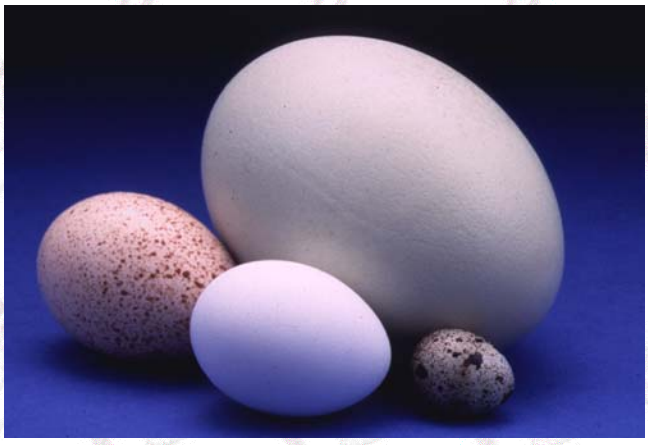


Thank You For You Attention!



EU Ref 2005

VIROLOGICAL FINDINGS IN SELECTED FREE-RANGE MULE DUCK FARMS AT HIGH RISK regarding AI infection

CHERBONNEL M., LAMANDÉ J., ALLÉE C., SCHMITZ A., LOUBOUTIN K., LE BRAS M.O., GUILLEMOTO C., PIERRE I., LE GALL G., PICAULT J.P., JESTIN V.

Purpose : Virological surveillance (I)

Previous data : problems with the results from serological surveillance year 2003-2004

Why virological surveillance (II)

- **What's the meaning of**

  → only 1 serum displaying a titre of 16 (out of 29 sera with a titre < 16

- **Some discrepancies between**

  - the negative results given by the CRL H5 antigens
    and
  - the positive results obtained with the French NRL H5 antigens*

*A/Dk/Fr/02166/2002 LP H5N3
*A/Ck/Fr/03 426/ 2003 LPH5N2

Principle of virological surveillance

- Target 30 farms (= 10 / zone of production)

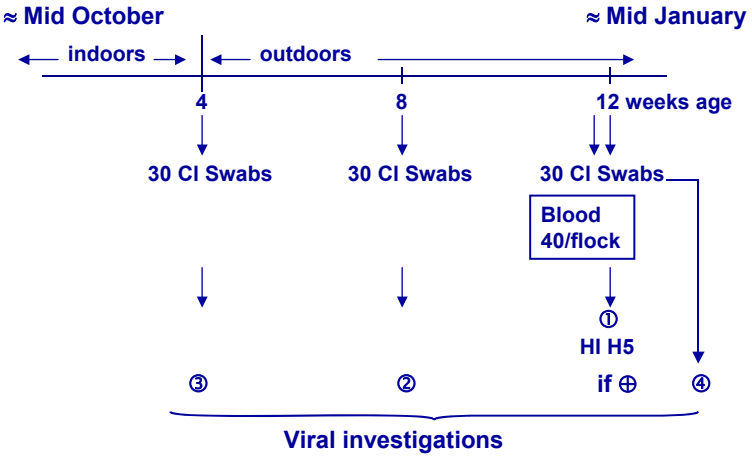
  that might be at high risk given the following criteria :

  → free-range
  → and ≠t ages in the same farm
  → and/or large quantity of wild bird in the neighboring
  → and/or several species in the same farm

- Winter time

- Focuse on H5 AIV surveillance

Experimental protocol (I)



NB : H5 HI = { H5N7 CRL ⊕ H5N2 CRL } { H5N3 NRL H5N2 NRL }

Experimental protocol (II)

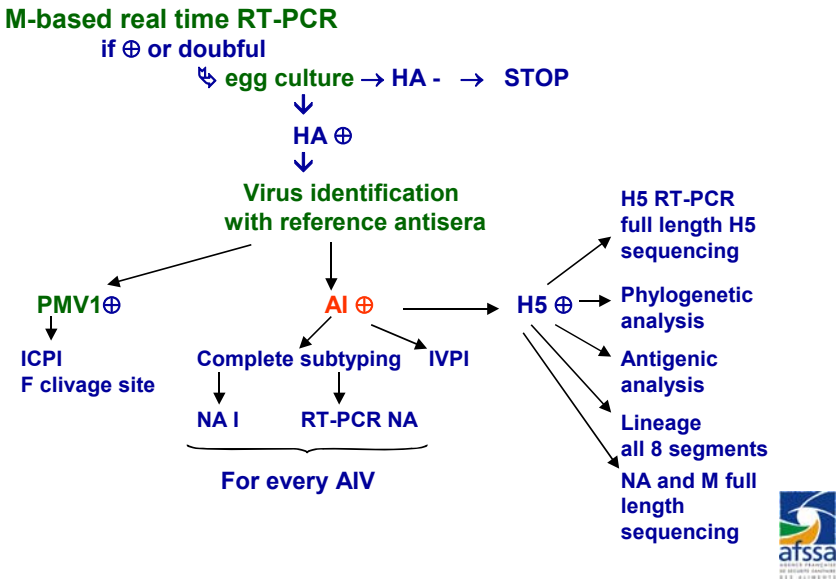
- Where H5 positive farms
- Decontamination
- Further investigations

} next flock

↳ HI

and virology cloacal swabs

Viral investigations (cloacal swabs)



Results

➢HI test

- **Zones 1 and 2 : 20/20 farms H5 –**
- **Zone 3 : 2/10 → Farms H5 –**

  6/10 → Farms H5 + (CRL and NRL Ag)

  2/10 → Farms H5 + (NRL Ag)
  H5 – (CRL Ag)

Results of virological surveillance in 8 H5 seropositive farms

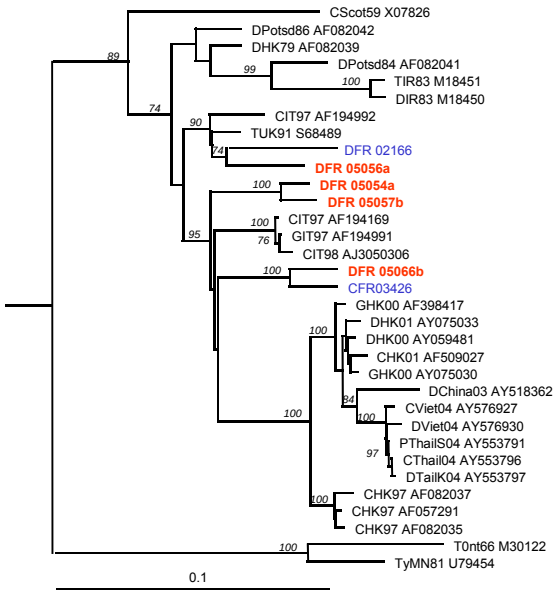
Farm	H5 positive sera (out of 40)	Virological results depending on the age (weeks)			
		4	8	12	IVPI/ICPI
1	2	-	-	-	/
2	2	H5N3	-	-	IVPI=0,0
3	2	H6N2	PMV1#	-	IVPI=0,0 ICPI=0,0
4	3	H5N2	-	-	IVPI=0,0
5	11	H6N2	H5N2	H11N9	IVPI=0,0
6	1	-	-	H6N8 PMV1#	IVPI=0,0 ICPI=0,0
7	6 (NRL)	PMV1#	-	-	ICPI≤0,4*
8	4 (NRL)	-	H5N1	-	IVPI=0,0

*To be repeated (bacterial contamination)
#: clivage site F gene = GKQGRL

H5 AIV genetic characteristics

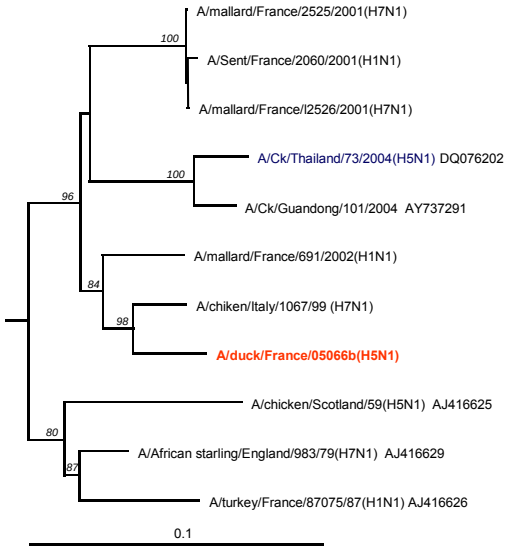
	HA		NA	M	NS1			PB1, PB2, PA & NP	
	Cleavage site	Addit. glycosylation sites	Deletion	Origin	Origin	aa 97	Subgroup A/B	Origin	Gene PB2: aa 627
A/duck/France/05054a/2005 (H5N3) LP	PQRETR	No	No	Avian	Avian	E Glu	A	Avian	E Glu
A/duck/France/05056a/2005 (H5N2) LP	PQKETR	No	No	Avian	Avian	E Glu	B	Avian	E Glu
A/duck/France/05057b/2005 (H5N2) LP	PQRETR	No	No	Avian	Avian	E Glu	A	Avian	E Glu
A/duck/France/05066b/2005 (H5N1) LP	PQRETR	No	No	Avian	Avian	E Glu	A	Avian	E Glu

Phylogenetic relationships HA gene (avian)



Outgroup: H3N2M55059 Bootstrap > 70%

Phylogenetic relationships NA gene (avian)



Outgroup: A/Herring Gull/Delaware/677/88(H2N8) L06585
Bootstrap > 70%

Antigenic relationships H5 AIV
Cross HI test with 8 H5 antigens (6 Fr and 2 CRL) and their antisera

1- 4 2005 H5 Fr gave high level of cross HI (between each other)

2- 2 previous H5 Fr more distant from H5N3 2005

3- H5N7 CRL can detect every Fr H5 antisera

4- H5N2 CRL gives low cross HI with H5N1 Fr 2005 (and H5N7 CRL)

Conclusion

- **Confirmation of our previous H5 serological results**
- **Confirmation of our previous ELISA results (≈ 80 % positive)**
- **Confirmation of previous data (D.J. Alexander and Stuart, 1982)**

= This is probably not a new situation, however this is of concern

Perspectives

- **Biosecurity measures enhanced**
  - ⇒ **Not to attract wild birds**
    Food and water indoors (or supplied at restricted times)
- **Virological surveillance to be continued**
- **Updating assessment of H5 vaccines efficacy in mule ducks against new Fr LPAI**

  ----→ **Prophylactic vaccination**

Acknowledgments

- **French Veterinary Authority (DGAL health animal division)**
  J. Francart
- **French veterinary services and the linked veterinarians**

----→

VARIABLE EFFECT OF VACCINATION AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA IN DIFFERENT BIRD SPECIES.

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INTRODUCTION

Highly pathogenic avian influenza (HPAI) is a disease of poultry with mortality that ranges up to 100%, caused by H5 or H7 avian influenza strains. Not only poultry but also a lot of other bird species are susceptible to HPAI. Natural infections as well as experimental infections have been documented in many different species (1,4,6). The observed disease symptoms vary from sub clinical to severe illness and death. Currently zoo birds are vaccinated against H5N1 in several European countries, in the recent past exotic birds have been vaccinated against H7N7 (The Netherlands 2003)(7) and H5N1 (Singapore)(5).

It is known that vaccination can prevent illness and mortality and transmission of HPAI H7N7 in chickens (8). However, not much is known about the effect of vaccination in exotic birds. The objective of this study was to establish whether these birds are protected by vaccination against infection, and more importantly, whether transmission of virus is reduced after vaccination.

Representatives of two common families were chosen to study the effect of vaccination in exotic birds: Golden Pheasants (Chrysolophus pictus, family Phasianidae) and Ringed Teals (Callonetta leucophrys, family Anatidae). Transmission experiments were performed with vaccinated and unvaccinated birds to quantify the effect of vaccination on transmission (8). Transmission experiments offer a way to look at the spread of virus under experimental conditions in different treatment groups. The vaccinated birds were challenged with HPAI H7N7 two weeks after a single vaccination. Disease symptoms, excretion of virus and transmission of virus were observed.

Our result show that teals and pheasants react very differently to infection and vaccination and it may not be easy to make general statements on the effectiveness of vaccination for semi feral birds.

