

# The pathology of experimental duck plague in Muscovy ducks

## Nghiên cứu bệnh học dịch tả vịt thực nghiệm ở vịt xiêm

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### Abstract

Duck plague is recognised chiefly as a devastating disease of domestic ducks. Muscovy ducks are also susceptible, but fewer studies of the disease have been conducted in this host. Domestic Pekin ducks and Muscovy ducks were experimentally infected with a virulent strain of duck plague herpesvirus isolated in Vietnam. Clinical signs, and gross and microscopic lesions, were similar in both Pekin and Muscovy ducks. Diarrhoea and laboured breathing were common signs in Muscovy ducks, but paralysis and subcutaneous oedema of the head were rare. Changes in the oesophageal mucosa of Muscovy ducks were not as severe as in Pekin ducks. Cloacal haemorrhage was severe in Muscovy ducks.

### Tóm tắt

Dịch tả vịt được thừa nhận là một bệnh gây chết hàng loạt ở các loài vịt nuôi. Vịt xiêm cũng mắc cảm với bệnh, nhưng bệnh dịch tả vịt (DTV) ít được nghiên cứu ở loài vật chủ này. Vịt và vịt xiêm được gây nhiễm thực nghiệm với một chủng virút DTV phân lập ở tỉnh Cần Thơ. Các triệu chứng lâm sàng và bệnh tích đại thể cũng như vi thể giống nhau ở vịt và vịt xiêm. Tiêu chảy và khó thở là những triệu chứng thường thấy ở vịt xiêm, nhưng liệt và phù đầu ít khi xuất hiện. Biến đổi ở niêm mạc thực quản vịt xiêm không nghiêm trọng bằng ở vịt. Mức độ xuất huyết ở ổ nhớp vịt xiêm là nghiêm trọng.

### Introduction

DUCK plague or duck virus enteritis is an acute and highly contagious infection caused by duck plague herpesvirus or Anatid herpesvirus in a variety of domestic and wild aquatic birds of the family *Anatidae* of the order *Anseriformes*.

The disease has been recognised in many flocks of domestic ducks all over Vietnam. There have been several studies on the disease and its control measures (Vu Dinh Tieu and Mai Anh, 1969; Tran Minh Chau, 1980; Pham Thi Lan Thu and Than Thi Hanh, 1989; Tran Dinh Tu, 1995; Tran Dinh Tu and

Kim Van Phuc, 1998, 1999; Nguyen Duc Hien, 1997). Duck plague has been recorded in geese (Le Hong Phong et al, 1986). However, information on duck plague in Muscovy ducks (*Cairina moschata*) is not readily available. The results of outbreak investigation during 2002 conducted by the Department of Animal Health of Can Tho Province showed that up to 15% of Muscovy ducks of the province were suspected of being infected with duck plague. To improve the diagnosis, we have conducted experimental infection of Muscovy ducks to observe some pathological characteristics. This report provides results recorded from the above experiment.

## Materials and methods

### Virus strain for challenge

The virus strain was isolated from a duck plague outbreak occurring in field rice scavenging ducks in O Mon district, Can Tho province in May 2002. The identity of the isolate was confirmed by duck plague antigen ELISA (Ag-ELISA). The virus strain was maintained in susceptible 6-week-old ducks. Liver and spleen from ducks showing typical signs and lesions of duck plague were collected and stored at  $-20^{\circ}\text{C}$  until further use.

### Preparation of inoculum

Samples of liver and spleen were thoroughly washed with PBS containing antibiotics. The samples were then ground into a 20% suspension (weight/volume) in PBS solution containing Penicillin 200 IU/mL and Streptomycin 200  $\mu\text{g}/\text{mL}$ . This suspension was diluted at  $10^{-4}$  for use as an inoculum.

### Pekin ducks and Muscovy ducks

White (Pekin) ducks and Muscovy ducks of local breed with dark-green feathers were hatched at Soha-farm Incubator from eggs purchased from local farmers. One-day-old ducklings were incubated in cages for 2 weeks and then transferred to a husked-floor, net-partitioned enclosure with playing ground and pool.

Prior to challenge, ducks were examined and found to be negative for DP ELISA antibodies. The experimental challenge was conducted at the Experimental Farm of the Department of Animal Health of Can Tho Province in O Mon District.

Muscovy ducks were challenged at 6 weeks and 10 weeks old. Each group comprised 10 birds. Five birds in a negative control group were inoculated with PBS. To ensure the disease was caused by duck plague virus, 10 white (Pekin) ducklings for challenge were raised simultaneously with Muscovy ducks. Five of the Pekin ducklings were vaccinated with duck plague vaccine 2 weeks prior to the challenge trial. Each duckling was inoculated with 1 ml of liver-spleen suspension containing virus at the dilution rate of  $10^{-4}$ . Each ml of liver-spleen suspension contained about  $10^3$  DLD<sub>50</sub> (previously determined in a different experiment).

After challenge, ducklings were observed twice daily for 14 days (at 0700 hours and 1500 hours) to record any clinical signs. A necropsy was conducted on the dead birds. Typical signs and lesions were photographed with a digital camera.

### Light microscopy and electron microscopy techniques

After necropsy of the dead birds and observation of gross lesions, 5–10 g of oesophagus tissue, proventriculus, small intestine, rectum, anus, liver and spleen were taken, thoroughly washed with PBS and soaked in a fixing solution for the following purposes:

1. Microscopic preparations were stained at the Pathological Operations Department, Tu Du Hospital. After being taken out of the fixing solution, the sample was processed by routine paraffin-embedding techniques. The sample was cut by Microtome into slices of 4–5  $\mu\text{m}$  thickness, then stained with Haematoxylin and Eosine (H and E). The sections were read and photographed at the Pathology–Infection Section, Faculty of Animal Husbandry and Veterinary Medicine, The HCMC University of Agriculture and Forestry. Microscopic lesions were observed at magnifications of 100 $\times$ , 400 $\times$  and 1000 $\times$  to identify inclusion bodies in the cell nucleus. The severity of lesions was divided into 5 levels: 0 (normal), 1 (mild), 2 (average), 3 (severe) and 4 (extremely severe).
2. Electromicroscopic preparations were made, photographed and the results read in the electron microscope room of the Central Institute of Hygiene and Epidemiology, Ha Noi.

### ELISA techniques

The DP antibody ELISA (Ab-ELISA) and Ag-ELISA were applied to identify antibodies present in the serum before the experiment and DP antigen in the liver of ducks which died after challenge, respectively. These techniques were studied and developed by the Australian Animal Health Laboratory (AAHL), then transferred to NAVETCO. After standardisation of these techniques to suit the conditions in Vietnam, ELISA techniques were further transferred to Can Tho Animal Health Department through ACIAR/AusAID projects' training courses on advanced laboratory diagnostic methods.

## Results and discussion

### Clinical signs and DP lesions in Muscovy ducks

To study the pathological characteristics of duck plague in Muscovy ducks, we used a local virulent virus strain isolated from a duck plague outbreak in scavenging ducks in O Mon District, Can Tho Province.

The experimental challenge was conducted in 6-week-old and 10-week-old Muscovy ducks with a

**Table 1.** Results of experimental challenge with duck plague virus.

Duck breed	Age (weeks)	No. of ducks	Challenge inoculum	Mortality rate (%)	No. of days that birds died
Muscovy	6	10	Duck plague virus	100	4–5
	10	10	Duck plague virus	100	3–5
	6	5	PBS	0	0
White (Pekin) ducks	10	5	Duck plague virus	100	4–5
	10	5	Vaccine + duck plague challenge virus	0	0

1 mL dose of liver–spleen suspension diluted in PBS at the dilution rate  $10^{-4}$  (about  $10^3$  DLD<sub>50</sub>) together with 5 white ducks vaccinated with DP vaccine 2 weeks ago and 5 unvaccinated white ducks, which served as a positive control group. Five Muscovy ducks injected with PBS only served as a negative control group (placebo). The results of the experiment are shown in Table 1.

The data in Table 1 indicate that the virus strain isolated from the outbreak caused experimental duck plague in Muscovy ducks. The mortality rate in this experiment was 100%.

