INTERIM REPORT 23 December 2005

Title:

Titration of Highly Pathogenic Avian Influenza H5N1 virus in commercial broiler chickens for the purpose of developing an infection model

Objective

Highly Pathogenic Avian Influenza (HPAI) H5N1 is involved in current outbreaks of disease in chickens and waterfowl in South-East Asia and Eastern Europe. Reduction of viral shedding by infected birds and in environmental viral load has emerged as a critical control point for management of HPAI H5N1 in these countries

NeuMedix Biotechnology PL has developed a tea-tree oil extract (Megabac) that has exhibited anti-viral activity *in vitro* against Highly Pathogenic Avian Influenza (HPAI) H5N1.

A challenge model for HPAI H5N1 infections in commercial broiler chickens is needed for evaluation of the Megabac product in efficacy studies. Development of the challenge model should incorporate clinical observations of birds challenged with various viral loads, assessment of the degree and duration of viral excretion, and measurement of seroconversion. This should permit selection of a suitable challenge dose of virus, observation parameters and strategic intervention points for assessment of viral excretion and/or seroconversion in subsequent studies.

Sponsor

NeuMedix Biotechnology

Personnel

All personnel are from the OIE and National reference Laboratory for Avian Influenza, *CSIRO AAHL, PB 24, Geelong, 3220, Australia ph. 03 5227 5300*

Deborah Middleton BVSc, MVSc, PhD	Veterinary pathologist
John Bingham BVSc, PhD	Veterinary pathologist
Paul Selleck BAgrSc	Avian virologist
Sue Lowther	Virologist
John Muschialli	Animal attendant
Don Carlson	Animal attendant

Experimental period

Experimental starting date: November 05
Experimental termination date: January 05

Test substance

Avian Influenza HPAI H5N1 (A/Ch/Vietnam/8/04)

Challenge strains

The challenge strain A/ Ch/ Vietnam/ 8/ 04 H5N1 was obtained from the Regional Animal Health Centre, Ho Chi Minh City, Vietnam. Infected allantoic fluid (passed three times in SPF chicken eggs) was diluted in sterile PBS in serial dilutions from 10^{-2} to 10^{-6} for administration to chickens. The challenge dose was 0.5 ml inoculum and it was administered intranasally (0.2ml), orally (0.2ml) and by eyedrop (0.1ml).

The median chicken infectious dose (CID_{50}) will be calculated according to the Reed and Muench formula on the basis of an agreed case description of an infected animal.

Test system

One set of 25 x 4 week old commercial broiler chickens, males and females were used. Chickens were brought into the Biosafety Level 3 containment animal facility two days prior to challenge to allow acclimatization to the facility. Only healthy chickens were used in this study. Australian commercial poultry is considered to be free of avian influenza, but screening of pre-challenge sera for antibody to avian influenza will be carried out by cELISA with follow-up testing by HI where indicated.

Birds ate commercial compound grower feed *ad libitum*. Birds drank water from the domestic water supply *ad libitum*.

The chickens were divided into 5 groups each of 5 birds. For reasons of space, two BSL3 animal rooms were required. One animal room housed one group of chickens; the other room housed four groups of chickens in separate pens. Temperature in the pens was 20-24 ºC. The group of birds given virus diluted to 10^{-2} (highest dose of virus) were housed in the first animal room; remaining groups were housed in the second animal room.

Facilities

The "in vivo" phase of the study, as well as the serology, was performed in *CSIRO AAHL, 5 Portarlington Rd, Geelong, 3220, Australia*. Virus isolations will be performed in *CSIRO AAHL, 5 Portarlington Rd, Geelong, 3220, Australia*.

Experimental design

All birds were individually identified by means of a numbered leg band prior to challenge. Once identified, chickens were randomly distributed in five groups, each of five birds.

Birds were observed daily for clinical symptoms throughout the study and twice daily during periods of acute disease (days 2 to 11 inclusive). All signs and mortality were recorded in the study file.
Following 2 days of acclimatization, each chicken had a blood sample collected from the wing vein for serology studies, and was challenged with 0.5 ml of a viral suspension of H5N1. Each group received a different dilution of infected allantoic fluid. Groups of birds receiving lower doses of allantoic fluid were handled first on the day of challenge and at all times thereafter.

Blood samples for serology studies were obtained from each surviving chicken 10 days after challenge and again on day 14. In order to monitor virus shedding after challenge, tracheal and cloacal swabs from each challenged bird was obtained on days 2, 3, 4, and 7 post-challenge. These samples may be used for virus isolation in SPF chicken eggs.

Procedures and Results

Challenge

Every chicken received a challenge dose of 0.5 ml by intra nasal, intraoral and eyedrop routes containing various dilutions of infected allantoic fluid. Challenge was performed by means of a 1ml graduated syringe.

Clinical signs

Birds were observed daily for clinical symptoms throughout the study. After the challenge, clinical signs were evaluated twice a day during periods of acute disease. Signs and mortality were recorded and are presented in Appendix 1. A summary of observations for the various groups of birds is presented below.