MATERIALS AND METHODS

Animals. Golden Pheasants (*Chrysolophus pictus*) and Ringed Teals (*Callonetta leucophrys*) were used. The pheasants and ducks were inoculated both intranasally and intratracheally with 0.1 ml diluted allantoic fluid containing 10^6 median egg infectious dose (EID_{50}) per ml. All animal experiments were undertaken in a high containment unit under BSL3+

conditions at the Central Institute for Animal Disease Control Lelystad. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

Viruses. The influenza virus used in this study was A/Chicken/Netherland/621557/03 H7N7. This virus was isolated on the index farm of the outbreak in the Netherlands in March 2003. The virus had an intravenous pathogenicity index (IVPI) of 2.93, as determined by the procedure described elsewhere (3). Briefly, ten chickens were injected intravenously with 0.1 ml of tenfold diluted allantoic fluid. Birds were examined at 24-hour intervals for ten days. At each observation each chicken was recorded normal (0), sick (1), severely sick (2) or dead (3). The index is calculated by adding up all scores and by dividing the total by 100. When the index is greater than 1.2 the avian influenza is considered highly pathogenic.

Vaccine. An inactivated oil emulsion H7N1 vaccine (A/Chicken/Italy/99) was used. A dosage of 0,5 ml was injected in the muscles of the leg.

Transmission experiments. Experiments were performed with unvaccinated pheasants and ducks and with vaccinated pheasants and ducks. The birds vaccinated were challenged two weeks after vaccination. All experiments were done in duplicate. The design of the experiments was as follows: five birds were placed in a cage. These five birds were inoculated with virus and 24 hours later five contact birds were added. The birds were monitored by taking tracheal and cloacal swabs daily during the first ten days and twice a week for the next 14 days. The experiment was terminated 24 days after the challenge.

Virus isolation. Swabs were submersed in 2 ml 2.95% tryptose phosphate buffer with 5 x 10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at -70°C until analyzed. Three embryonated chicken eggs incubated for 9 days were inoculated with 0.2 ml per egg. After 72h the allantoic fluid was harvested and a Haemagglutination Assay (HA) was performed following standard procedures. When at least one of the eggs was positive in the HA assay the swab was considered to be positive.

Statistical analysis. The analysis of the transmission experiments is based on a stochastic SEIR epidemic model in which individuals are susceptible (S), latently infected (i.e. infected but not yet infectious) (E), infected and infectious (I), and recovered and immune or dead (R). Throughout, the analyses are aimed at estimation of the (basic) reproduction ratio. The reproduction ratio (denoted by R) is defined as the mean number of infections that would be caused by a single infected individual in a large population of susceptible animals. If $R>1$, an infected animal infects on average more than 1 susceptible animal, and a chain reaction of infections may occur. If $R<1$, a prolonged chain reaction of infections is not possible, and the epidemic comes to a halt.

In this paper the reproduction ratio was estimated using the final size of the transmission experiments. The final size of an experiment is given by the number of contact animals that has been infected when the infection chain has ended. Our (maximum likelihood) estimates of the reproduction ratio are based on the final size distributions as determined in (2). We refer to (8) for a detailed description of the statistical analysis. All calculations are carried out using the software package Mathematica 5.2.

RESULTS

Transmission in unvaccinated golden pheasants and ringed teals. All inoculated pheasants and teals became infected, and they spread the virus to all contact birds. The golden pheasants showed severe signs of illness, eight of the inoculated animals died and four of the contact animals died. In the groups with the teals four developed conjunctivitis, no other symptoms were observed, and all animals survived. The estimate of the reproduction ratio based on the final size method with an exponentially distributed infectious period is $R_{exp} > 1.5$ with 95% confidence in both species (Table 1).

Transmission in vaccinated golden pheasants and ringed teals. All vaccinated challenged pheasants and all contact pheasants became infected. None of them showed signs of illness. The estimate of the reproduction ratio based on the final size method with an exponentially distributed infectious period is $R_{exp} > 1.5$ with 95% confidence (Table 1). In teals nine of the ten inoculated birds became infected and no contact infections were demonstrated. The estimate of the reproduction ratio based on the final size method with an exponentially distributed infectious period is $R_{exp} < 0.70$ with 95% confidence. When the unvaccinated and vaccinated teals are compared, it appears that there is a significant difference in the reproduction ratio of vaccinated and unvaccinated teals ($P<0.001$) (Table 1).

DISCUSSION

An infection with HPAI in pheasants and teals leads to very different symptoms: while the pheasants showed severe morbidity and high mortality, only conjunctivitis was seen in some of the teals. This implies that in pheasants an infection with H7N7 HPAI will readily be detected by disease symptoms while in other species an infection with the same virus can easily go unnoticed. Despite the differences in appearance of the infection, our results show that the virus spreads extensively in both species. These findings have important implications for monitoring and surveillance strategies.

A single vaccination against HPAI protects both pheasants and ducks against morbidity and mortality, but the difference in the effect on transmission was striking. In teals transmission was significantly reduced after the vaccination while in pheasants transmission was not influenced by a single vaccination. This also has important implications for control strategies, as it implies that pheasants can become silent spreaders after vaccination.

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Table 1. Overview of the statistical analyses of the experiments.

Group	Final size	R [A]	p H0:$R \geq 1$	p H0:$R_v=R_c$ [B]
Unvaccinated pheasants	5,5	> 1.5	1	
Vaccinated pheasants	5,5	> 1.5	1	1
Unvaccinated teals	5,5	> 1.5	1	
Vaccinated teals	0,0	< 0.70	0.017	<0.001

[A] one-sided 95% confidence interval.

[B] R_v, reproduction ratio amongst vaccinated birds; R_c, reproduction ratio amongst unvaccinated birds.

VAR

Evaluation of the potential use of a M2e-specific ELISA for DIVA testing

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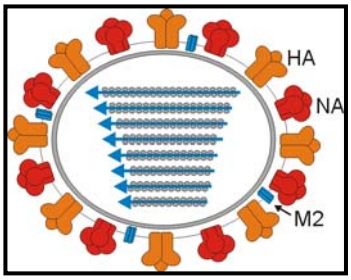
Avian Virology & Immunology Unit

Veterinary and Agrochemical Research Centre (VAR)

BELGIUM

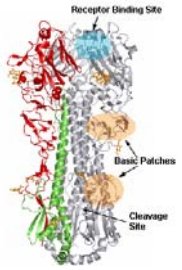
Extracellular Influenza A virus antigens



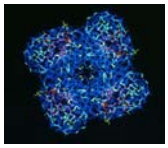
Three integral membrane proteins of the influenza A:

- •Hemagglutinin (HA)
- •Neuraminidase (NA)
- •M2 protein (M2)

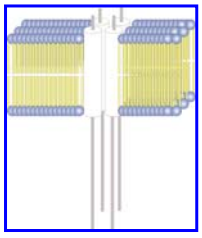
HA



NA

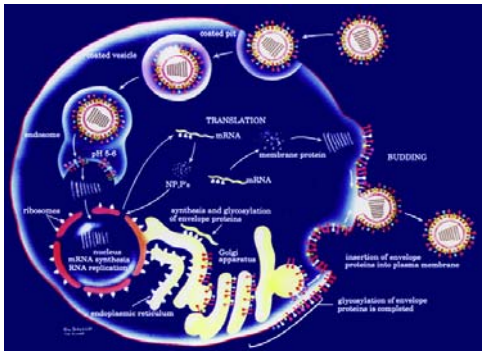


M2



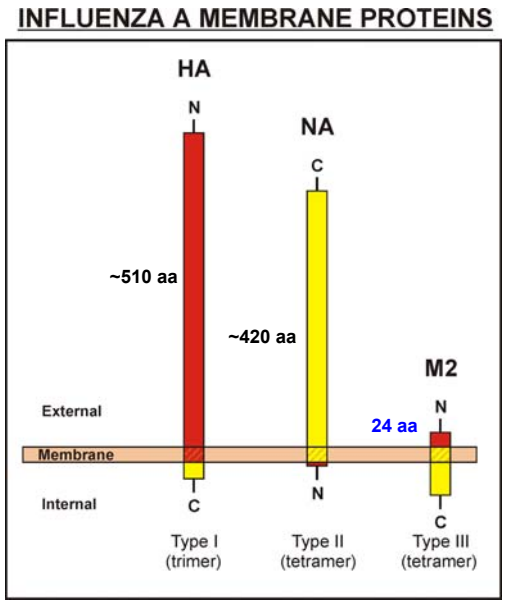
from Xavier Saelens

Extracellular Influenza A virus antigens fonctions



- Hemagglutinin (HA)
  - Viral Attachment and penetration of genetic material
- Neuraminidase (NA)
  - Viral dissemination

- Present a rapid mutation rates
- Induce a protective immune response

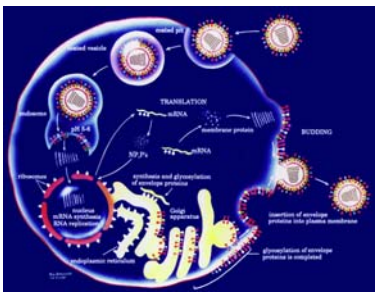


from Xavier Saelens

M2 protein:

- The smallest of the three viral membrane proteins
- Non glycosylated
- Exists as a homotetramer formed by two disulfide linked dimers
- 97 amino acids long, with a extracellular domain of 24 aa (M2e) at the N-terminus
  - Highly conserved within AI unlike HA & NA

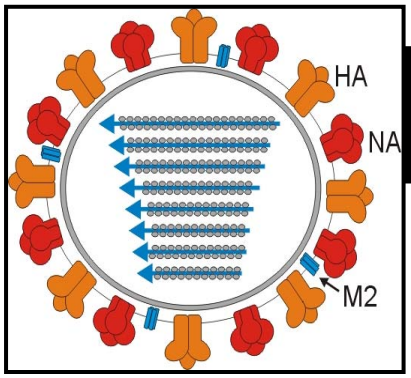
M2 protein fonction



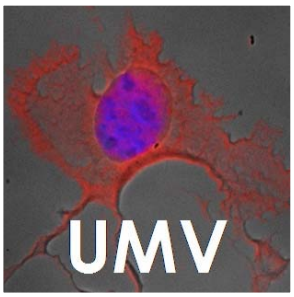
•Forms a proton-selective ion channel, playing a important role in facilating viral entry. M2 protein mediates an influx of protons into virions, which facilitates dissociation of the matrix protein (M1) from viral ribonucleoprotein.

• Plays also a role, late in infection preventing conformational change of the HA molecule during its maturation.

•Identified as the target of the antiviral drug, amantadine hydrochloride.



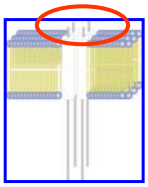
~400 HA
~100 NA
~ 10 M2



- Present in small quantities on the surface of mature virions.
- Expressed abundantly at the apical surface of infected cells, with a ratio of approximatively two M2 molecules per HA trimer.

? Suggests that M2 may be partially excluded from budding virus particules.

The extracellular domain of M2 protein (M2e)



highly conserved as shown by alignment of the sequence of M2e, isolated from different human strains of influenza A virus.

consensus M2e sequence	SLLTEVET PIRNEWGCRCNDSS D
A/Brevig_Mission/1/1918 H1N1	SLLTEVET PTRNEWGCRCNDSS D
A/Puerto Rico/8/1934 H1N1	SLLTEVET PIRNEWGCRCNGSS D
A/Chile/13/1957 H2N2	SLLTEVET PIRNEWGCRCNDSS D
A/Japan/170/1962 H2N2	SLLTEVET PIRSEWGCRCNDSS D
A/An Arbor/7/1967 H2N2	SLLTEVET PIRNEWGCRCNDSS N
A/Aichi/2/68 H3N2	SLLTEVET PIRNEWGCRCNDSS D
A/England/878/1969 H3N2	SLLTEVET PIRNEWGCRCNDSS N
A/Caracas/1/1971 H3N2	SLLTEVET PIRKEWGCRCNDSS D
A/Taiwan/3/71 H3N2	SFLTEVET PIRNEWGCRCNDSS D
A/Aichi/69/1994 H3N2	SLLTEVET PIRNEWECRCNGSS D
A/Wuhan/359/95 H3N2	SLPTEVET PIRSEWGCRCNDSS D
A/Wisconsin/10/98 H1N1	SLLTEVET PIRNGWECKCNDSS D

from Xavier Saelens

Genetic Constraints on the M2e-sequence

•The low degree of structural variation in M2e is certainly in part due to constraints resulting from its genetic relation to M1, the most conserved protein of the virus.