On the second day of challenge, ducks showed signs of tiredness, poor appetite, diarrhoea and failure to move. Other common typical signs of duck plague were at lower frequency in Muscovy ducks. Some of the clinical signs and their frequency of appearance in experimentally infected Muscovy ducks are shown in Table 2.

**Table 2.** Duck plague signs observed in Muscovy ducks.

Signs	Frequency (%)	
	6-week-old Muscovy ducks	10-week-old Muscovy ducks
Diarrhoea	100	100
Poor appetite	100	100
Extreme thirst	100	100
Exhaustion	80	100
Difficulty breathing	60	70
Wing paralysis	20	30
Leg paralysis	10	20
Swollen head	10	20
Minor head swelling	10	10
Lachrimation	20	20
Nasal mucus discharge	10	20

In addition to signs of the digestive and respiratory tracts, signs of oedema and nervous disorder were at a lower rate. This was probably due to the short period of disease development (acute form). The ducks that died were necropsied to determine the observed lesions (Table 3).

Generally speaking, duck plague lesions in Muscovy ducks are similar to those in Pekin ducks with major signs of haemorrhage and necrosis in the digestive tract. Most ducks that died after being challenged had haemorrhagic and necrotic lesions in the oesophagus (Figure 1), intestine, rectum and cloaca (Figure 2).

Some Muscovy ducks had inflammation and ulcers with pseudo-membrane on the longitudinal fold of the oesophagus or cloaca and a reddened ring on the intestinal mucosal surface. However, the apparent frequency of these duck plague lesions in Muscovy ducks is lower than in Pekin ducks.

#### Studies on microscopic and ultrastructural lesions caused by duck plague virus in Muscovy ducks

The examination of microscopic specimens made from 8 body parts of Muscovy ducks that died on day 5 after experimental challenge with duck plague virus showed that the common microscopic lesions observed were hyperaemia, haemorrhage, inflammation, necrosis and detachment of epithelial cells. The severity of pathological changes depended on the organ and location where specimens were taken, as shown in Table 4.

As expected from the gross lesions described above, lesions of haemorrhage and hyperaemia were present in almost all visceral organs. Inflammation and necrosis were seen in the trachea, oesophagus, intestine, cloaca and liver. Microscopic lesions in some organs were as follows:

*Oesophagus and trachea.* The blood vessels in the mucous membranes of the oesophagus showed hyperaemia and severe haemorrhage. There were many foci of red blood cells on the surface of the epithelium and in the mucous membranes. The trachea showed detachment of epithelium and haemorrhage, but these lesions were not as severe as in white ducks.

*Small and large intestines.* Hyperaemia was observed in all layers of both the large and small intestines from the mucous membrane to the intestinal muscle.



**Figure 1.** Caseous material and haemorrhage along the longitudinal folds of the oesophagus of a Muscovy duck with duck plague.

**Table 3.** Duck plague lesions observed in Muscovy ducks.

Lesion type	Frequency (%)	
	6-week-old Muscovy ducks	10-week-old Muscovy ducks
Haemorrhagic oesophagus, covered with pseudo-membrane	70	60
Haemorrhagic proventriculus	90	70
Haemorrhagic gizzard	70	100
Haemorrhagic intestine	100	100
Haemorrhagic, inflammatory rectum	70	100
Haemorrhagic cloaca, covered with pseudo-membrane	70	80
Haemorrhagic, necrotic liver	100	100
Spleen hyperaemia	100	90
Haemorrhagic trachea	40	30
Congested lung	60	60
Chest cavity with fluid	10	10
Haemorrhagic heart membrane, muscle	80	90
Haemorrhagic cardiac coronary fat	10	10
Hemorrhagic kidney	60	40
Haemorrhagic, congested testes or ovaries	60	60
Haemorrhagic pharynx	10	10
Haemorrhagic conjunctiva	10	30
Head subcutaneous mucus	20	30

Petechial haemorrhages were commonly seen in the mucous membrane.

*Cloaca.* The mucous membrane of the anus was haemorrhagic. The haemorrhage obscured histological structures of the cloaca. This indicates that the cloaca is an important organ in the diagnosis of duck plague in Muscovy ducks.

*Liver and spleen.* Hyperaemia and haemorrhage were prominent in all lobes of the liver. The hepatic parenchyma showed coagulative necrosis and fat was severely degenerated. Muscovy liver is a target organ of duck plague virus.

Splenic parenchyma was also badly damaged with hyperaemia and haemorrhage. The lymphoid follicles

were also degenerated. This indicates that the spleen is also a target organ of duck plague virus.

*Inclusion bodies.* Intra-nuclear inclusion bodies were commonly seen in the epithelium of anal and oesophageal mucosa.

*Electron microscopy.* Ultrastructural studies were undertaken on the oesophagus. Herpesvirus particles

were observed in the nucleus and in the cytoplasm, with enveloped particles measuring 180–200 nm.

### Conclusion

In summary, the gross and microscopic pathological signs in the visceral organs of Muscovy ducks affected by duck plague were similar to those in Pekin ducks infected with the virus. However, the severity

**Table 4.** Microscopic lesions in the visceral organs of Muscovy ducks that died of experimental duck plague on day 5.

Organs	Lesion type				
	Hyperaemia	Haemorrhage	Inflammation	Necrosis	Other
Trachea	1–3 <sup>a</sup>	1–3	1	0	2 (detachment of epithelium)
Oesophagus	2–3	2–3	0	2	0
Proventriculus	2–4	2–4	0	0	0
Gizzard	2–4	2–4	0	0	2 (epithelium desquamated)
Intestine	1–3	1–3	1–3	2–3	0
Cloaca	4	4	3	3	0
Liver	2–4	2–4	0	1–4	2–3 (fat degenerated)
Spleen	2–4	3–4	0	0	3 (damaged parenchyma) 3 (damaged lymphoid follicles)

<sup>a</sup>Severity of lesion: 0 = normal, 1 = mild, 2 = average, 3 = severe, 4 = extremely severe.



**Figure 2.** Haemorrhage of the cloaca in a Muscovy duck infected with duck plague virus.

of changes in the oesophageal mucosa of Muscovy ducks was not as severe as in Pekin ducks. Lesions such as hyperaemia, haemorrhage, inflammation and necrosis occurred mainly in the mucous membranes of the digestive tract, and the liver and spleen.

Clinical signs of diarrhoea and difficult breathing were common in Muscovy ducks, but paralysis and head subcutaneous oedema were rare.

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# Adaptation of duck plague virus to chicken embryo fibroblast cell culture for vaccine production

## Thích ứng chủng virút vắc xin dịch tả vịt vào môi trường tế bào xơ phôi gà để sản xuất vắc xin

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### Abstract

A Chinese strain of duck plague virus, adapted to growth in embryonated duck eggs, has been used for two decades to produce vaccine in Vietnam. A project funded by the Australian Centre for International Agricultural Research sought to produce an improved vaccine. The seed virus strain underwent 15 serial passages in embryonated chicken eggs, and 12 passages in chicken embryo fibroblast cell cultures. Adaptation to chicken embryos was indicated by decreased survival time of the embryos and increased titres of virus in allantoic fluid. Adaptation to chicken fibroblasts was indicated by more rapid production of cytopathic changes and increased titres of virus in culture supernatants. The subsequent (13<sup>th</sup>) passage was stored as a master seed and a pilot vaccine was produced. This vaccine was shown to be safe in ducklings and to protect against challenge with virulent duck plague virus. The new vaccine was more potent than the previous duck embryo vaccine and will be cheaper to produce.