10^{-2}
Three of five birds were clinically depressed 24 hrs (Day 1) post-challenge; all birds in this group had died 36hrs post-challenge (Day 2).
10^{-3}
Five out of five birds had died 36hrs (Day 2) post-challenge without being observed to exhibit clinical signs of disease.
10^{-4}
One bird was depressed and sitting with its eyes closed 36hrs (Day 2) post-challenge; this bird was euthanased by cervical dislocation. Two birds were found dead on Day 4, one on Day 6, and the fifth bird was noted to be ill on Day 7. This last bird initially appeared to be recovering from HPAI but was ultimately euthanased by cervical dislocation on day 11 post-challenge.
10^{-5}
All birds remained clinically well throughout the study and were euthanased by cervical dislocation on Day 14 post-challenge.

10^{-6}

All birds remained clinically well throughout the study and were euthanased by cervical dislocation on Day 14 post-challenge.

Serology

Blood samples were obtained from each bird involved in the study before the challenge and from surviving birds 10 and 14 days after the challenge. Sufficient blood to perform all serologic tests was collected from the wing vein by venepuncture.

Samples were identified individually with the date of collection, individual bird identification and treatment group. Sera were harvested and stored at minus 20°C or lower until they were tested. All sera were tested by HI test for antibodies to H5. Serum samples left after all serology tests are completed were stored at minus 20°C or lower temperature.

Serology results are presented in Appendix 2.

All birds were negative for antibodies to H5 prior to challenge. One bird in Group 10^{-4} that survived to day 10 had seroconverted to H5. Remaining surviving birds in Groups 10^{-5} and 10^{-6} were negative for antibodies to H5 on both Days 10 and 14 after challenge.

Virus shedding after challenge

After challenge, tracheal and cloacal swabs from each surviving challenged bird were obtained on days 2, 3, 4 and 7 post-challenge. These samples were identified with the type of specimen, the date, the bird identification number and the treatment group. These samples may be used for virus isolation in SPF eggs.

After collecting tracheal and cloacal samples, samples were placed in isotonic phosphate buffered saline (PBS), pH 7.0 - 7.4, containing antibiotics. Samples have been stored at -80°C pending analysis.

Evaluation of results to date:

10^{-2}

Death of birds in this group was attributed to peracute HPAI H5N1. No samples were available for assessment of viral shedding as all birds died prior to scheduled sample collection. Collection of samples at earlier time points is not advised due to possible sample contamination by the challenge inoculum.

10^{-3}

Death of birds in this group was attributed to peracute HPAI H5N1. No samples were available for assessment of viral shedding as all birds died prior to scheduled sample collection. Collection of samples at earlier time points is not advised due to possible sample contamination by the challenge inoculum.

10^{-4}

All birds developed clinical signs or mortality consistent with HPAI H5N1 between 2 and 7 days post-challenge. One bird surviving to day 10 had already seroconverted to H5 consistent with primary infection having occurred. **It is proposed that tracheal and cloacal swabs be processed from this trial group to provide data to contribute to the evaluation of infection status and time course of infection in these birds and to assist in the design of the Megabac efficacy study.**

10^{-5}

All birds remained clinically well throughout the study and were euthanased by cervical dislocation on Day 14 post-challenge. No birds had seroconverted to H5 by day 14 and it is concluded that infection was not established in this group of birds. Attempts at virus isolation from swabs collected from this group of birds are unlikely to provide additional useful information and is not recommended at this stage.

10^{-6}

All birds remained clinically well throughout the study and were euthanased by cervical dislocation on Day 14 post-challenge. No birds had seroconverted to H5 by day 14 and it is concluded that infection was not established in this group of birds. Attempts at virus isolation from swabs collected from this group of birds are unlikely to provide additional useful information and is not recommended at this stage

At present, a dilution of stock virus of 10^{-4} administered at a dose of 0.5ml appears to be a suitable challenge dose for the Megabac efficacy study. **The specific amount of virus present in this dose has yet to be determined by back titration of the stock virus. At this time a Minimum Chicken Infectious $Dose_{50}$ will also be calculated and, after considering all available data, a final decision made as to the challenge dose to be used in subsequent studies.**

Unforeseen occurrences:

Currently, no unforeseen circumstances have occurred that will impact on the outcome of the study.

End of the study:

After bleeding on the 14th day after challenge, all surviving chickens were euthanased by cervical dislocation. Their carcasses were incinerated.

Summary of events

For the set of 25 chickens:

Age:	Event:
Day -2	Housing the bird in suitable floor pens.
Day 0	Bleeding of all birds, challenge with AI virus.
Days 1 to 14	Record daily mortality and clinical signs post-challenge. Tracheal and cloacal swabs days 2, 3, 4 and 7. Bleed survivors on day 10 and day 14.

Final report

A final report including recommendations regarding a suitable challenge dose of virus and sampling interventions for subsequent studies will be written and it will be offered to Neumedix Biotechnology.