•M2 is encoded by a spliced RNA of the viral gene segment 7, which codes also for M1. The splicing removes most of the nucleotides that code for M1.

•M1 and M2 share the same initiation codon for protein synthesis. The first part of M2e is on the same open reading frame and the second part, in a different reading frame.

(after Lamb e.a., PNAS 78, 4170, 1981)

Immunogenicity of M2 protein

- M2 appears as weakly immune, absence of M2e-specifc Abs (no pressure for change).
- Human: Antibodies against the M2 protein seem absent from acute-phase sera but become detectable in sera of patients after they recover from an influenza. However, the response is not generated concisely.
- Mice and ferrets: The immunity induced by the M2e was protective and also broad-spectrum due to its highly conservative. Abs to M2e reduced the replication level of influenza A virus in the lung of mice, reduce morbidity and mortality.

Potential influenza vaccine based on the M2e peptide

? The protective efficacy of M2e-specific immunity has been confirmed in various types of vaccine constructs and vaccination modalities in mice, ferrets and Rhesus monkeys (Fan et al . 2004; De Filette et al. 2005)

M2e sequence differs according to host

from Xavier Saelens

Evaluation of the potential use of a M2e-specific ELISA for DIVA testing

- M2 is a minor component of purified virus. Currently licensed inactivated influenza virus vaccines would not be expected to induce significant M2-specific immunity.

M2e specific Ab should be low or absent in the vaccinated chickens

- M2 is expressed abundantly at the surface of infected cells. Thus after infection, the level of M2-specific Abs should increase and become detectable by a M2e-specific ELISA.

M2e specific Ab should be present in the infected chickens

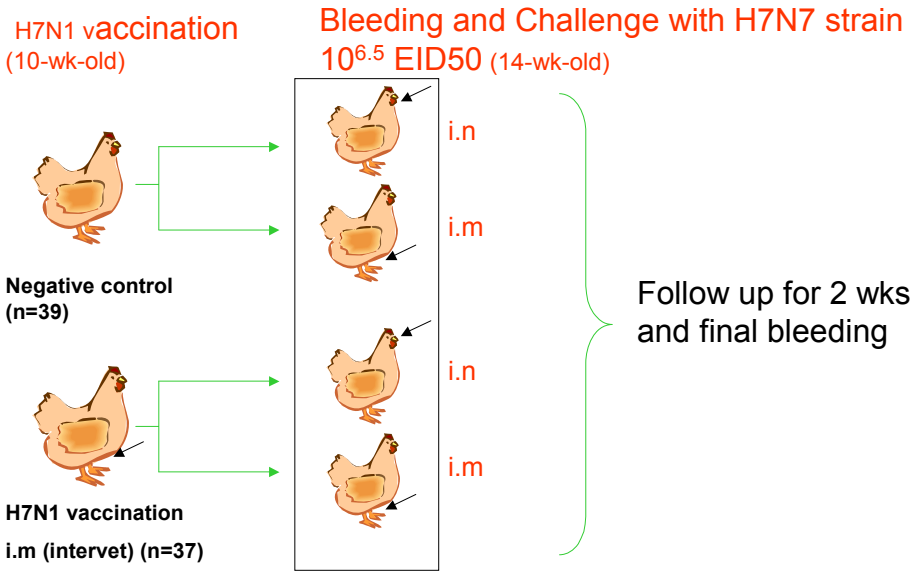
- The sequence M2e is highly conserved between different avian influenza strain (consensus sequence).

Development of a single M2e-specific ELISA for different avian influenza strains

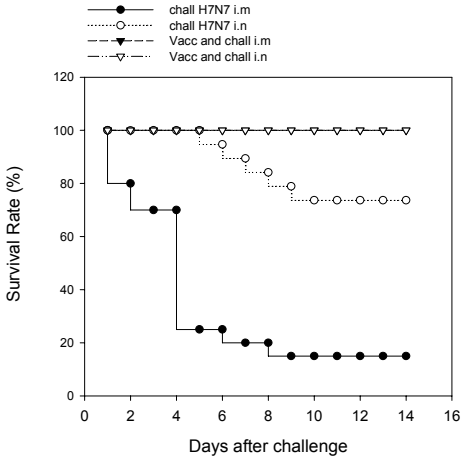
Development of a M2e-specific ELISA

- Coating of the peptide corresponding to the first 17 aa of the human M2e, without the first Methionine
- Blocking of the nonspecific binding
- Incubation of the diluted chickens sera (control, vaccinated and infected)
- Revelation

Protocole of H7N1 vaccination and H7N7 challenge on layer chickens



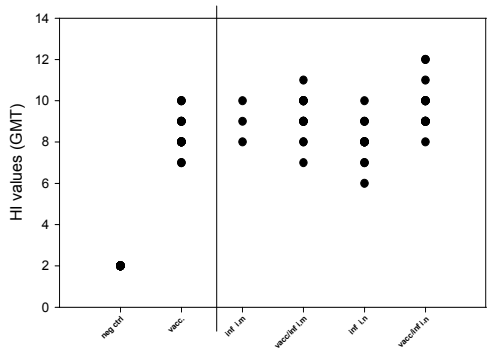
Survival post challenge of vaccinated and negative chickens



Five days after i.m H7N7 challenge, 50 % of control chickens died to reach 15% of final survival at day 14. On the contrary, after i.n challenge, 75% of chickens survived.

After H7N1 vaccination, all animals are protected against the challenge, independly of the route of inoculation of the challenge

Serological response: inhibition of hemagglutination



Prechallenge Post challenge

Before the challenge,

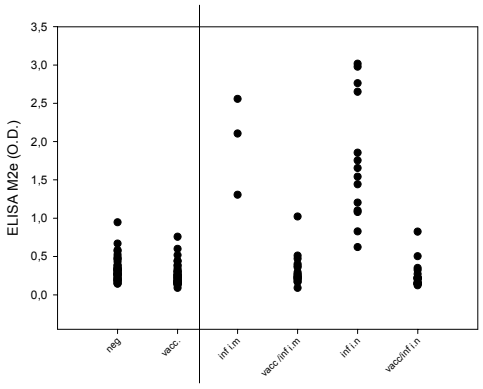
All vaccinated chickens presented high HI values

Post challenge,

Control chickens that survived the challenge, presented high HI values. No significant difference appears between i.n and i.m challenge.

Vaccinated and challenged chickens presented HI values comparable to that before challenge. No boost effect of challenge can be observed.

M2e-specific serological response



Prechallenge Post challenge

Before the challenge,

All vaccinated chickens did not present M2e-specific Abs, like the control group.

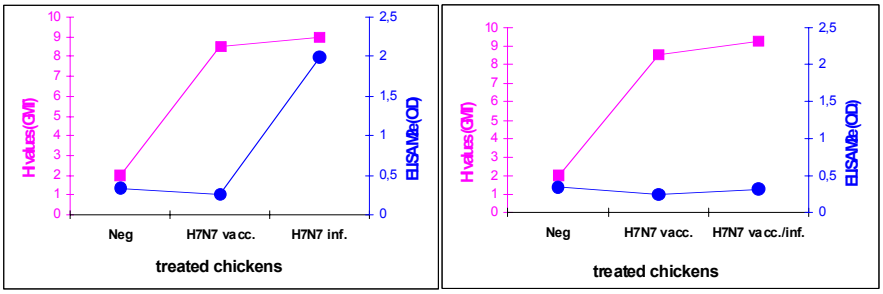
Post challenge,

Control chickens that survived the challenge, presented high levels of M2e-specific Abs.

No difference appears between i.n and i.m challenge.

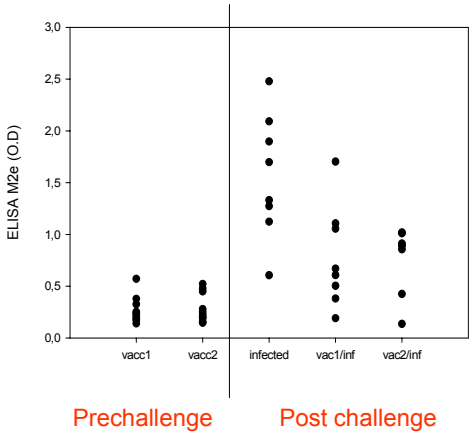
Most vaccinated /challenged chickens did not present M2e-specific Abs, like the control group.

In summary:



- With HI values, no difference can be observed between vaccinated , vaccinated/infected and infected groups
- With M2e specific ELISA, difference can be done between vaccinated and infected groups but not between vaccinated/infected and vaccinated groups

M2e-specific serological response after H5 vaccination and challenge H5N1 on ducks



Before the challenge,

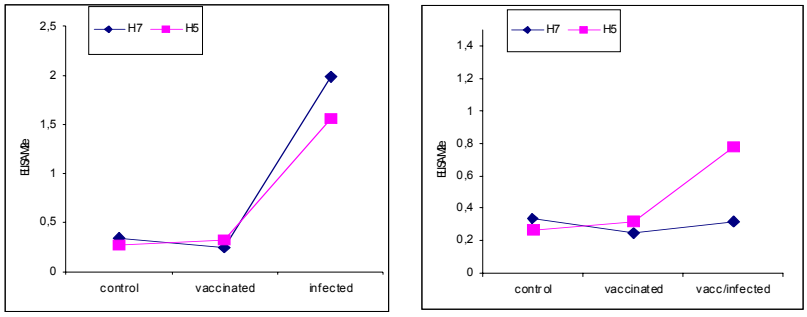
All vaccinated ducks did not present M2e-specific Abs, like the control group.

Post challenge,

Control ducks presented high levels of M2e-specific Abs.

Vaccinated /challenged ducks present M2e-specific Abs, level significatively different that vaccinated and infected groups.

In summary:



- With M2e specific ELISA, difference can be done between vaccinated , infected groups and vaccinated/infected in the case of H5 experiment but not in the case of H7experiment.

M2e sequence differs according to host

	2							10								20							24
Human	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G E	C	R	C	N	D G	S	S	D
%	100	100	100	100	100	100	100	100	100	98	98	100	100	100	76 24	100	98	100	100	76 24	100	100	100
Swine	S	L	L	T	E	V	E	T	P	I	R	N	G	W	E	C	R	C	N	D	S	S	D
Avian	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	K	C	S	D	S	S	D

Increase the sensibility, /specificity of of a M2e-specific ELISA

- Coating of the peptide corresponding to the first 17 aa of the avian M2e, without the first Methionine
- Blocking of the nonspecific binding
- Incubation of the diluted chickens sera (control, vaccinated and infected)
- Revelation

Isolation and characterisation of highly pathogenic H5N1 virus from Thai eagles smuggled into Europe

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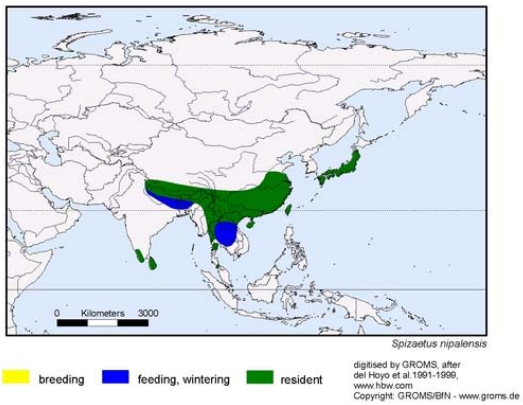
The story

- Mon 18/10/2004: Thai man <Bangkok-Vienna-BXL apprehended at BXL airport by the anti-drug group
- 2 birds of prey (*Spizaetus Nipalensis*) in a hand luggage (sports bag) with open zipper
- Birds wrapped in a cotton cloth with head free and inserted in a wicker tube
- Mr. P: "present for his brother" living in Antwerp

Eagles tested positive for H5N1 imported illegally into Europe from Thailand

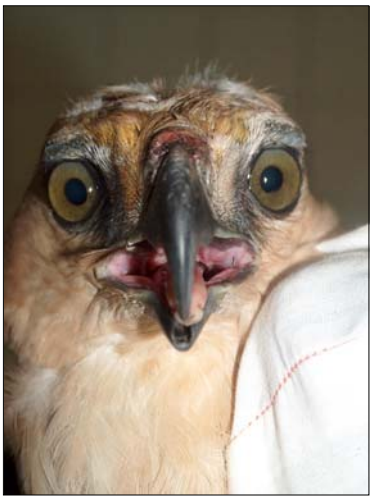
Crested mountain Hawk Eagle
(Spizaetus Nepalensis)