### Tóm Tắt

Virút dịch tả vịt (DTV), chủng Trung quốc thích ứng phát triển trên phôi vịt, đã được sử dụng trong hai thập niên vừa qua để sản xuất vắc xin ở Việt nam. Chương trình hợp tác nghiên cứu DTV được tài trợ bởi Trung tâm Nghiên cứu Nông nghiệp Quốc tế Úc (ACIAR) đã cố gắng cải tiến vắc xin này. Chủng virút vắc xin đã được cấy truyền 15 đời trên phôi trứng gà và 12 đời tiếp theo trên môi trường tế bào xơ phôi gà. Sự thích ứng của virút vào phôi gà được chỉ rõ bởi rút ngắn thời gian sống của phôi và tăng hiệu giá virút trong nước trứng. Sự thích ứng trên tế bào xơ phôi gà thể hiện bằng rút ngắn thời gian tạo biến đổi bệnh lý tế bào (CPE) và tăng hiệu giá virút ở dịch nuôi cấy tế bào. Đời tiếp truyền thứ 13 được giữ lại làm giống gốc và một lô vắc xin thử nghiệm được thực hiện. Lô vắc xin này được chứng minh là an toàn và bảo vệ được vịt con khi công cường độc với virút độc lực cao. Vắc xin mới này có hiệu lực, an toàn và rẻ hơn so với vắc xin trước đây được sản xuất trên phôi trứng vịt.

### Introduction

THE National Veterinary Company (NAVETCO) of Vietnam has been producing a live duck plague vaccine in embryonating duck eggs for more than 20 years. The vaccine strain is an old isolate from China. The vaccine strain has not been well

characterised and vaccine production was not based on a seedlot system. Growth in embryonated duck eggs was recognised to pose some risk of transfer of agents pathogenic for ducks. As part of a project funded by the Australian Centre for International Agricultural Research (ACIAR) an improved vaccine was developed. The Chinese vaccine strain was

shown to protect against current Vietnamese isolates of duck plague virus, and it was adapted to growth in chick embryo cell cultures. The latter study is described below.

### Materials and methods

**Vaccine virus** The (unnamed) vaccine strain of duck plague virus was obtained from China in the 1970s and had been maintained by serial passage in embryonated duck eggs since then. Safety tests conducted on each batch of vaccine indicated that the virus was not pathogenic for ducklings.

**Virulent challenge virus** The challenge virus was isolated in North Vietnam in 1969. It had been maintained since then by serial passage in ducks. Efficacy tests on each batch of vaccine indicated that the Chinese vaccine strain was efficacious against this challenge strain.

**Chicken embryonated eggs** Embryonated eggs were obtained from a commercial chicken flock.

**Chicken embryo fibroblast monolayers** Chicken embryo fibroblasts were obtained by trypsinisation of 10-day-old embryos. Cultures were grown in plastic flasks in medium consisting of minimum essential medium (MEM) with 10% foetal calf serum (FCS) and antibiotics. Confluent monolayers were maintained in MEM with 2% FCS and antibiotics.

**Ducklings** One-day-old ducklings were obtained from a commercial hatchery and reared in isolation until 3 weeks of age.

**Adaptation** The methods for adaptation of the vaccine strain were based on those described by Bordolai et al. (1994), Dardiri (1969), Kalaimathi and Janakiram (1989) and Tantaswasdi (1987). The Chinese vaccine virus was adapted to growth in embryonated chicken eggs, and then to growth in cultured chicken embryo fibroblasts (CEF). Vaccine master seed was produced from CEF-adapted virus and tested for safety and efficacy.

**Adaptation to embryonated chicken eggs** Reconstituted vaccine was diluted with an equal volume of Hank's balanced salt solution (HBSS) and inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. Fifteen serial passages were performed. Allantoic fluid at each passage level was tested for the presence of DP virus by antigen capture ELISA (Dang Hung et al., 2004) and PCR (Kim Van Phuc et al., 2004). At passage levels 5, 7, 11, 13 and 15 the viral content was titrated in embryonated chicken eggs and expressed as 50% egg infectious doses (EID<sub>50</sub>).

Allantoic fluid from the 15th passage was tested for safety and efficacy. Five 2-month-old ducklings

were inoculated subcutaneously with undiluted allantoic fluid. Two weeks later these ducklings, and 5 uninoculated control ducklings, were challenged by intramuscular injection of 10<sup>5.5</sup> 50% lethal doses (LD<sub>50</sub>) of challenge virus.

**Adaptation to CEF** Allantoic fluid from the 15th passage in embryonated chicken eggs was diluted 1 in 10 in HBSS and used to inoculate confluent cultures of CEF. Twelve serial passages were made. The presence of duck plague virus was confirmed at each passage by antigen capture ELISA. Viral titres were determined at passage levels 5, 7, 9, 11 and 12 in CEF cultures and expressed as 50% cell culture infectious doses (CCID<sub>50</sub>).

**Preparation of a master seed and experimental vaccine** Master seed was prepared from the subsequent (13th) passage in CEF cultures. The infected cell culture fluid was stored at -80°C. Portions were thawed and one was titrated as 10<sup>7.2</sup> CCID<sub>50</sub>/ml in CEFs. Another was diluted from 10<sup>-1</sup> to 10<sup>-3</sup> in HBSS (equivalent to 100, 10 and one doses) and each dilution was injected subcutaneously into 5 ducklings, which were 3 weeks old. Another 5 ducklings were maintained as controls. Two weeks later, all ducklings were challenged by intramuscular injection of 10<sup>5.5</sup> LD<sub>50</sub> of virulent challenge virus.

Master seed was diluted 10<sup>-2</sup> in HBSS and used to infect CEF monolayers to produce experimental vaccine (passage 14). Cell culture fluid was harvested after freezing and thawing, mixed with an equal volume of 10% skim milk and freeze dried. Vaccine was reconstituted and titrated in CEF cultures. A potency test was conducted. Three-week-old ducklings, 10 per group, were vaccinated by subcutaneous injection of vaccine dilutions ranging from 10<sup>-2</sup> to 10<sup>-9</sup>. Ten ducklings in a ninth group were not vaccinated. Two weeks later all ducklings were challenged by intramuscular injection with 10<sup>5.5</sup> LD<sub>50</sub> of virulent virus.

## Results

### Adaptation to embryonated chicken eggs

The Chinese vaccine strain was passed 15 times in chicken embryos. During the first 4 passages (CE<sub>1</sub>-CE<sub>4</sub>), the virus killed chicken embryos at 5-8 days post inoculation and the lesions were slight haemorrhages on the head, wings and legs. From the 11th passage (CE<sub>11</sub>) onwards, the time to kill the embryos shortened to 4-5 days after inoculation, with specific duck plague lesions presenting as petechial haemorrhages on the whole body, and necrosis on the liver. The virus titres increased gradually. Duck plague virus was detected by antigen ELISA and PCR at each passage. The results are shown in Table 1.

**Table 1.** Effect of serial passage of duck plague vaccine strain in chicken embryos on the death time of embryos and virus titre.

Passage level	Time to death of embryos (days)	Antigen ELISA	PCR	Titre (EID <sub>50</sub> /ml)
CE <sub>5</sub>	5-7	+	+	10 <sup>5.2</sup>
CE <sub>7</sub>	4-7	+	+	10 <sup>5.5</sup>
CE <sub>11</sub>	4-5	+	+	10 <sup>5.8</sup>
CE <sub>13</sub>	4-5	+	+	10 <sup>6.2</sup>
CE <sub>15</sub>	4-5	+	+	10 <sup>6.5</sup>

#### Immune response to chick embryo adapted virus

The duck plague vaccine at the 15th passage in chick embryos was tested for immunogenicity in ducks. Five control ducks died from the 3rd to 5th day with typical duck plague lesions after challenge. The five vaccinated ducks remained healthy and showed no signs of duck plague. The survivors developed duck plague antibody which was detected by duck plague indirect antibody ELISA at titres of 3200 to 6400.

#### Adaptation in chicken embryo fibroblast (CEF)

There was no clear cytopathic effect (CPE) in CEF cell culture up to the 3rd passage (CEF<sub>3</sub>). From the 4th to 7th passage (CEF<sub>4</sub>-CEF<sub>7</sub>) CPE was observed after 72 hours and from CEF<sub>8</sub> to CEF<sub>12</sub>, CPE was observed after 48 hours with typical duck plague virus CPE. The changes observed were the formation of syncytia, enlarged cells and cell death. The virus titres increased gradually from CEF<sub>5</sub> to CEF<sub>12</sub>. Results are shown in Table 2.