Appendix 1
Clinical observations and mortality record sheet

Chick No.	Day 1	2	3	4	5	6	7	8	9	10	11
10^{-2} 3	s	d	*	*	*	*	*	*	*	*	*
11	n	d	*	*	*	*	*	*	*	*	*
16	s	d	*	*	*	*	*	*	*	*	*
17	n	d	*	*	*	*	*	*	*	*	*
23	s	d	*	*	*	*	*	*	*	*	*
10^{-3} 4	n	d	*	*	*	*	*	*	*	*	*
18	n	d	*	*	*	*	*	*	*	*	*
20	n	d	*	*	*	*	*	*	*	*	*
21	n	d	*	*	*	*	*	*	*	*	*
22	n	d	*	*	*	*	*	*	*	*	*
10^{-4} 2	n	n	n	d	*	*	*	*	*	*	*
5	n	n	n	n	n	n	s(c)	s(c)	s(c)	s(c)	d(e)
7	n	n	n	d	*	*	*	*	*	*	*
14	n	d(e)	*	*	*	*	*	*	*	*	*
24	n	n	n	n	n	d	*	*	*	*	*
10^{-5} 6	n	n	n	n	n	n	n	n	n	n	n
10	n	n	n	n	n	n	n	n	n	n	n
12	n	n	n	n	n	n	n	n	n	n	n
13	n	n	n	n	n	n	n	n	n	n	n
15	n	n	n	n	n	n	n	n	n	n	n
10^{-6} 1	n	n	n	n	n	n	n	n	n	n	n
8	n	n	n	n	n	n	n	n	n	n	n
9	n	n	n	n	n	n	n	n	n	n	n
19	n	n	n	n	n	n	n	n	n	n	n
25	n	n	n	n	n	n	n	n	n	n	n

s=depression
n=normal
c=congested feet, comb and wattles
e=euthanased
d=found dead
*=no further record

After bleeding on the 14th day after challenge, all surviving chickens were euthanased by cervical dislocation. Their carcasses were incinerated.

Summary of events

For the set of 25 chickens:

Age:	Event:
Day -2	Housing the bird in suitable floor pens.
Day 0	Bleeding of all birds, challenge with AI virus.
Days 1 to 14	Record daily mortality and clinical signs post-challenge. Tracheal and cloacal swabs days 2, 3, 4 and 7. Bleed survivors on day 10 and day 14.

Final recommendations

The Chicken Infectious $Dose_{50}$ for A/ Ch/ Vietnam/ 8/ 04 H5N1 in 4 week old broiler birds was calculated to be $10^{3.9}$ EID_{50} / 0.5ml. The viral dose administered to the 10^{-4} group of birds in this study was $10^{4.4}$ EID_{50} /0.5ml, representing approximately 3 Infectious $Dose_{50}$.

As there will be some between-animal variation around this endpoint, it is recommended that a pilot study to form a preliminary assessment of the biological efficacy of Megabac against infection with H5N1 HPAI in broiler birds be carried out using a slightly higher challenge dose than that employed in the 10^{-4} group for the titration study described above. As the pilot study will be using a small number of birds, we need to reduce the chance that some control birds (not treated with Megabac) do not become infected with the challenge virus. Accordingly, a challenge dose of $10^{4.9}$ EID_{50} / 0.5ml (representing 10 Chicken Infectious $Dose_{50}$) is proposed for the pilot study.

Appendix 2: Record of serological results

Chicken		HI day 0	HI day 10	HI day 14
10^{-2}	3	<2	ND	ND
	11	<2	ND	ND
	16	<2	ND	ND
	17	<2	ND	ND
	23	<2	ND	ND
10^{-3}	4	<2	ND	ND
	18	<2	ND	ND
	20	<2	ND	ND
	21	<2	ND	ND
	22	<2	ND	ND
10^{-4}	2	<2	ND	ND
	5	<2	32	ND
	7	<2	ND	ND
	14	<2	ND	ND
	24	<2	ND	ND
10^{-5}	6	<2	<2	<2
	10	<2	<2	<2
	12	<2	<2	<2
	13	<2	<2	<2
	15	<2	<2	<2
10^{-6}	1	<2	<2	<2
	8	<2	<2	<2
	9	<2	<2	<2
	19	<2	<2	<2
	25	<2	<2	<2

ND = not done, no specimen available

Appendix 3: Record of virus isolation results- 10^{-4} bird group

Chicken N°	Trachea				Cloaca			
	Day 2	Day 3	Day 4	Day 7	Day 2	Day 3	Day 4	Day 7
2	neg	**pos**	ND	ND	neg	neg	ND	ND
5	neg	neg	neg	**pos**	neg	neg	neg	neg
7	neg	neg	ND	ND	neg	neg	ND	ND
14	**pos**	ND	ND	ND	**pos**	ND	ND	ND
24	neg	C	**pos**	ND	neg	neg	neg	ND

ND= not done, no specimen available
C= bacterial contamination; no result