Distribution

Diagnosis

- Birds were seized and killed humanely (cfr Thai origin: Import of birds & products from several Asian countries in EU forbidden (DG SANCO Decision 2004/122/EC)
- Symptomatology: no clinical signs
- Necropsy at VAR:
  - Bilateral pneumonia in one eagle
- Inoculation of lung suspension to embryonnated eggs
- Egg mortality < 2 days
- Isolation of an haemagglutinating agent



Pictures courtesy of P. Meuleneire

Diagnosis

- Typing as H5N1:
  A/crested eagle/Belgium/01/2004 (H5N1)
- Confirmed by RT-PCR
- IVPI = 2.94
- HA cleavage site sequence : 6 basic residues:
  KRRKKR
- Phylogenic analysis of a 645 bp HA sequence:
  - identity score of 0.992 with strain A/Ck/Thailand/9.1/2004 (H5N1)

Public health measures

- 25 people in direct or indirect contact with eagles (veterinarian, lab staff, Thai passenger & brother):
  - oseltamivir prophylaxis on 24/10,
  - 2 nasal + 1 throat swab: all H5 negative (RT-PCR)
- The custom veterinarian who sacrificed the birds developed bilateral conjunctivitis 3 days after handling the birds: tear swab negative
- At reception of passengers list:
  - French Community: traced & checked (no symptoms),
  - Flemish community: tracing judged unnecessary (low risk, incubation period)

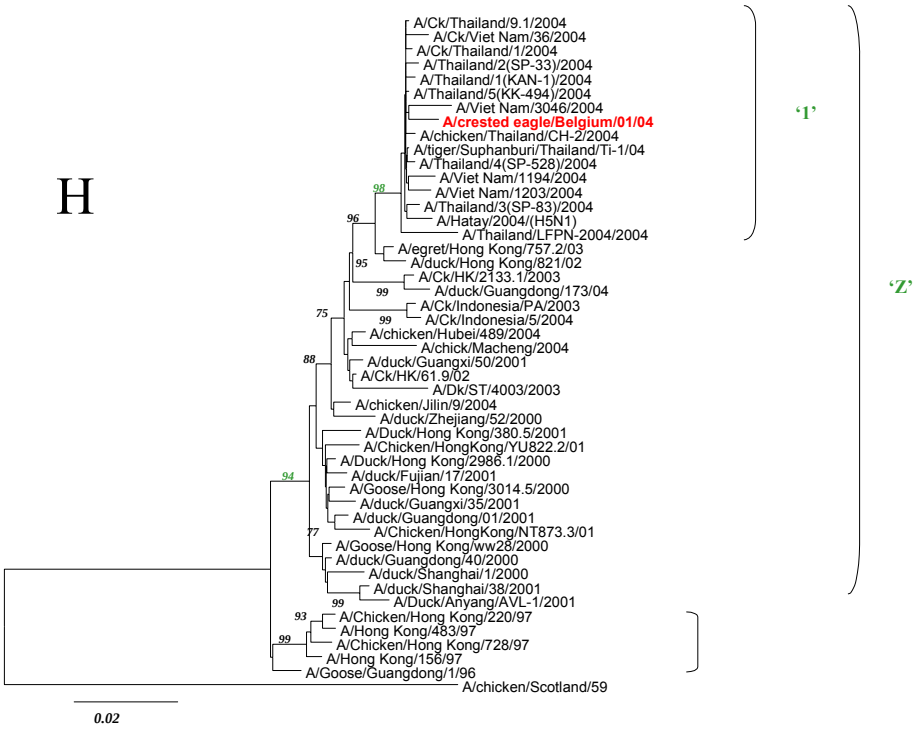
Veterinary measures

- Following the tracing of birds that had passed through the customs inspection centre during the at-risk period, the Federal Agency for the Safety of the Food Chain euthanised several batches of birds, notably:
  - 2 parrots at the customs inspection centre,
  - 200 parrots in a quarantine centre,
  - 450 exotic passerine birds in another quarantine centre.
- The sacrificed birds were brought to the VAR for further testing (RT-PCR and virus isolation on embryonnated egg or cell culture).
- All the RT-PCR and isolation tests were negative for the H5N1 strain.

- NP = +
- 18S = +
- H5 = +
- H7 = -
- HA cleavage site: 6 basic AA: highly pathogenic

H5N1

- NP = -
- 18S = +
- H5 = -
- H7 = -


H
A/Ck/Thailand/9.1/2004
A/Ck/Viet Nam/36/2004
A/Ck/Thailand/1/2004
A/Thailand/2(SP-33)/2004
A/Thailand/1(KAN-1)/2004
A/Thailand/5(KK-494)/2004
A/Viet Nam/3046/2004
A/crested eagle/Belgium/01/04
A/chicken/Thailand/CH-2/2004
A/tiger/Suphanburi/Thailand/Ti-1/04
A/Thailand/4(SP-528)/2004
A/Viet Nam/1194/2004
A/Viet Nam/1203/2004
A/Thailand/3(SP-83)/2004
A/Hatay/2004/(H5N1)
A/Thailand/LFPN-2004/2004
A/egret/Hong Kong/757.2/03
A/duck/Hong Kong/821/02
A/Ck/HK/2133.1/2003
A/duck/Guangdong/173/04
A/Ck/Indonesia/PA/2003
A/Ck/Indonesia/5/2004
A/chicken/Hubei/489/2004
A/chick/Macheng/2004
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A/Dk/ST/4003/2003
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A/duck/Zhejiang/52/2000
A/Duck/Hong Kong/380.5/2001
A/Chicken/HongKong/YU822.2/01
A/Duck/Hong Kong/2986.1/2000
A/duck/Fujian/17/2001
A/Goose/Hong Kong/3014.5/2000
A/duck/Guangxi/35/2001
A/duck/Guangdong/01/2001
A/Chicken/HongKong/NT873.3/01
A/Goose/Hong Kong/ww28/2000
A/duck/Guangdong/40/2000
A/duck/Shanghai/1/2000
A/duck/Shanghai/38/2001
A/Duck/Anyang/AVL-1/2001
A/Chicken/Hong Kong/220/97
A/Hong Kong/483/97
A/Chicken/Hong Kong/728/97
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A/Goose/Guangdong/1/96
A/chicken/Scotland/59
0.02
'1'
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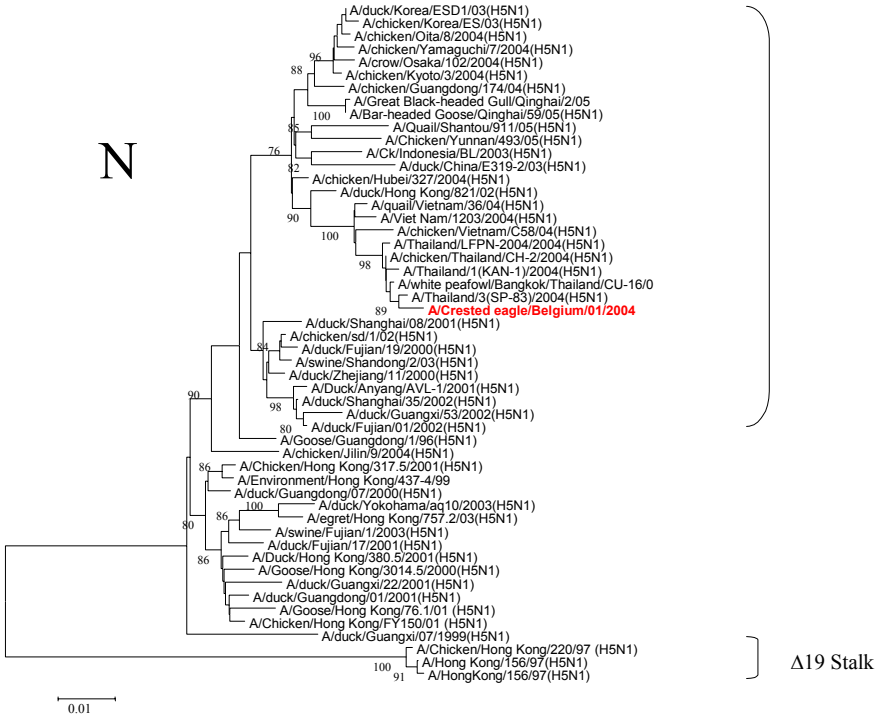
N
A/duck/Korea/ESD1/03(H5N1)
A/chicken/Korea/ES/03(H5N1)
A/chicken/Oita/8/2004(H5N1)
A/chicken/Yamaguchi/7/2004(H5N1)
A/crow/Osaka/102/2004(H5N1)
A/chicken/Kyoto/3/2004(H5N1)
A/chicken/Guangdong/174/04(H5N1)
A/Great Black-headed Gull/Qinghai/2/05
A/Bar-headed Goose/Qinghai/59/05(H5N1)
A/Quail/Shantou/911/05(H5N1)
A/Chicken/Yunnan/493/05(H5N1)
A/Ck/Indonesia/BL/2003(H5N1)
A/duck/China/E319-2/03(H5N1)
A/chicken/Hubei/327/2004(H5N1)
A/duck/Hong Kong/821/02(H5N1)
A/quail/Vietnam/36/04(H5N1)
A/Viet Nam/1203/2004(H5N1)
A/chicken/Vietnam/C58/04(H5N1)
A/Thailand/LFPN-2004/2004(H5N1)
A/chicken/Thailand/CH-2/2004(H5N1)
A/Thailand/1(KAN-1)/2004(H5N1)
A/white peafowl/Bangkok/Thailand/CU-16/0
A/Thailand/3(SP-83)/2004(H5N1)
A/Crested eagle/Belgium/01/2004
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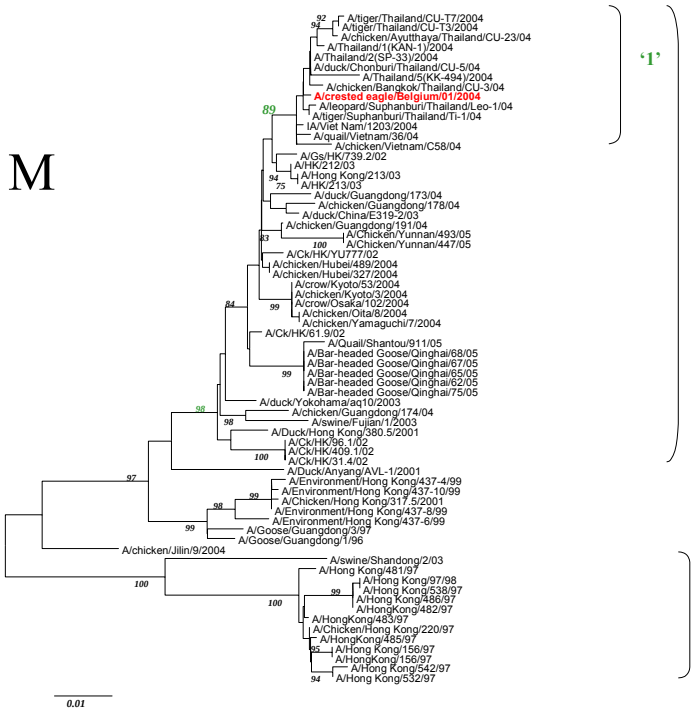
A/duck/Guangxi/53/2002(H5N1)
A/duck/Fujian/01/2002(H5N1)
A/Goose/Guangdong/1/96(H5N1)
A/chicken/Jilin/9/2004(H5N1)
A/Chicken/Hong Kong/317.5/2001(H5N1)
A/Environment/Hong Kong/437-4/99
A/duck/Guangdong/07/2000(H5N1)
A/duck/Yokohama/aq10/2003(H5N1)
A/egret/Hong Kong/757.2/03(H5N1)
A/swine/Fujian/1/2003(H5N1)
A/duck/Fujian/17/2001(H5N1)
A/Duck/Hong Kong/380.5/2001(H5N1)
A/Goose/Hong Kong/3014.5/2000(H5N1)
A/duck/Guangxi/22/2001(H5N1)
A/duck/Guangdong/01/2001(H5N1)
A/Goose/Hong Kong/76.1/01 (H5N1)
A/Chicken/Hong Kong/FY150/01 (H5N1)
A/duck/Guangxi/07/1999(H5N1)
A/Chicken/Hong Kong/220/97 (H5N1)
A/Hong Kong/156/97(H5N1)
A/HongKong/156/97(H5N1)
0.01
Δ20 Stalk
Δ19 Stalk



Comparative pathogenicity

3 months old layers

H7N7 IM route	H7N7 ON route	H5N1 IM route	H5N1 ON route
$10^{6,5}$ ELD_{50}	$10^{6,5}$ ELD_{50}	$10^{6,5}$ ELD_{50}	$10^{6,5}$ ELD_{50}
85 % mortality 10 days pi	26 % mortality 10 days pi	100 % mortality 1 day pi	100 % mortality 2 days pi

Comparative pathogenicity

6-week-old Pekin ducks

H5N1 by ON route
$10^{6,5}$ ELD_{50}
Somnolence-apathy 3 days pi Nervous signs starting on the 4th day 100 % mortality 8 days pi

Discussion

- Although *S. Nipalensis*, a CITES-listed species, frequently occurs in H5N1 problem regions in Thailand (see previous map), no details are currently available that may explain how the birds went infected.
- One possibility is that they have been fed with infected chicken carcasses shortly prior to their departure to Europe. This may explain why no clinical symptoms were observed.
- Back in Thailand, the smuggler was caught by the police and given a penalty of 5000 Bahts and waiting for punishment but maintained having bought the eagles in the Bangkok's Sunday market.
- Alternatively, some avian wildlife may have a higher resistance to the disease.