**Table 2.** Serial passage of chicken embryo adapted duck plague vaccine in chick embryo fibroblasts (CEF).

Passage in CEF	CPE	Time CPE observed (hours)	Antigen ELISA (O.D. <sub>450</sub> )	PCR	Titre (CCID <sub>50</sub> /0.1 ml)
CEF <sub>1</sub>	-	120	0.967-1.047	+	nd
CEF <sub>2</sub>	±	96	1.074-1.075	+	nd
CEF <sub>3</sub> -CEF <sub>4</sub>	+	72	1.134-1.139	+	nd
CEF <sub>5</sub>	+	72	1.140-1.145	+	10 <sup>5.2</sup>
CEF <sub>7</sub>	+	72	1.186-1.158	+	10 <sup>5.7</sup>
CEF <sub>9</sub>	+	48	1.194-1.198	+	10 <sup>6.2</sup>
CEF <sub>11</sub>	+	48	1.195-1.196	+	10 <sup>6.7</sup>
CEF <sub>12</sub>	+	48	1.163-1.277	+	10 <sup>6.8</sup>

#### Results of safety and potency testing of master seed virus

The titre of the master seed was 10<sup>7.2</sup> CCID<sub>50</sub>/ml. During 2 weeks of observation, the duck groups

vaccinated with 100 doses, 10 doses and 1 dose of master seed, all ducks looked active and were eating and drinking well, with smooth feathers and no diarrhoea. After challenge with 10<sup>5.5</sup> LD<sub>50</sub> of virulent duck plague virus, the vaccinated duck groups remained healthy and showed no clinical signs of duck plague. Results are shown in Table 3.

**Table 3.** Results of safety and potency testing of master seed virus.

Dose of master seed (virus dilution)	No. of survivors/No. of challenged ducks
100 doses (10 <sup>-1</sup> )	4/5*
10 doses (10 <sup>-2</sup> )	5/5
1 dose (10 <sup>-3</sup> )	5/5
Control (0)	0/5

\*One duck in this group died during transportation. At necropsy, there were no lesions of duck plague.

#### Results of quality testing of the new duck plague freeze-dried vaccine

The vaccine before freeze-drying contained 10<sup>7.2</sup> CCID<sub>50</sub>/ml and reconstituted vaccine after freeze-drying contained 10<sup>6.7</sup> CCID<sub>50</sub>/ml. Two weeks after challenge, the control group and vaccinated group at 10<sup>-9</sup> dilution all died with typical duck plague lesions. The vaccinated groups from 10<sup>-2</sup> to 10<sup>-6</sup> dilutions remained healthy and were 100% ELISA antibody positive. At the dilution 10<sup>-7</sup>, only 1 duck was ELISA positive and 5 ducks were protected against challenge even though 4 of these were completely ELISA negative. At the 10<sup>-8</sup> dilution, 1 duck was ELISA positive and this duck was protected. The results are shown in Table 4. The vaccine was calculated to contain 10<sup>7.1</sup> 50% protective doses (PD<sub>50</sub>) per ml.

**Table 4.** Response of ducklings to various doses of cell culture adapted duck plague vaccine.

Vaccine dilution	No. of ELISA antibody positive ducks/No. ducks in group	No. of survivors/No. of challenged ducks <sup>a</sup>
10 <sup>-2</sup>	10/10	10/10
10 <sup>-3</sup>	9/9	9/9
10 <sup>-4</sup>	10/10	10/10
10 <sup>-5</sup>	10/10	10/10
10 <sup>-6</sup>	10/10	10/10
10 <sup>-7</sup>	1/10	5/10
10 <sup>-8</sup>	1/10	1/10
10 <sup>-9</sup>	0/10	0/10
Control	0/10	0/10

<sup>a</sup>Ducks were challenged with virulent duck plague virus 2 weeks after vaccination.

## Discussion

The original Chinese strain of duck plague herpesvirus underwent biological changes as it adapted to replication in embryonated chicken eggs and in the CEF cultures. With serial passage the survival time of embryos and the time taken to produce visible CPE was reduced. In both systems the yield of virus increased with increasing levels of passage.

The vaccine remained safe and efficacious. Ducklings receiving even 100 times the normal vaccine dose remained clinically normal, indicating a large margin of safety with this vaccine. Vaccinated ducklings were protected against the standard, virulent challenge virus and this encouraged further trials of the vaccine in the field.

With lower doses of the vaccine, some ducklings that had produced no antibodies detectable by ELISA were resistant to challenge with virulent duck plague herpesvirus. Similar observations have been made by Tantaswasdi (1987), who suggested that some other immune mechanisms might be involved. Cell-mediated immunity can be suggested. This is a characteristic of resistance to herpesvirus infections suggested by Toth (1970) and Dardiri (1975).

The new cell culture vaccine contained  $10^{7.1}$  PD<sub>50</sub>/ml, 10 times greater than the value for the original duck egg vaccine ( $10^{6.1}$  PD<sub>50</sub>/ml). This increased potency is an added advantage of the new vaccine. Potentially the new vaccine will also be safer, as the possibility of transmitting pathogens that may be present in duck eggs is eliminated.

## Conclusion

Through 15 serial passages in chicken embryo (CE) and 12 passages in chicken embryo fibroblasts (CEF), the vaccine strain has been completely adapted to chicken cell cultures. The pilot batch of vaccine has been produced and tested for sterility, safety and potency. The results showed that the new vaccine is safe and potent.

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# Laboratory trials of a new duck plague vaccine produced in chicken embryo fibroblast cell cultures

## Các thử nghiệm vắc xin phòng bệnh dịch tả vịt sản xuất trên môi trường tế bào xơ phôi gà ở điều kiện phòng thí nghiệm

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### Abstract

A series of laboratory trials was conducted to determine the efficacy, safety and stability of a new cell culture adapted vaccine for duck plague. The vaccine induced full protection against challenge with virulent duck plague virus in 3- to 4-week-old ducks. The mean protective titre of the vaccine was approximately  $10^7$  DPD<sub>50</sub>/ml and it was decided that a recommended dose should contain  $10^3$  DPD<sub>50</sub>. The protection against virulent challenge induced by a single recommended dose of vaccine lasted at least 6 months after vaccination. The vaccine was completely safe in experimental ducks. Ducks that received 10 or 100 times the recommended dose showed no clinical signs. Freeze-dried vaccine retained its efficacy for at least 9 months when stored at 2–8°C and for 120 hours when stored at room temperature (25–35°C), despite some loss of infectivity titre at both temperatures. Reconstituted vaccine retained its efficacy for up to 10 hours when stored in an icebox (0–4°C) or at room temperature.

### Tóm tắt

Một loạt thử nghiệm trong điều kiện phòng thí nghiệm được thực hiện để xác định độ an toàn, hiệu lực và tính bền vững của vắc xin dịch tả vịt (DTV) thích ứng trên môi trường tế bào. Vắc xin đã tạo được sự bảo hộ hoàn toàn chống lại virút cường độc khi tiêm chủng cho vịt 3-4 tuần tuổi. Liều bảo hộ trung bình của vắc xin tương đương  $10^7$  DPD<sub>50</sub>/ml và một liều vắc xin được đề nghị chứa ít nhất  $10^3$  DPD<sub>50</sub>. Sự bảo hộ tạo ra bởi một liều vắc xin kéo dài ít nhất 6 tháng sau tiêm chủng. Vắc xin rất an toàn ở vịt thí nghiệm. Vịt sau khi được tiêm chủng 10 hoặc 100 liều vắc xin đều không có bất kỳ dấu hiệu lâm sàng nào. Vắc xin đông khô giữ nguyên hiệu lực ít nhất 9 tháng khi bảo quản ở 2-8°C và trong 120 giờ khi bảo quản ở nhiệt độ phòng (25-35°C), dù rằng hiệu giá virút có giảm chút ít ở cả hai điều kiện bảo quản. Vắc xin sau khi pha lỏng vẫn duy trì hiệu lực khi được bảo quản ở trong thùng lạnh hoặc nhiệt độ phòng tới 10 giờ.