Discussion

- The only other report of H5N1 in wild birds of prey consists in a single peregrine falcon found dead in Hong Kong (OIE 2004).
- There are also two reports of AI infections of falcons with H7 HPAI:
  - Manvell *et al.*, Avian Pathology, 2000: Isolation of a HPAI virus of H7N3 subtype from a peregrine falcon dying in the United Arab Emirates.
  - Magnino *et al.*, Veterinary Record, 2000: During the HPAI outbreaks in Italy in 2000, an H7N1 virus was isolated from a saker falcon that died three days after normal hunting activity The raptor was presented with a sudden onset of depression, weakness and anorexia the day after normal hunting activity and died 2 days later without further clinical signs.

Conclusions

- Wild birds of prey have never been demonstrated as being involved in the dissemination of HPAI viruses but are most likely dead-end (top of pyramid) in the epidemiology of HPAI, eating infected carcasses.
- Anyway, illegal movements of birds of prey represent a significant threat for the introduction of HPAI.
- Hunting with falcons is practiced in several countries around the world.
- Here, a Belgian falconer who offered 7500 Euro for each bird had ordered the eagles & already owned birds of the same species.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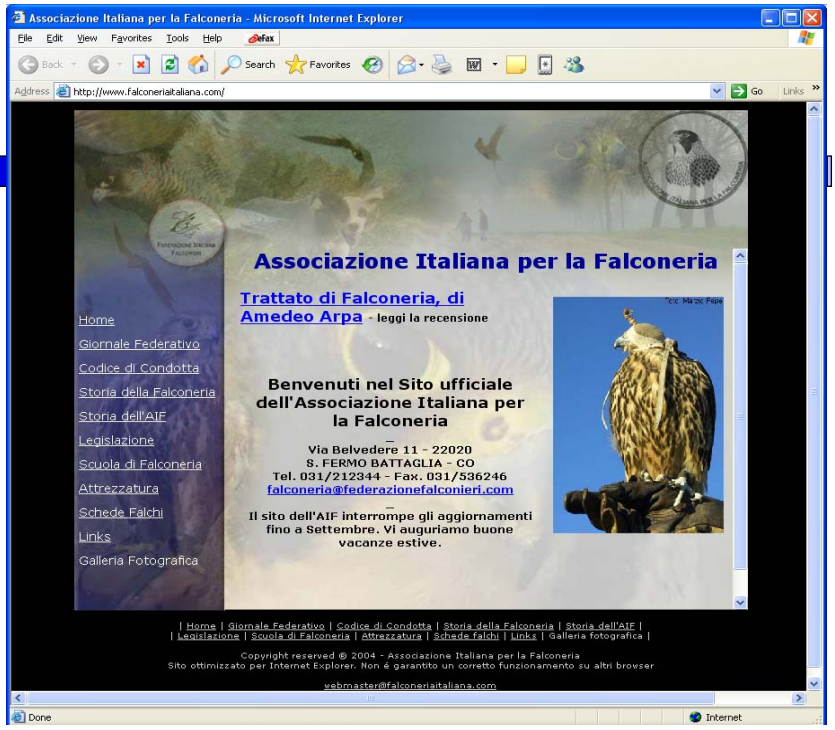
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Conclusions

- These two birds detected by customs may reflect a much larger underlying problem of bird smuggling into European Union member states.
- They easily remain undetected because airport scanners only detect metal objects.
- Specific methods for the systematic detection of live animals (e.g. dogs) should be considered at EU airports and borders. This is now under consideration in Belgium.

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- VAR: D. morales, M. Gonze, M. VdBroeck
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- Airport custom services: A. Graus, P. Meuleneire
- VLA Weybridge: D. Alexander, I. Brown, R. Manvell

Bracing Real Time RT-PCR's Achilles heel

11th EU NRLs
AI-NDV Meeting
Brussels 2005

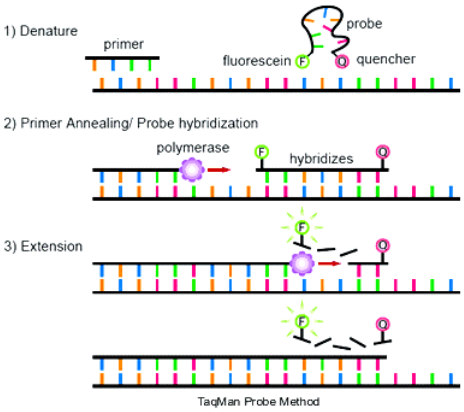
Steven Van Borm
B. Lambrecht
T. van den Berg
M.Steensels
M.Boschmans

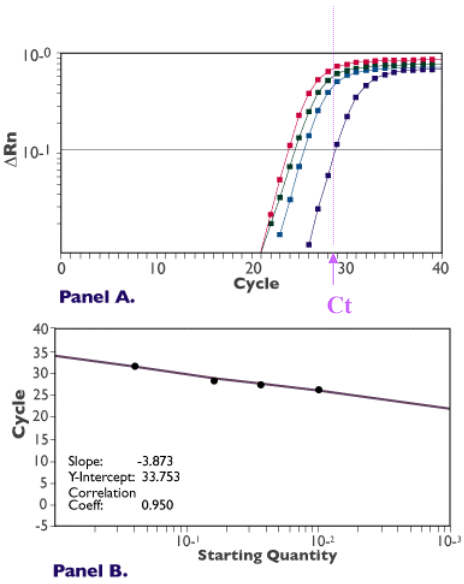
applying controls to a diagnostic setting

The Styx of Molecular Biology

- PCR: powerful technique allowing fast + sensitive diagnostic testing
- Achilles heel:
  - Theoretical sensitivity of 1 NA template: contamination – false positive results
  - DNA Polymerases sensitive to inhibition of enzymatic activity – false negative results
- Adapted to 'real time' fluorescence detection of template amplification

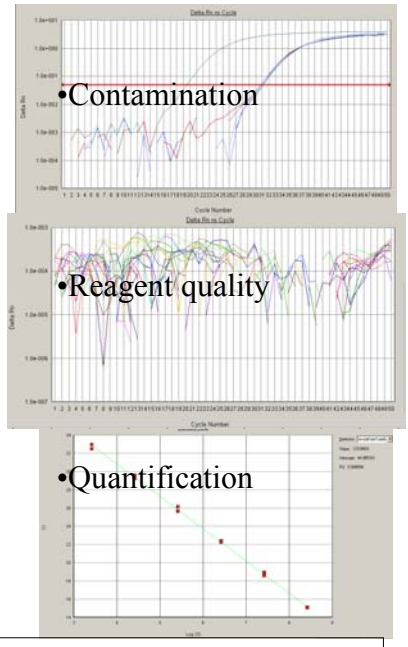
Real time RT-PCR





External controls

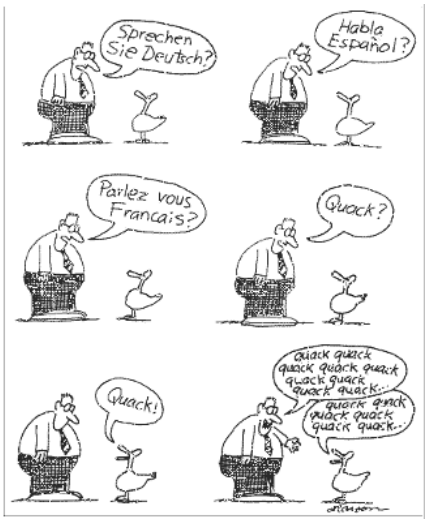
- Negative control
  - Water
  - Proven negative sample (SPF)
- Positive control
  - Synthetic RNA
  - Proven (VI) positive sample
- Standard
  - Using synthetic RNA dilutions as a standard curve for quantification



Inhibition of enzyme activity ? Sample quality/preservation?
→ "internal controls"

Internal controls: confusion

- « Internal » control
- « Endogenous » control
- « Exogenous » control
- « normalizer » control
- « housekeeping gene »



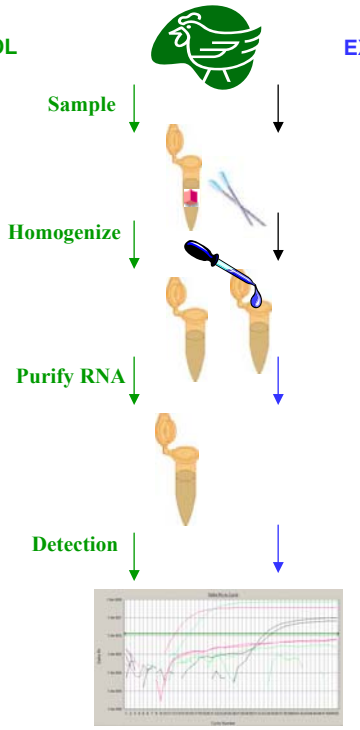
ENDOGENOUS CONTROL

•naturally present in each experimental sample

•Typically "housekeeping gene"

•Control for:
- •RNA extraction efficiency
- •Matrix associated inhibitors
- •**Sample quality / preservation ("nuclease context" of the sample)**

•Quantification normalizer if properly validated (gene or virus copies per normalizer copy)



EXOGENOUS CONTROL

•characterised RNA or DNA spiked into each sample at a known concentration

•Typically in vitro construct. Some commercially available constructs exist

•Control for:
- •RNA extraction efficiency
- •Matrix associated inhibitors

Real time RT-PCR detection of AI & NDV in our lab

- Influenza A. Matrix gene (if Q: in vitro transcribed RNA)

Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. Garber, K. Lohman, L. T. Daum, and D. L. Suarez. 2003. Development of real time RT-PCR for the detection of avian influenza virus. *Avian Diseases* 47: 1079-1082.

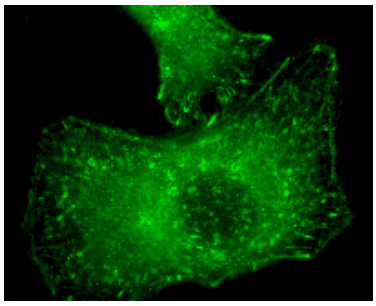
- NDV. Matrix gene (if Q: in vitro transcribed RNA)

Wise, M. G., D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, D. R. Kapczynski, and E. Spackman. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle Disease virus RNA in clinical samples. *J. Clin. Microbiol.* 42: 329-338.