### Introduction

DUCK plague is a highly contagious disease of anseriform birds which causes significant economic loss to producers in Vietnam. It can be controlled by vaccination (Tran Minh Chau, 1980). The vaccine currently produced by the National Veterinary Company (NAVETCO) is cultured in embryonated duck eggs and has been shown to be effective in pro-

tecting ducks throughout Vietnam from duck plague. However, obtaining duck eggs of a quality sufficient for vaccine production is difficult. Furthermore, the use of embryonated duck eggs may pose a risk of transmission of pathogenic agents. To overcome these problems, the Australian Centre for International Agricultural Research funded a collaborative project involving NAVETCO researchers and Australian scientists to develop a new vaccine

adapted to growth in chicken embryo cell cultures (Nguyen Thi Kim Dinh et al., 2004).

The objectives of the work reported here were to test the safety and efficacy of the vaccine, to determine the duration of immunity and to test the stability of the vaccine under various storage conditions.

## Materials and methods

### Vaccine trials

Two batches of cell-culture adapted duck plague vaccine (lots 110899 and 230500) were used in the trials. Ducks were obtained from a commercial hatchery at 1-day of age and raised in isolation until 3–4 weeks of age. The duck plague antigen-capture ELISA and the duck plague antibody ELISA were performed as described by Morrissy et al. (2004), with reagents supplied by the Australian Animal Health Laboratory, Geelong, Australia.

*Trial 1: Protective dose.* To determine the 50% duck protective dose (DPD<sub>50</sub>), vials from 2 vaccine batches were reconstituted and diluted from 10<sup>-1</sup> to 10<sup>-9</sup> in sterile phosphate buffered saline (PBS). One ml of each dilution was inoculated intramuscularly into groups of 10 ducks. Two weeks post inoculation, all inoculated ducks along with 10 control ducks were challenged by intramuscular inoculation of 1 ml of liver suspension containing at least 10<sup>5.5</sup> 50% duck lethal doses (DLD<sub>50</sub>) of virulent duck plague virus. Ducks were observed twice daily for 14 days. The experiments were repeated 3 times for each batch of vaccine. The protective titre was calculated by the method of Reed and Muench (1938).

*Trial 2: Safety test.* Groups of 5 ducks (3- to 4-week-old) were inoculated intramuscularly with 1x, 10x or 100x the recommended dose of vaccine (10<sup>3</sup> DPD<sub>50</sub>) and observed for 14 days for the presence of clinical signs. Two batches of vaccine were tested, with a total of 30 ducks used in the trial.

*Trial 3: Duration of immunity.* To determine the duration of immunity, 60 ducks were inoculated intramuscularly with the recommended vaccine dose and 30 ducks remained as unvaccinated controls. At 1, 2, 3, 4, 5 and 6 months after vaccination, 10 vaccinated ducks and 5 control ducks were selected randomly and blood samples collected to determine duck plague ELISA antibody titres (Dang Hung et al., 2004). At each time point, the 15 ducks were challenged intramuscularly with 10<sup>5.5</sup> DLD<sub>50</sub> of virulent duck plague virus.

*Trial 4: Thermostability of freeze-dried vaccine.* To determine the thermostability of the vaccine, two groups of 100 vials from the same batch of vaccine

(lot 110899) were stored at 2–8°C or at at room temperature (25–35°C). The vaccine stored at 2–8°C was tested at monthly intervals from 0 to 9 months and the vaccine stored at room temperature was tested at 6-hourly intervals until 24 hours and then at 24-hour intervals from 24 to 144 hours. On each testing occasion, 5 vials of vaccine were reconstituted and pooled, and the virus was titrated in chicken embryo fibroblast cells. An *in vivo* protection test was performed on each occasion using a volume of vaccine equivalent to 1 recommended dose and using 10<sup>5.5</sup> DLD<sub>50</sub> of virulent duck plague virus for the challenge at 2 weeks post vaccination.

*Trial 5: Thermostability of reconstituted vaccine.* Ten vials of freeze-dried vaccine were reconstituted and pooled, then divided into 12 aliquots. Six aliquots were stored in an ice box (at 0–4°C) and 6 aliquots were stored at room temperature (at 25–35°C). At 2-hourly intervals from 0 to 10 hours, 2 aliquots from each of the 2 storage conditions were pooled and inoculated intramuscularly into 5 ducks, with the volume used equivalent to 1 vaccine dose of freshly reconstituted vaccine. Five ducks remained unvaccinated controls. Two weeks after vaccination, the vaccinated and control ducks were bled for determination of ELISA antibody titre, and then challenged with virulent duck plague virus.

## Results and Discussion

### Trial 1: Determination of 50% duck protective dose

The mean protective titre of 2 batches of duck plague cell culture vaccine, each tested 3 times, was approximately 10<sup>7</sup> DPD<sub>50</sub>/ml (Table 1). It was decided that a recommended dose of vaccine should contain at least 10<sup>3</sup> DPD<sub>50</sub>.

**Table 1.** Determination of the protective titre of 2 batches of vaccine.

Batch of vaccine	Log DPD <sub>50</sub> /ml			
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Mean value
Lot 110899	7.09	7.23	7.20	7.17
Lot 230500	6.62	7.00	6.49	6.70

This trial showed that the new cell culture adapted vaccine has a higher protective titre than the older duck egg-based vaccine, which reportedly had values of 10<sup>5.63</sup> DPD<sub>50</sub>/ml (Do Van Dung, 2000) or 10<sup>5.27</sup> DPD<sub>50</sub>/ml (Tran Minh Chau, 1980). This means that the new vaccine can be diluted further than the old

vaccine, to produce more vaccine doses from any given volume of vaccine concentrate.

### Trial 2: Safety test

All ducks that were vaccinated with 1, 10 or 100 doses of the new vaccine remained healthy for the entire 2-week observation period (Table 2). The ducks drank and fed normally and showed no clinical signs. No deaths were recorded.

**Table 2.** Results of the safety test of the new cell culture adapted duck plague vaccine.

No. of doses of vaccine	Batch of vaccine	No. healthy ducks/no. tested
1	Lot 110899	5/5
	Lot 230500	5/5
10	Lot 110899	5/5
	Lot 230500	5/5
100	Lot 110899	5/5
	Lot 230500	5/5

### Trial 3: Duration of immunity

The protective immunity induced in 3- to 4-week-old ducks by a single vaccination of 1 recommended dose ( $10^3$  DPD<sub>50</sub>) lasted for at least 6 months (Table 3). On most occasions, all vaccinated ducks survived challenge with virulent duck plague virus, whereas all unvaccinated control ducks died. At months 4 and 5 post vaccination, 2 ducks and 1 duck, respectively died following challenge. However, duck plague did not appear to be the cause of these deaths and no duck plague virus antigen was found at post mortem.

**Table 3.** Duration of protective immunity and ELISA antibody titres induced by the cell culture adapted duck plague vaccine.

Time post vaccination (months)	No. survived/No. challenged		Mean antibody titre $\pm$ standard deviation ( $\log_2$ )	
	Vaccinated ducks	Controls	Vaccinated ducks (n = 10)	Controls (n = 5)
1	10/10	0/5	9.87 $\pm$ 0.84	0.04 $\pm$ 0.02
2	10/10	0/5	8.07 $\pm$ 0.54	0.04 $\pm$ 0.02
3	10/10	0/5	7.66 $\pm$ 0.71	0.03 $\pm$ 0.01
4	8/10*	0/5	6.86 $\pm$ 0.45	0.03 $\pm$ 0.02
5	9/10**	0/5	5.91 $\pm$ 0.50	0.03 $\pm$ 0.01
6	10/10	0/5	4.86 $\pm$ 0.44	0.04 $\pm$ 0.05

\* Two ducks died three days after challenge, but duck plague virus antigen was not detected.

\*\* One duck died three day after challenge, but duck plague virus antigen was not detected.

The mean ELISA antibody titres of vaccinated ducks declined gradually over a 6-month period from 9.8  $\log_2$  at 1 month post vaccination to 4.8  $\log_2$  at 6 months post vaccination (Table 3).

### Trial 4: Stability of freeze-dried vaccine

The infectivity titre (determined at various intervals) of the freeze-dried vaccine stored at 2–8°C and at room temperature (25–35°C) is shown in Tables 4 and 5, respectively. The level of protection afforded by the vaccine at each time interval is also shown. When stored at 2–8°C, the vaccine lost almost 2 logs of titre over a 9-month period and when stored at 25–35°C a loss of 1.5 logs occurred over 72 hours. However, vaccine stored at 2–8°C for 9 months or at 25–35°C for 5 days (120 hours) still afforded 100% protection.

**Table 4.** Virus titres and protection level of freeze-dried duck plague vaccine stored at 2–8°C.

Storage time (months)	Mean titre* ( $\log_{10}$ TCID <sub>50</sub> /ml)	No. survived/no. challenged	
		Vaccinated	Controls
0	7.02	10/10	0/5
1	6.89 $\pm$ 0.14	10/10	0/5
3	6.81 $\pm$ 0.05	10/10	0/5
4	6.62 $\pm$ 0.12	10/10	0/5
5	6.43 $\pm$ 0.09	10/10	0/5
6	6.20 $\pm$ 0.23	10/10	0/5
8	5.64 $\pm$ 0.05	10/10	0/5
9	5.52 $\pm$ 0.06	10/10	0/5

\* Values are the mean titre  $\pm$  standard deviation of 5 vials of vaccine.

**Table 5.** Virus titres and protection level of freeze-dried duck plague vaccine stored at 25–35°C.

Storage time (hours)	Infectivity titre ( $\log_{10}$ TCID <sub>50</sub> /ml)	No. survived/no. challenged	
		Vaccinated	Controls
0	6.87	5/5	0/5
6	6.36	5/5	0/5
12	6.36	5/5	0/5
24	5.64	5/5	0/5
48	5.63	5/5	0/5
72	5.34	5/5	0/5
96	ND	5/5	0/5
120	ND	4/4	0/5
144	ND	3/4	0/5

\* values are the mean titre  $\pm$  standard deviation of 5 vials of vaccine.

ND: Not done (No titration).

### Trial 5: Stability of reconstituted duck plague vaccine

Pooled sera from groups of 5 ducks inoculated with reconstituted vaccine that had been stored in an icebox (0–4°C) or at room temperature (25–35°C) for various time intervals were tested in the duck plague antibody ELISA. The results of the ELISA testing and virulent virus challenge tests are shown in Table 6. The data show that the reconstituted cell culture vaccine induced 100% protection against challenge following storage for up to 10 hours in either an icebox or at room temperature. The ELISA antibody responses in ducks vaccinated with the reconstituted vaccine were similar at each sampling occasion from 0 to 10 hours of vaccine storage. These results indicate that the reconstituted vaccine may be kept for up to 10 hours without losing efficacy.

**Table 6.** ELISA antibody titres and protective immunity induced by reconstituted duck plague vaccine that was stored in an icebox or at room temperature.

Storage time (hours)	Ice box (0–4°C)		Room temperature (25–35°C)	
	No. survived/ no. challenged	OD*	No. survived/ no. challenged	OD*
0	5/5	0.61	5/5	0.45
2	5/5	0.42	5/5	0.41
4	5/5	0.37	5/5	0.27
6	5/5	0.57	5/5	0.47
8	5/5	0.46	5/5	0.59
10	5/5	0.57	5/5	0.37
Control	0/5	0.03	0/5	0.03

\* OD: the OD value in the duck plague antibody ELISA of pooled sera from 5 ducks diluted 1/50.

### Conclusions

These laboratory trials demonstrated that the new cell culture adapted duck plague vaccine is effective and safe. The duration of protective immunity induced by a single vaccination is at least 6 months. Freeze-dried vaccine can be stored for up to 9 months at 2–8°C and up to 5 days at room temperature with no loss of efficacy. Reconstituted vaccine can be stored for up to 10 hours in an icebox or at room temperature with no loss of efficacy.

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# Serological and immunological responses of ducklings vaccinated at 1 and 21 days of age with lyophilised live duck plague vaccine

## Đáp ứng huyết thanh và miễn dịch của vịt con được tiêm chủng vắc xin dịch tả vịt đông khô vào lúc 1 và 21 ngày tuổi

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### Abstract

The indirect antibody ELISA developed at AAHL was used to study the decline in maternal antibodies in ducklings, and the effect of these antibodies on responses to duck plague vaccination and challenge with virulent virus. Levels of maternal antibodies were uniform in ducklings from a commercial hatchery with vaccinated breeding stock, and diverse in ducklings from market hatcheries. By 3 weeks of age, no ducklings had detectable levels of maternal antibody. Maternal antibodies did not protect against intramuscular challenge with duck plague virus, but high levels did interfere with the protective response to vaccination. Ducklings vaccinated at 1 day of age were resistant to challenge before ELISA antibodies became detectable. In ducklings 4 weeks of age and older, levels of vaccine-induced antibody did correlate with protection.

### Tóm tắt

Ứng dụng phương pháp ELISA gián tiếp phát hiện kháng thể kháng virút dịch tả vịt (DTV) của Phòng Thí nghiệm Thú y Úc (AALH) để nghiên cứu sự biến động kháng thể mẹ truyền (KTM) ở vịt con và tác động của kháng thể này đối với đáp ứng sau tiêm chủng vắc xin và thử thách cường độc ở vịt con. Mức kháng thể mẹ truyền ở vịt con mua từ lò ấp của các trại vịt giống có tiêm chủng vắc xin tương đối đồng nhất hơn vịt con của các lò ấp khác. Vào khoảng 3 tuần tuổi không có vịt con nào còn kháng thể mẹ truyền có thể phát hiện được. KTM tuy không bảo vệ được vịt con khi công cường độc nhưng có ảnh hưởng đến hiệu quả bảo hộ sau tiêm chủng. Vịt con được tiêm chủng vắc xin vào lúc một ngày tuổi đã có thể đề kháng lại công cường độc trước khi kháng thể ELISA đạt tới mức phát hiện được. Ở vịt con 4 tuần tuổi hoặc lớn hơn, mức kháng thể tạo ra bởi vắc xin tương quan với mức bảo hộ.

### Introduction

DUCK plague or duck virus enteritis was first detected in North Vietnam in 1962, and in some southern provinces in the 1980s. A live, lyophilised duck plague vaccine, based on an old Chinese strain of duck plague virus, has been produced by the National Veterinary Company (NAVETCO) for many years. There have been few scientific studies

with the vaccine, in part because the usual assay for neutralising antibodies utilises embryonated duck eggs and is time consuming and complicated. The recent development at AAHL, Geelong, Australia of an Enzyme-Linked Immunosorbent Assay (ELISA) (Dang Hung et al., 2004) for the detection of antibodies against duck plague virus offers a rapid and convenient tool. The indirect antibody ELISA was used to measure the antibody response to vaccination

and the maternal transfer of antibodies. Correlations were sought with resistance to artificial challenge with virulent duck plague virus, and with ability to respond to vaccination

### Materials and methods

**Ducks.** Ducks of either the commercial super-meat breed or of local breeds were obtained at 1 day of age from commercial or market hatcheries.

**Duck plague live virus vaccine.** The current duck plague vaccine produced in duck embryonated eggs inoculated via the chorio-allantoic membrane by NAVETCO was used in this experiment and contains  $10^{3.1}$  50% duck protective doses (DPD<sub>50</sub>) per vaccine dose.

**Virulent duck plague challenge virus.** This virus has been maintained by passage in ducks for more than 30 years. The challenge dose, delivered by intramuscular injection, was  $10^{3.1}$  50% duck lethal doses (DLD<sub>50</sub>) for ducklings to 3 weeks of age, and  $10^{5.5}$  DLD<sub>50</sub> for older ducks.

**Indirect Antibody ELISA.** The test kit was kindly supplied by AAHL. All sera were initially diluted 1 in 50, followed by serial 2-fold dilutions. The diluent was phosphate buffered saline (PBS). The titres of ELISA antibodies were expressed as  $\log_{10}$  of the reciprocals of the highest dilution with an optical density (OD)  $\geq 0.2$ .

#### Maternal antibody in ducklings from a market hatchery

Markets are the main source of ducklings for private smallholders in Vietnam. Hatcheries obtain embryonated duck eggs from various sources. A total of 100 one-day-old ducklings were purchased from a market hatchery. Ducklings were identified by wing tags and bled at 1, 7 and 14 days of age. ELISA antibody titres were determined.