- Development & validation of universal avian Endogenous internal control

Endogenous internal control

- 2 housekeeping genes 18S and β actin
- Design of oligonucleotides to bird-conserved sequences.
- 18S: frequent contamination false positives (2 different primer sets tested): too conserved ? + higher level of transcription?
- β actin
  - Single-copy gene
  - Moderate transcription level
  - Essential in cytoskeleton

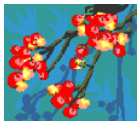


β actin alignment and oligos

β actin endogenous internal control for RRT-PCR: validation

- Universal avian?
- Tissues?
- Swabs?
- Quantitative? (development of a synthetic standard RNA)
- Multiplexing? (two step RT-PCR multiplexing OK. Technical difficulties with one-step)

Universal Avian endogenous internal control

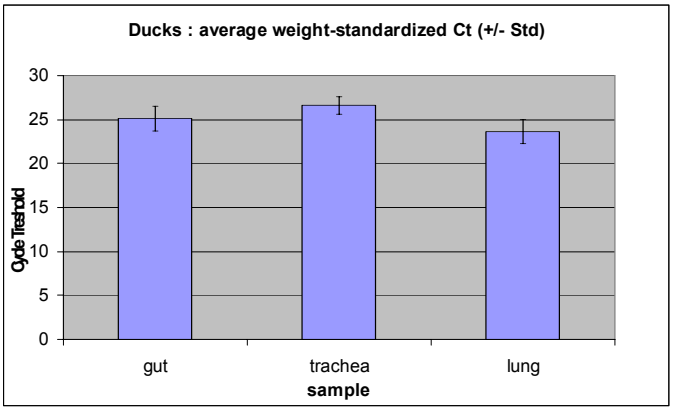
Familia (Ordo)	Species	Sample type	Result
Anatidae (Anseriformes)	*Anas plathyrhynchos* domesticated variant	Trach, lung, intestine, brain, oral swab, cloacal swab	+
	Anser anser domesticus	Trach	+
	Cygnus olor	Trach, lung	+
Columbidae (Columbiformes)	*Columba livia domestica*	Trach	+
Accipitridae (Falconiformes)	*Spizaetus nipalensis*	Trach, lung	+
Phasianidae (Galliformes)	*Gallus gallus domesticus*	Trach, lung, liver, intestine, oral swab, cloacal swab	+
	Phasianus Colchicus	Trach, lung	+
	Pavo cristatus	Lung	+
	Perdix perdix	Trach, lung	+
	Meleagris gallopavo	Trach	+
	Coturnix coturnix	Trach+lung	+
Rallidae (Gruiformes)	*Gallinula chloropus*	Lung, trachea	+
Corvidae (Passeriformes)	*Pica pica*	Lung, trachea, brain	+
	Corvus corone	Lung liver trachea heart	+
	Garrulus glandarius	brain	+
Fringillidae (Passeriformes)	*Serinus canarius*	brain	+
	Carduelis carduelis	Liver, lung, kidney	+
Turdidae (Passeriformes)	*Turdus merula*	Trachea , lung	+
Psittacidae (Psittaciformes)	*Agapornis cana*	Cloacal swab	+
	Psittacus erithacus	Cloacal swab	+
	Melopsittacus undulatus	Lung , trach	+
	Lathamus discolor	Brain	+
Struthionidae (Struthioniformes)	*Struthio camelus*	lung	+
Musophagidae (Musophagiformes)	*Corythaeola cristata*	Feces	+




(N=24)

No sign. Difference in β actin CT between different tissues

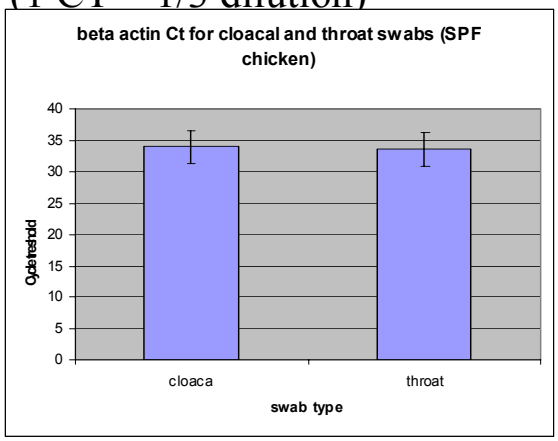
- Duck: constant weight standardized CT for different tissues



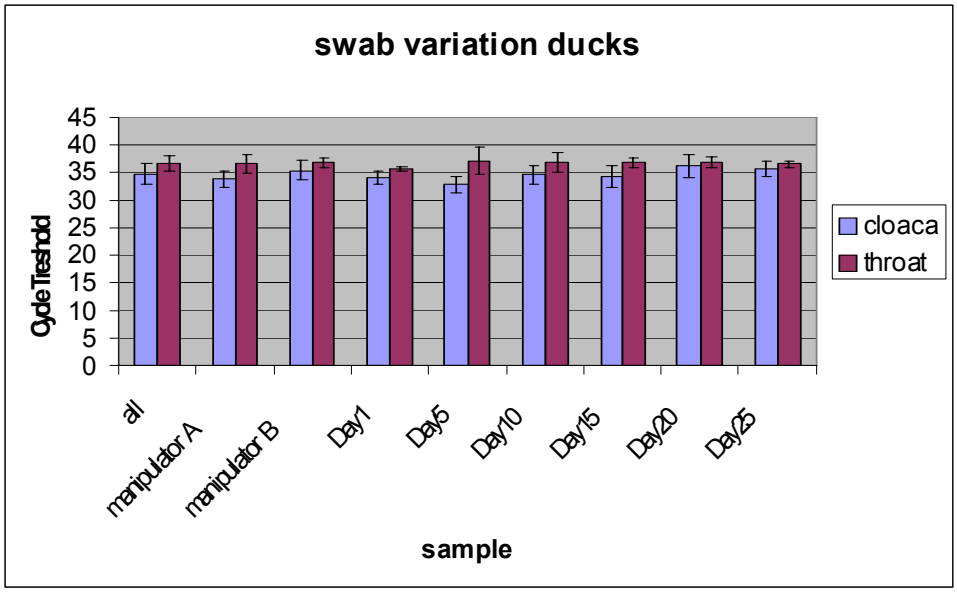
Swabs?

- Variation in swabbing: chicken

(1 CT ~ 1/3 dilution)

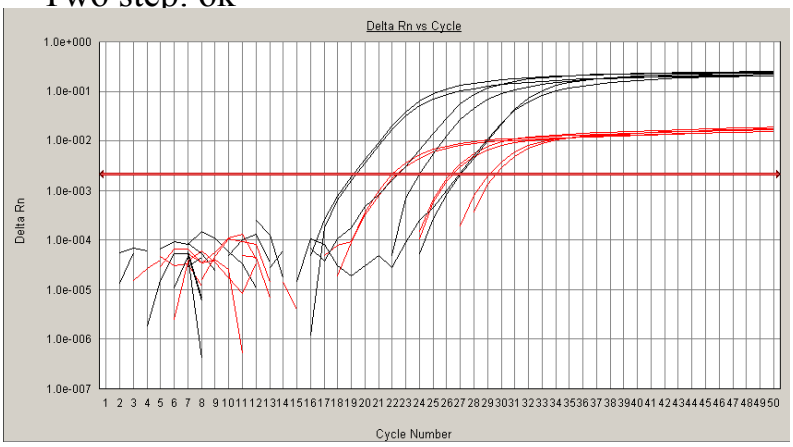


Swabs:



Multiplexing?

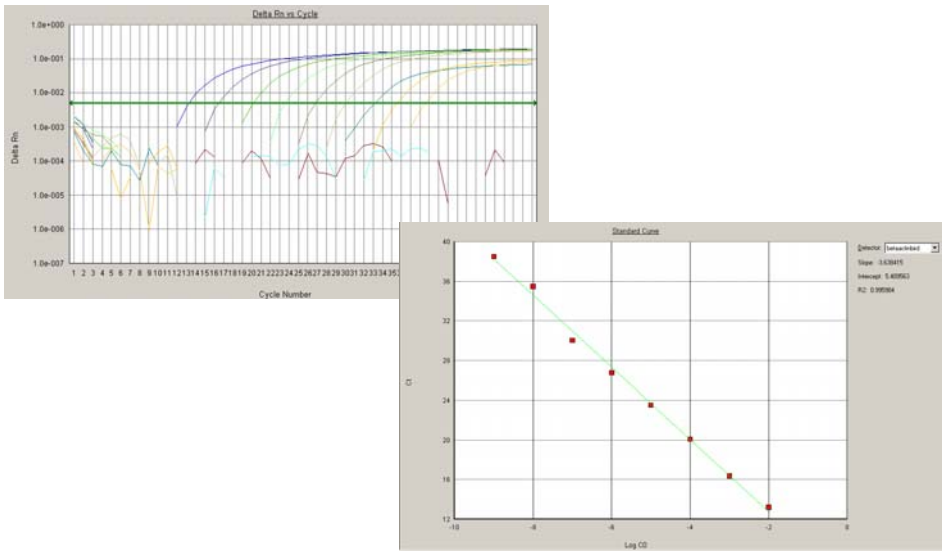
- Two step: ok



- Does not work in one step RT-PCR yet

Absolute quantification of β actin:

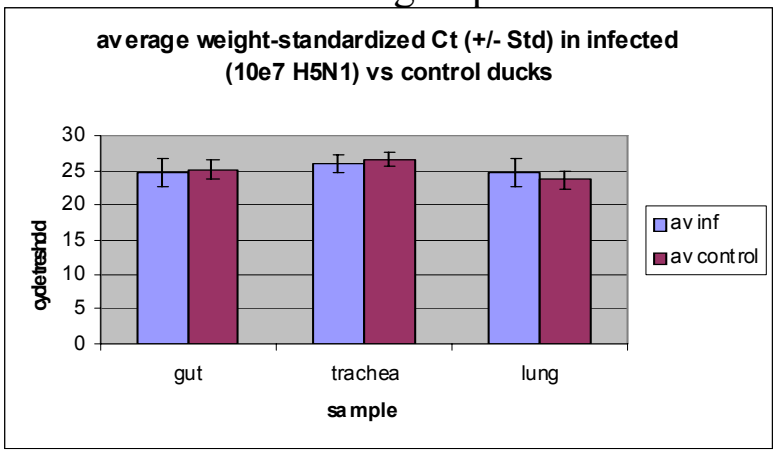
in vitro transcribed beta actin RNA



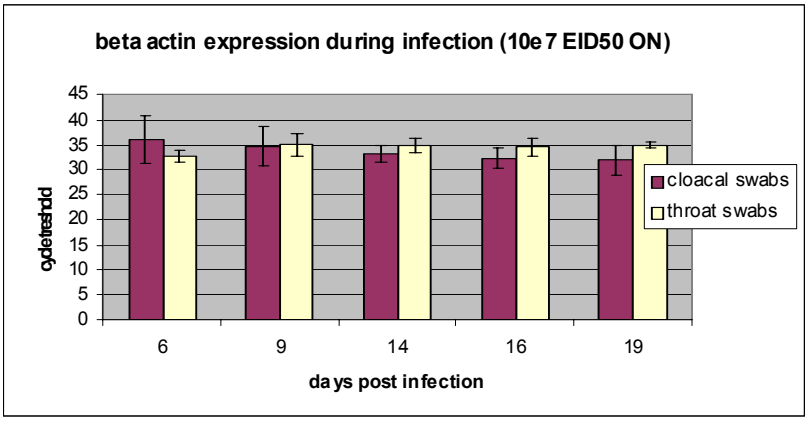
Relative quantification as a solution for variation in swab samples??

- Is β actin a good normaliser for influenza infection experiments?
- i.e.: is β actin gene expression influenced by influenza infection/disease?
- Our first results seem to suggest so:
  - weight standardized CT's seem identical between control and infected SPF chicken groups
  - β actin CTs seem to stay constant during infection

weight standardised CT's seem identical between control and infected SPF chicken groups



β actin CTs seem to stay constant during infection



discussion

- Universal avian endogenous control
- Qualitative control for tissue and swab
- Absolute quantification
- Possible normalizer/relative quantification of gene expression/viral RNA
- Duplex RRT-PCR Influenza& β actin (2step)

discussion

- Literature: some examples

Gene	Specificity	Ref
α2 (VI) collagen	Chicken	Islam et al 2004 J.Vir.Meth.119,103
18S rRNA	Human>chicken	Iván et al 2005 Can.J.Vet.Res. 69, 135
18S, β actin, 28S,GAPDH,TBP, β-2-microglobulin	Chicken (CEF), VAL NORM gene expression and virus quantitation studies	Li et al 2005 Vet. Microbiol. Sept10 Epub ehead of print
Yersinia bacteria	Exogenous control	Lund et al 2004 J.Clin.Microbiol. 42, 5125
…		

- Future priorities
  - Check and validate relative quantification possibilities for the swabbing variation problem.
  - 1 step multiplex detection NDV, Flu, β actin (qualitative)
  - Discussion, uniformisation, ring testing at the European level: lots of different "internal controls" are being used currently.

acknowledgements

•**Avian Virology and Immunology Unit**
•Thierry van den Berg
•Marc Boschmans
•Mieke Steensels
•Helena Ferreira
•Whole team …

The EU Diagnostic Manual for avian influenza

I.H. Brown, D.J. Alexander, R.J. Manvell, J. Banks and M. Slomka

CRL, VLA Weybridge, UK

Changes to the Directive:

- In the current Directive the techniques section forms part of the legal document
  - Therefore unable to revise in the light of advances in techniques
- In the new Directive the 'EU diagnostic manual' is an annex.
  - This gives the opportunity to modify the recommended protocols in the light of new advances

Proposed mechanism for review

- Review date is not formal
- Revisit at each National laboratories meeting
- Acceptance of a new technique/protocol would require the agreement of Member States
  - The 'new' technique would have to pass a standard proficiency trial against other accepted tests
  - Dossier of validation data would be required

Goals for a diagnostic test

- Specific
- Sensitive
- Reproducible
- Rapid
- Adaptable
  - Laboratory level
  - 'Field'
- Scale
- Competitively priced

Challenge is to apply advances in molecular diagnostics to AI

- Molecular detection methods offer the advantage of high sensitivity and for avian influenza may also provide genetic material suitable for the determination of the HA cleavage site by nucleotide sequencing.

'New' technologies

- Real time RT-PCR (RRT/PCR)
- Nucleic acid sequence based amplification (NASBA)
- DNA microarray

- Robotics
- Field application

- Aim is to harmonise AI molecular tests throughout EU
  - Only use tests that perform to the required standard
- EU diagnostic manual will give guidelines and protocols for recommended tests
- For molecular tests a range of protocols e.g. RT/PCR and RRT/PCR should be included in the manual to take account of the differing resources available to member states.

Choice of recommended protocols

- EU AVIFLU project - consortium involving 5 National AI Laboratories (DK, F, IT, NL, UK):
- Two ring trials
  - Evaluated a number of RT/PCR and RRT/PCR protocols

Aim of AI PCR Ring Trials

1) Establish an agreed approach to AI diagnostic PCR in the EU.

2) Focus on AI detection in clinical samples e.g. cloacal, tracheal or buccal swabs

Approach:

Each of six participating national reference laboratories were asked to use their chosen PCR methods to investigate a blind panel of 10 reconstituted clinical specimens and answer the following questions:

- Is there AI virus in the specimen, yes / no?
- If yes, is it H5 / H7 AIV?
- If H5 / H7, pathotype as LPAI / HPAI by cleavage site sequencing

PCR Ring Trial 2

17 blind samples with AI (or other) RNA present at clinical levels.

Each of six participating national reference laboratories to use recommended protocols from Trial 1, plus any other method of its choice and answer the same questions

- Is there AI virus in the specimen, yes / no?
- If yes, is it H5 / H7 AIV?
- If H5 / H7, pathotype as LPAI / HPAI by cleavage site sequencing

Results summary

M gene PCRs:

Sensititvity: Very good by both conventional & RealTime approaches.
Specificity: Superior by RealTime PCR.

H5 & H7 PCRs:

Sensititvity: Varied considerably between different protocols in the first trial.

In the second trial, where a recommended protocol was used, good correlation was obtained between all the participating laboratories

For RealTime PCR, sensitivity is good for H5 but deficient for H7.

Specificity: Very good by both conventional & RealTime PCRs for both H5 & H7.

Findings/Recommendations:

- RRT/PCR offers significant advantages over conventional RT/PCR and should be used where possible
- The M gene RRT/PCR test for all subtypes of AI (*Spackman et al.*, 2002) was the most sensitive, specific and robust test and will be recommended. A conventional RT/PCR adaptation of this test was also found to be appropriate
- H5 and H7 specific tests are less sensitive than the M gene test. However, protocols for conventional RT/PCR and RRT/PCR have been identified and may be recommended for use alongside the M gene test after further validation.
  - Where the M test is positive and H5/H7 tests are negative further tests will be required to identify the virus subtype e.g. virus isolation and antigenic characterisation

Quality Control & Validation Issues

- Internal Control?
  - For PCR tests to be validated there is often a requirement to include internal controls for each sample. Especially for single e.g. tests on human specimens.
- Propose the use of external controls – a dilution series of positive control. Applied per plate or test.
- This approach would be justified because there will be a recommendation for the number of samples that should be tested to give confidence that any positive animals will be detected
- Protocols that are recommended will have been fully validated

Proficiency testing

- Administered by the CRL
- Member states would be required to demonstrate their proficiency before using Molecular tests.
- Competency tested in annual ring trials
- Probes and Primers should be checked by the CRL biannually to ensure that all recent AI strains can be detected.

Avian Influenza in Kazakhstan

OIE mission September 6-11, 2005

Giovanni Cattoli & Stefano Marangon

Reference Laboratory for Newcastle Disease and Avian Influenza,
Istituto Zooprofilattico Sperimentale delle Venezie
Legnaro –Padova, Italy

AVIAN INFLUENZA IN KAZAKHSTAN

OIE mission carried out from 6 to 11 September 2005

- Seven AI outbreaks were notified in Kazakhstan in July – August 2005 in 4 regions
- The Central Veterinary Department of Kazakhstan requested an OIE mission to:
  - assess the epidemiological situation
  - audit animal health policies for diagnosis and control
  - recommend further actions

KAZAKHSTAN POULTRY POPULATION SIZE

Kazakhstan is the world's 9th largest country

30 million poultry are reared in Kazakhstan:

- 12 millions in 64 large poultry operations (among these 15 with more then 30,000 birds)
- 18 millions in villages

Poultry holdings and villages sparsely distributed in the country

Trade among poultry farms and villages appeared to be limited

Presence of markets for live poultry

PUBLIC VETERINARY SERVICE

The Veterinary Department, Ministry of Agriculture, co-ordinates the activity of:

- Regional Veterinary Inspection Offices
- State Veterinary Border Control Offices
- Veterinary Disease Control Offices
- Veterinary Laboratories (MAC RK and RSE)

More then 3,000 veterinarians at Regional, sub-regional, village and market level

AVIAN INFLUENZA IN KAZAKHSTAN

First AI outbreak suspected on 22/07/2005

- Private poultry farm with 3,411 birds (2,350 geese, 450 ducks and 611 chickens)
- High mortality (chickens), depression and nervous signs (ducks and geese)
- ELISA for type A antibody test: 38/39 positive blood samples (27/07/2005)
- Depopulation completed on 28/07/2005

AVIAN INFLUENZA IN KAZAKHSTAN

First AI outbreak in Pavlodar region (Golubovka)

- Samples taken from 4-5 sick geese and 1 sick wild duck
- H5N1 HPAI virus isolated at Otar laboratory (SRAI)
- The farm was located close to a small lake, where wild waterfowl were present, 2 km away from the village of Golubovka
- The village was put under restriction and no other cases of AI were detected

AVIAN INFLUENZA IN KAZAKHSTAN

Seven AI outbreaks were detected from 22 July to 17 August 2005 in 4 regions

- All the outbreaks were detected in village poultry and they were not epidemiologically correlated
- Only one outbreak was not located close to a lake
- Geese, ducks and chickens were present in all the affected villages
- H5N1 virus was isolated only from the first three outbreaks

AVIAN INFLUENZA IN KAZAKHSTAN

Eradication measures enforced

- Stamping out of all birds in 5 out of 7 affected villages
- A total of 13,438 birds were immediately killed and destroyed on the spot
- Enforcement of restriction measures (movement restriction, checking and disinfection points, etc.)
- Compensation was paid to farmers
- No vaccination was applied

AVIAN INFLUENZA IN KAZAKHSTAN

Control and prevention measures

- Disease awareness increased *(slide con poster e leaflet)*
- AI crisis units were instituted in each region
- Domestic ducks and geese not allowed to feed in rivers and lakes
- Trade of live poultry and poultry products among villages has been prohibited
- Poultry farms put under official veterinary surveillance

AVIAN INFLUENZA IN KAZAKHSTAN

Origin of the outbreaks

- The CVA reported that contact with infected wild waterfowl was the likely origin of the infection
- H5N1 virus was reported to have been isolated from one sick wild duck found close to the second outbreak site
- No other AI tests were carried out from wild waterfowl
- No high mortality was reported in wild waterfowl

MIGRATORY FLYWAYS OVER KAZAKHASTAN

- Wild waterfowl arrive from the south in April-May
- Wild ducks breed in lakes and ducklings are hatched towards the end of May
- The winter migration takes place in October-November
- The virus should have been introduced in the country during the spring migration and should have been maintained and amplified in the resident wild bird population until July

AVIAN INFLUENZA IN KAZAKHSTAN

Comments

- No systematic monitoring was carried out in wild waterfowl due to the limited diagnostic capabilities of the laboratories
- H5N1 virus has been detected only in one sick wild duck found close to the first outbreak site. The bird could have been infected from diseased domestic poultry

AVIAN INFLUENZA IN KAZAKHSTAN

Human health implications

- Only one human case was suspected (first outbreak)
- The man, with clinical signs of pneumonia, was hospitalised
- All the tests gave negative result for AI virus of the H5N1 subtype
- No other cases of AI in humans have been reported

THE VETERINARY LABORATORIES OF KAZAKHSTAN

National Centre of Veterinary Diagnostic and Methodology (2 branches: Astana and Almaty)

- research, monitoring and confirmation of infectious animal diseases

Republican Veterinary Laboratory (215 labs)

- basic diagnostic service for animal infections

Scientific Research Agricultural Institute (SRAI, Otar)

- research on diagnostic tests and molecular biology

VETERINARY LABORATORIES

The National Centre of Veterinary Diagnostic and Methodology and the Republican Veterinary Laboratory carried out only antibody ELISA tests for type A viruses (*commercial kit – not validated for duck & geese*)

Scientific Research Agricultural Institute received samples from three outbreaks and performed the following tests:

- PCR for type A avian influenza virus
- H5 specific PCR
- sequence of the cleavage site
- virus isolation in chicken fibroblasts
- HI test (for typing)

VETERINARY LABORATORIES

Major drawbacks

- Lack of reference materials (reference strains and antisera) used for PCR reactions, HI and Neuramminidase-inhibition test
- Complete data about sequencing was not provided
- Identification of virus strains isolated in the outbreaks was not confirmed by using the standard OIE/EU procedures

CONCLUSIONS

- Stamping out and restriction measures were effectively applied to control and eradicate the disease
- Surveillance and follow-up systems were effective for the early detection and the rapid elimination of AI outbreaks
- The possible introduction of the infection from wild waterfowl should have been confirmed by the isolation of the H5N1 HPAI virus from wild waterfwol

RECOMMENDATIONS

- The CVA should identify one National Reference Laboratory
- Permanent links must be created between OIE Laboratory network and such laboratory in order to provide reagents, training and exchange of technical and scientific information

RECOMMENDATIONS

The reference diagnostic laboratory must:

- have all the necessary equipment, reagents end expertise to perform basic diagnostic tests for screening and identification of AI viruses (e.g. PCR for type A, H5 and H7)
- perform AI diagnostics in accordance to OIE/EU standard procedures

RECOMMENDATIONS

- Monitoring and preventive measures put into place in Kazakhstan should be continued
- Virological monitoring of wild waterfowl should be carried out to identify the possible origin of infection and evaluate the risk of further outbreaks

TECHNICAL REPORT OF THE COMMUNITY REFERENCE LABORATORY FOR AVIAN INFLUENZA, 2004

I. LEGAL FUNCTIONS AND DUTIES

The functions and duties are specified in Annex V of Council Directive 92/40/EEC (Official Journal of the Communities No L 167 of 22.6.1992).

II. OBJECTIVES FOR THE PERIOD JANUARY – DECEMBER 2004

1. Characterising viruses submitted to the Laboratory by Member States and third countries listed in Commission Decisions 95/233/EC and 94/85/EC. This will, at the request of the European Commission or the submitting National Laboratory or at the discretion of the Reference Laboratory, include:

    a) Determining the intravenous pathogenicity index (IVPI)

    b) Antigenic typing of viruses and both haemagglutinin and neuraminidase subtypes

    c) Determining the amino acid sequence at the haemagglutinin cleavage site of H5 and H7 subtype viruses

    d) Limited phylogenetic analysis to assist in epidemiological investigations.

***Work Plan**: The number of viruses received will be dependent on the outbreaks occurring and those viruses submitted, as a guide the numbers received since 1988 are shown in Table 1.*

Table 1: Number of submissions to the CRL by year since 1988.