#### Maternal antibody in ducklings from a commercial hatchery

Three batches of one-day-old ducklings were purchased from a commercial hatchery with vaccinated breeding stock. Blood samples were collected at 1, 7, 14 and 21 days of age. From each batch, 20 blood samples were collected and then pooled in groups of 5 at each time point. These pooled sera were titrated for indirect ELISA antibodies.

#### Effect of maternal antibody on challenge with virulent duck plague virus

Groups of 5 or 10 ducklings from the commercial hatchery were challenged at 1, 2, 7, 12, 17 or 21 days of age with virulent duck plague virus. Indirect ELISA antibody titres were determined on the day of

challenge. A further 10 ducklings served as uninoculated controls. Dead ducklings were autopsied, and the antigen capture ELISA (Dang, 2004) was performed on spleen homogenate to detect the presence of duck plague virus.

#### Effect of maternal antibody on response to vaccination

Ducklings from the market hatchery were used. Ducklings in 1 group were vaccinated once at 1 day of age; ducklings in the second group were vaccinated twice, at 1 and 21 days of age. Control, unvaccinated ducklings comprised a third group. Groups of ducklings were challenged with virulent duck plague virus at 1, 2, 3, 4, 6 or 8 weeks of age after determination of the mean indirect ELISA antibody titres.

### Results

#### Maternal antibody in ducklings from a market hatchery

The results are shown in Table 1. Ducklings from this source showed wide fluctuations in titres of maternally derived antibody, with highest values of 3.2. One third of the one-day-old ducklings lacked detectable antibodies. By 14 days of age there were no detectable antibodies in any of the birds that were tested.

**Table 1.** Maternal antibody titre in ducklings from a market hatchery.

Days of age	Number of samples	ELISA titre (X $\pm$ SD)	Range
1	100	1.78 $\pm$ 1.25	0–3.2
7	23	0.78 $\pm$ 1.02	0–2.6
14	18	0	

#### Maternal antibody in ducklings from a commercial hatchery

Table 2 shows the results. Antibody levels were relatively uniform and were not detectable by 3 weeks of age.

**Table 2.** Maternal antibodies in ducklings from a commercial hatchery.

Batch	Age of ducklings (days)			
	1	7	14	21
1	3.31 <sup>a</sup>	2.60	1.70	0
2	3.20	2.30	1.70	0
3	3.20	2.15	0	0

<sup>a</sup> Mean ELISA antibody titre. Sera from 20 birds were

pooled in groups of 5 at each time point.

### Effect of maternal antibody on challenge with duck plague virus

Despite the presence of maternal antibodies at 2, 7 and 12 days of age, all the challenged ducklings died 3–7 days after challenge (Table 3). All birds contained duck plague virus in spleen samples tested by antigen capture ELISA. Control ducklings remained normal.

**Table 3.** Effect of maternal antibody on challenge with virulent duck plague virus.

Age of duckling (days)	ELISA antibody titre	Number died/ Number challenged
1	0	10/10
2	3.0	10/10
7	2.6	10/10
12	1.7	10/10
17	0	10/10
21	0	5/5
Control	0	0/10

### Effect of maternal antibody on response to vaccination

The results are shown in Table 4. A single vaccination produced a moderate antibody response, and moderate levels of protection which fell with age. Eighteen of 24 birds survived challenge in the first 3 weeks of life but only 5 of 14 survived challenge at 4–8 weeks of age. Higher levels of antibody resulted from revaccination at 3 weeks of age, with absolute protection at 4 and 6 weeks. Unvaccinated ducklings all died when challenged. More detailed analysis of the results appears in Tables 5, 6 and 7.

**Table 5.** Response to duck plague virus challenge in ducklings lacking ELISA antibody titres at the time of challenge.

	Duck number	Ab titre before vaccination (maternal ab)	Antibody titre at time of challenge (week)						Status
			1	2	3	4	6	8	
Challenged at 1–3 weeks of age	68	2.6	0						S
	801	2.3	0						S
	785	1.7	0						S
	323	0	0						S
	859	0	0						S
	867	0	0						S
	708	0	0	0					S
	793	2.9	0						S
	712	2.6	0						S
	369	2.3	0						D
	197	2.3	0						S
	465	0	0						S
	709	3.2			0				D
	230	2.9			0				S
244	2.6			0				S	
239	2.3			0				S	
Challenged at 4–8 weeks of age	794	3.2				0			D
	104	2.3				0			D
	100	2.3				0			D
	796	ND					0		D
	164	2.6						0	D
	786*	2.6							D

0 — Serum with OD <0.2 at the first dilution; S — Survived; D — Died; ND — Not done.

\* Duck vaccinated twice (at 1 and 21 days of age). All other ducks were vaccinated once (at 1 day of age).

**Table 4.** Immune response of ducklings vaccinated with duck plague vaccine at 1 and 21 days of age.

Age at vaccination (days)	Mean ELISA antibody titre and protection against challenge at weeks of age					
	1	2	3	4	6	8
1	0.37 <sup>a</sup> (7/9) <sup>b</sup>	1.58 (7/10)	0.52 (4/5)	0.80 (2/5)	1.72 (2/5)	1.57 (1/4)
1 & 21				2.50 (5/5)	2.60 (5/5)	2.20 (3/5)
none	0.40 (0/10)	0 (0/4)	0 (0/5)	0 (0/4)	0 (0/4)	0 (0/5)

<sup>a</sup> mean ELISA antibody titre.

<sup>b</sup> (No. survived/ No. challenged).

**Table 6.** Response of ducklings to challenge in the first 3 weeks post vaccination: influence of maternal antibodies on the efficacy of vaccination at 1-day-old.

	Duck number	Ab titre before vaccination (maternal ab)	Ab titre at time of challenge (week)			Status
			1	2	3	
	68	2.6	0		S	
	801	2.3	0		S	
	785	1.7	0		S	
	708	0	0		S	
	859	0	0		S	
	323	0	0		S	
	867	0	0		S	
Maternal Ab titre ≤2.6	712	2.6	0		S	
	369	2.3	0		D	
	197	2.3	0		S	
	788	2	1.7		S	
	845	1.7	2.3		S	
	790	0	3.2		S	
	465	0	0		S	
	244	2.6		0	S	
	239	2.3		0	S	
	224	0		2.6	S	
	236	3.2	1.7		D	
	783	2.9	1.7		D	
Maternal Ab titre >2.6	798	3.2	1.7		D	
	112	3.2	2		D	
	793	2.9	0		S	
	709	3.2		0	D	
	230	2.9		0	S	

0 — Serum with OD <0.2 at the first dilution.

S — Survived.

D — Died.

The response to challenge of vaccinated ducklings lacking antibody at the time of challenge is shown in Table 5. For challenge during the first 3 weeks of

life, 14 of 16 ducklings survived. All 6 antibody-free ducklings challenged at weeks 4–8 died.

Table 6 considers the response of vaccinated ducklings to challenge in the first 3 weeks post vaccination. The ducklings are divided into those with maternal antibody titres ≤2.6 and those with higher titres. Antibody responses were poor in both groups but protection levels were higher in the former group (survival of 16 of 17 challenged ducklings) than in the latter (2 of 7).

The responses of ducklings to challenge at 4–8 weeks of age are recorded in Table 7. A comparison is made between ducklings with an antibody titre ≥2.0 at the time of challenge and those with lower titres. Eighteen of 19 ducks with antibody titres ≥2 at the time of challenge survived, while none of 10 birds with titres <2 survived.

**Table 7.** Response of ducks to challenge with duck plague virus at 4 weeks or longer after vaccination.

	Duck number	Ab titre before vaccination (maternal ab)	Ab titre at time of challenge (week)			Status
			4	6	8	
	723	2.6	2		S	
	789	2.9	2		S	
	852*	0	2.6		S	
	838*	2	3.2		S	
	704*	2.6	2.3		S	
	449*	2.9	2.3		S	
	486*	3.2	2		S	
	770	ND		2.3	S	
Antibody titre ≥2 at time of challenge	234	0		2.9	S	
	877*	ND		2	S	
	305*	ND		2	S	
	776*	ND		3.2	S	
	262*	0		2.9	S	
	769*	ND		2.9	S	
	227	0		2.9	S	
	765*	ND		3.2	S	
	720*	3.2		2	D	
	784*	1.7		3.2	S	
	722*	2.3		2.6	S	
	104	2.3	0		D	
	794	3.2	0		D	
	100	2.3	0		D	
Antibody titre <2 at time of challenge	796	ND		0	D	
	772	ND		1.7	D	
	768	ND		1.7	D	
	80	≥3.2		1.7	D	
	764	2.6		0	D	
	786*	2.6		0	D	
	237	2		1.7	D	

0 — Serum with OD<sub>450</sub> <0.2 at the first dilution.