1988	1989	1990	1991	1992	1993	1994	1995
401	188	113	154	199	294	385	605
1996	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**
284	227	285	357	704	316	333	464	426

The haemagglutinin and neuraminidase subtypes of all influenza viruses submitted will be determined. IVPI tests will be done at the request of the submitting laboratory or the Commission. The amino acids at the haemagglutinin cleavage site of all viruses of H5 and H7 subtype will be deduced by nucleotide sequencing. For selected viruses sequencing will be extended into other areas of the H gene to allow phylogenetic analyses.

***% Resources**: 60 %*

WORK DONE: The viruses submitted in 2004 were characterised as shown in Table 2.

Table 2: Identification of viruses submitted to the reference laboratory in 2004

Virus identification	Number
Paramyxoviruses	***173***
Influenza A viruses	***181***
H1N1	2
H3N?	2
H3N2	2
H3N6	3
H3N8	4
H4N6	5
H5N?	3
H5N1	23
H5N2	6
H5N3	2
H5N9	1
H6N5	1
H6N6	1
H6N8	1
H7N?	3
H7N1	10
H7N3	26
H9N2	72
H9N8	1
H10N?	5
H10N7	7
H11N9	1
others	***72***
reovirus	1
poxvirus	7
untyped	52
virus not viable	12

In addition to conventional typing of the viruses submitted a total of 33 AI viruses was subjected to nucleotide sequencing and the amino acids at the haemagglutinin cleavage site deduced. Of these 25 had multiple basic amino acids and therefore were HPAI viruses, 8 had amino acid motifs consistent with virus of low pathogenicity.

The presence of basic amino acids at the haemagglutinin cleavage site is now well accepted as demonstrating virus virulence for AI viruses and intravenous pathogenicity index [IVPI] tests to assess the virulence of the submitted viruses were only done at the request of the submitting country. In all 43 IVPI tests were done on request.

Estimated actual resources: 54%

2. Maintain and distribute virus repository and reagents necessary for virus characterisation.

***Work Plan**: Maintenance of existing repository will continue. All viruses submitted to the CRL will be added to the repository after characterisation. Most viruses will be maintained in a frozen state, but selected, representative viruses will be freeze dried. Reagents such as polyclonal chicken antisera, and control antigens will be maintained at levels previous demands have indicated to be necessary to enable characterisation of all 15 H and all 9 N subtypes.*

***% Resources**: 6 %*

WORK DONE: The AI viruses received were added to the repository. Reagent stocks were maintained, at least at previous levels [Table 3] and during the year the following were supplied:

ANTIGENS: 40 x 1.0ml of influenza A agar gel precipitin antigen, 7 x 1.0ml of H1 antigen, 1500 x 1.0ml of H5 antigen, 10 x H6 antigen, 345 x 1.0ml of H7 antigen, 135 x 1.0ml of H9 antigen, 2 x H13 antigen,

ANTISERA: 95 x 0.5ml of influenza A agar gel precipitin antiserum, 11 x 0.5ml H1 antiserum, 8 x 0.5ml H2 antiserum, 11 x 0.5ml H3 antiserum, 8 x 0.5ml of H4 antiserum, 261x 0.5ml of H5 antiserum, 30 x 0.5ml H6 antiserum, 257 x 0.5ml of H7 antiserum, 6 x 0.5ml H8 antiserum, 37 x 0.5ml of H9 antiserum, 8 x 0.5ml H10 antiserum, 6 x 0.5ml of H11 antiserum, 6 x 0.5ml antiserum, 7 x 0.5ml H13 antiserum, 6 x 0.5ml H14 antiserum and 6 x 0.5ml H15 antiserum .

32 x 0.5ml ampoules of SPF chicken serum were also supplied.

***Estimated actual % resources**: 4%*

Table 3. Stocks of polyclonal chicken sera and virus antigens for HI tests held at the Reference Laboratory.

Type	Serum		Antigen	
	Quantity[a]	HI titre[b]	Quantity[a]	HA titre[b]
SPF	100	<1		
H5	300	7	400	6
H7	450	6	350	7

[a] *Number of freeze-dried ampoules containing 0.5 ml of serum or antigen at the indicated titre.*

[b] *HI and HA titres are expressed as $\log_2$. The SPF serum had an HI titre of <1 to each antigen.*

3. Prepare and distribute antisera, antigens and reagents for the inter-laboratory comparison tests.

***Work Plan**: Antisera and antigens to be used in the comparison tests will be prepared, freeze-dried and dispatched to the National Laboratories in time for results to be reported at the next annual meeting.*

***% Resources**: 6 %*

WORK DONE: Antigens were prepared and dispatched to EU National Laboratories and those of EFTA and accession countries [total 31 laboratories]

***Estimated actual % resources**: 4%*

4. Analysis of results submitted by National Laboratories for the inter-laboratory comparison tests.

***Work Plan**: As in previous years, results submitted by the National Laboratories will be analysed and presented at the annual meeting.*

***% Resources**: 3 %*

WORK DONE: *Results were received, analysed and an oral presentation made at the Annual Meeting in 2004. A written report will appear in the proceedings.*

***Estimated actual % resources**: 2%*

5. Conduct work to evaluate reported problem areas in diagnosis.

***Work Plan**: Staff of the CRL will be available for consultation by National Laboratories, problem sera and other reagents will be received from National Laboratories for testing and evaluation.*

***% Resources:** 2 %*

WORK DONE: Staff of the CRL were consulted on an ad hoc basis.

***Estimated actual % resources**: 1%*

6. Supporting by means of information and technical advice National Avian Influenza Laboratories and the European Commission during epidemics.

***Work Plan**: Staff of the CRL will be available for consultation and will forward all relevant information to the National Laboratories or the Commission, as appropriate.*

***% Resources:** 3 %*

WORK DONE: Staff of the CRL were consulted on numerous occasions by other National Laboratories representatives of member states and the Commission. In addition CRL staff took part in the following consultation groups and meetings

1. OIE ad hoc group on avian influenza [D. Alexander]
2. EFSA Working group on avian influenza [D. Alexander]
3. EU Commission Working Group on Revision of the Directive for the Control of Avian Influenza [I. Brown]
4. EU Mission to Canada on HPAI [R. Manvell]
5. EU AI survey guideline revision, Brussels, [I. Brown]
6. FAO emergency meeting re Asian AI epidemic, Rome, [I. Brown]
7. FAO technical meeting on AI, Bangkok, [I. Brown]
8. TAIEX seminar for new EU member states, Brussels, [I. Brown]
9. EU influenza research meeting, Brussels, [I. Brown]
10. FAO meeting on establishing AI surveillance and lab networks in Asia, Bangkok, [I. Brown]
11. TAIEX (EU) laboratory visit and simulation exercise ND/AI, Latvia, [I. Brown]
12. TAIEX (EU) laboratory visit and simulation exercise ND/AI, Hungary [I. Brown]
13. OIE/FAO/WHO meeting to establish formal connection between veterinary and human influenza networks, Padova, [I. Brown]
14. FAO/OIE endorsed meeting in Geelong, Australia, Global research agenda for AI, [I. Brown]
15. WHO/OIE/FAO meeting on AI research agenda and animal-human network, [I. Brown]

Estimated actual % resources**: **10%

7. Prepare the programme and working documents for the Annual Meeting of National Avian Influenza Laboratories.

***Work Plan**: The organisation of the Annual Meeting in collaboration with the Commission's representative will be done as in previous years.*

***% Resources:** 2 %*

WORK DONE: In collaboration with the Commission's representatives the Annual Meeting was organised and held at VLA Weybridge in September/October 2004.

Estimated actual % resources**: **4%

8. Collecting and editing of material for a report covering the annual meeting of National Avian Influenza Laboratories.

***Work Plan**: Receive and collate submissions edit and produce report of 2003 proceedings before 2004 Annual meeting. Receive and collate submissions of 2004 meeting.*

***% Resources**: 3 %*

WORK DONE: Proceedings of the 2003 meeting were produced before the 2004 meeting.

***Estimated actual % resources**: 2%*

9. In the light of the occurrence of influenza in birds and other animals keep under review the possible zoonotic impact arising from the risk of reassortment between influenza viruses.

***Work Plan**: Analyse data as it becomes available*

***% Resources:** 2%*

WORK DONE: This was done through CRL staff membership of the WHO/FAO/OIE animal-human influenza network and the various consultations indicated in section 6. In addition close watch was kept on situations relating to spread of AI viruses from birds to humans and this is reflected in the publication outputs from CRL staff.

***Estimated actual % resources**: 2%*

10. Finalise work carried out in respect to the surveys in poultry and wild birds during 2003 and revise survey guidelines for surveys to be carried out during 2004.

***Work Plan**: Produce final survey report and ensure guidelines are adopted*

***% Resources: 4**%*

WORK DONE: The CRL analysed all results received from member states and produced a final survey report. This report contained a detailed epidemiological analyses that was used to inform discussions for revision of the guidelines at a specific meeting held in Brussels with representatives of all member states. The revised guidelines were adopted in decision doc 2004/615/EC.

***Estimated actual % resources**: 6%*

11. Carry out work in relation with surveys for avian influenza to be implemented by Member States during 2004.

***Work Plan**: Produce and distribute panel of reagents, supply technical support.*

% Resources: 8%

WORK DONE: The CRL produced and held an enlarged panel of reagents. All national laboratories were supplied with reagents for the conduct of the survey. Technical support was provided to the programme including direction on application of the survey and verification of results by CRL if required.

Estimated actual % resources: 8%

12. Preparation and publications of articles and reports associated with above work.

% Resources: 1%

WORK DONE:

RELEVANT PUBLICATIONS IN 2003

1. SUAREZ, D.L., SENNE, D.A., BANKS, J., BROWN, I.H., ESSEN, S.C., LEE, C.W., MANVELL, R.J., MATHIEU-BENSON, C., MARENO, V., PEDERSEN, J., PANIGRAHY, B., ROJAS, H., SPACKMAN, E. & ALEXANDER, D.J. (2004). Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerging Infectious Diseases 10, 1-13. http://www.cdc.gov/ncidod/eid/vol10no4/03-0396.htm
2. CAPUA, I. & ALEXANDER, D.J. (2004). Avian influenza – Recent Developments Avian Pathology 33, 393-404.
3. ALEXANDER, D.J. (2004). Highly pathogenic avian influenza (fowl plague). Chapter 2.1.14. OIE Manual of Standards for diagnostic tests and vaccines, 5th ed volume I. OIE : Paris pp 258-269.
4. ALEXANDER, D.J. & MANVELL, R.J. (2004). Technical Report of the Community Reference Laboratory for avian influenza 2002. Proceedings of the Joint 9th Annual Meetings of the National Laboratories for Newcastle Disease and Avian Influenza of EU Member States, Brussels, 2003 pp8-13.
5. ALEXANDER, D.J. & MANVELL, R.J. (2004). Country Reports on avian influenza based on responses to the questionnaire. Proceedings of the Joint 9th Annual Meetings of the National Laboratories for Newcastle Disease and Avian Influenza of EU Member States, Brussels, 2003 pp 113-129.
6. ALEXANDER, D.J. & MANVELL, R.J. (2004). Comparative tests for antigen identification in different National Laboratories 2003. Proceedings of the Joint 9th Annual Meetings of the National Laboratories for Newcastle Disease and Avian Influenza of EU Member States, Brussels, 2003 pp 241-244.
7. ALEXANDER, D. & CAPUA, I. (2004). Avian influenza. State Veterinary Journal 14, 3-8.
8. VAN REETH, K., BROWN, I.H., ESSEN, S.C. & PENSAERT, M.B. (2004). Genetic relationships, serological cross-reaction and cross-

protection between influenza A virus subtypes endemic in European pigs. Virus Research 103, 115-124.

9. KO, L-S, LAU, L-T BANKS, J., AHERNE, R., BROWN, I., COLLINS, R., CHAN, K-Y., XING, J. & YU, A.C.H. (2004). Nucleic acid sequence-based amplification methods to detect avian influenza virus. Biochemical and Biophysical Research Communications 313, 336-342
10. PHIPPS, L.P., ESSEN, S.C. & BROWN, I.H. (2004) Genetic subtyping of Influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. Journal of Virological Methods 122, 119-122.
11. OLSEN, C.W, BROWN, I.H., EASTERDAY, B.C. & VAN REETH, K. (2004) Swine influenza. IN Diseases of Swine 9th edition. Iowa State University Press, Ames.

***Estimated actual % resources**: 1%*