S — Survived

D — Died.

ND — Not Done.

\* Duck vaccinated twice (at 1 and 21 days of age). All other ducks were vaccinated once (at 1 day of age).

## Discussion

The AAHL indirect antibody ELISA test for antibody to DP virus proved to be convenient and efficient. It was used to study the decline of maternally derived antibody in ducklings. Antibody levels were very diverse in ducklings from market hatcheries. Fertile eggs are sourced from various producers, and their layers probably have varied histories of vaccination, or of exposure to field virus. Antibody levels in ducklings from a commercial hatchery were more uniform and probably reflected uniform exposure of layers to vaccination. Whatever the initial levels of anti DP virus antibodies, they fell to below the level of detection in 2 or 3 weeks.

Levels of maternal antibody in commercial hatchery ducklings were not protective against artificial challenge with duck plague virus. This finding agrees with those of Tran Minh Chau (1980) and Balla (1984). This suggests that ducklings should be vaccinated against duck plague at as young an age as possible, depending on immunological maturity and any possible inhibition of vaccine virus by maternal antibody. Vaccination of breeders cannot be expected to confer temporary protection on their progeny.

Vaccination of ducklings at 1 day of age conveyed some protection during the first 3 weeks of life. During this period, 18 of 24 ducklings survived challenge, compared with 0 of 19 unvaccinated controls. Protection levels fell over the next 5 weeks if ducklings were not revaccinated. Revaccination at 3 weeks of age resulted in increased levels of antibody and high levels of protection against challenge. In ducklings that survived challenge, antibody levels rose to above pre-challenge levels (data not shown).

However, the presence of maternal antibody did interfere with the response to vaccination. Ducklings with maternal ELISA antibody titres of 2.6 or higher at the time of vaccination showed higher mortality rates following challenge than those with lower titres.

Thus, a maternal antibody titre of 2.6 in the indirect ELISA could be considered as the level that will interfere with a protective response to vaccination.

In the first 3 weeks after vaccination, protection against challenge did not depend on the development of antibodies detectable by ELISA. Ducklings with no detectable antibodies survived challenge infection. Other elements of the immune response must have been involved. Cell-mediated immunity and the protection of interferon could be suggested. The cell-mediated immune system is known to play an important role in herpesvirus diseases (Bela Toth, 1985; Islam et al., 1987; Jansen 1964; Sarmah and Sarmah, 1996). At 4 weeks or longer after vaccination, levels of ELISA antibody were predictive of protection. With the current test, a titre of 2.0 was a good indication of resistance to artificial challenge.

The ideal time for initial vaccination will be influenced by the age at which immunological maturity develops, and the age at which interference from maternal antibodies disappears.

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# Field trials of a cell culture adapted duck plague vaccine

## Thử nghiệm vắc xin dịch tả vịt thích ứng trên tế bào trong điều kiện sản xuất

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### Abstract

Following successful laboratory trials with a new vaccine against duck plague (duck virus enteritis) produced in cultured chick fibroblasts, NAVETCO undertook extensive field trials with the new vaccine. The pilot field trial involved 800 vaccinated meat ducks. The vaccine caused no clinical reactions, and 2–8 weeks after vaccination most ducks had developed ELISA antibodies and resisted artificial challenge. Most ducks challenged 1 week after vaccination were also protected, before ELISA antibodies became detectable. A subsequent extensive regional trial involved more than 170 000 ducks in 221 flocks. Detailed observations on all these ducks showed only 3 instances of possible reaction to the vaccine. A high proportion of ducks in 17 flocks sampled 3 or 8 weeks after vaccination had detectable ELISA antibodies to duck plague virus. Challenge trials on 28 ducks indicated that these antibodies were correlated with protection. The Ministry of Agriculture and Rural Development has approved the new vaccine for use in Vietnam

### Tóm tắt:

Tiếp theo các thử nghiệm thành công vắc xin mới phòng bệnh dịch tả vịt được sản xuất trên tế bào xơ phôi gà trong điều kiện phòng thí nghiệm, Navetco đã tiến hành thử nghiệm rộng rãi vắc xin mới trong điều kiện sản xuất. Thử nghiệm thực địa sơ bộ được thực hiện với tiêm chủng 800 vịt thịt. Vắc xin không gây ra các phản ứng lâm sàng, và 2-8 tuần tiêm chủng đa số vịt phát triển kháng thể ELISA và đề kháng với công cường độc. Phần lớn vịt được công cường độc 1 tuần sau khi tiêm chủng cũng được bảo vệ, trước khi kháng thể ELISA có thể phát hiện được. Thử nghiệm tiếp theo trên diện rộng bao gồm hơn 170.000 vịt trong 221 đàn. Các quan sát chi tiết tất cả những đàn vịt này chỉ thấy có 3 trường hợp phản ứng có thể do vắc xin. Có một tỷ lệ cao những vịt trong 17 đàn lấy mẫu vào 3 hoặc 8 tuần sau khi tiêm chủng có kháng thể ELISA đối với virút dịch tả vịt. Thử thách cường độc 28 vịt đã chỉ rõ các kháng thể này tương quan với sự bảo hộ. Bộ Nông nghiệp và PTNT đã chấp nhận cho sử dụng vắc xin dịch tả vịt mới ở Việt nam..

### Introduction

IN VIETNAM, the disease which causes the greatest loss to farmers is duck plague. Apart from appropriate hygienic methods, management, and nutrition, vaccination is the most effective and economical way to prevent the disease and restrict damage. The

National Veterinary Company (NAVETCO) produces and supplies hundreds of millions of doses of duck egg embryo duck plague vaccine every year, contributing greatly to the control of the disease. However, being produced from embryonated eggs, this vaccine has some disadvantages such as instability, high price and possibility of contamination.

During the cooperative research program between Vietnamese and Australian scientists (which has been mainly sponsored by ACIAR since 1995) the vaccine virus has been adapted to growth in cultures of chick embryo fibroblasts (Nguyen et al., 2004). The cell culture vaccine has shown good results when tested under experimental conditions (Do Van Dung et al., 2004). In order to determine the safety and effectiveness under field conditions, we subjected the vaccine to both pilot and extensive regional trials in 2 provinces in the Mekong Delta.

## Materials and methods

### Preparation

An agreement was negotiated between NAVETCO and sub-departments of the Department of Animal Health (DAH). The purpose of the field trial was explained and procedures were agreed. Extension exercises were then undertaken, involving departmental staff and farmers who would be involved in the trials.

The properties of the new vaccine were explained, and advice given on its preservation and use. Vaccination techniques were demonstrated.

### Trial flocks

Flocks to be used in the field were identified. Flocks of various ages and breeds were selected, and husbandry conditions varied from industrial through semi-industrial to free-range. Both laying flocks and meat flocks were involved.

Meat ducks were vaccinated once at 10–21 days of age, receiving a single subcutaneous dose of vaccine. Blood was collected before vaccination and at intervals after vaccination. Arrangements were made for purchase and challenge of vaccinated and unvaccinated ducks.

Layer ducks were usually vaccinated before breeding. Flocks already in production and already vaccinated were revaccinated. Vaccine was administered by subcutaneous or intramuscular injection. Blood samples were collected before and after vaccination as indicated.

### Vaccine

The cell culture vaccine was supplied in 500 dose bottles. Each dose contained at least  $10^3$  50% cell culture infectious doses (CCID<sub>50</sub>).

### Determination of safety

Vaccine safety was assessed by close monitoring of flocks for 2 weeks after vaccination. DAH sub-department staff and NAVETCO veterinarians com-

pleted recording sheets on duck numbers and flock health.

### Determination of efficacy

Antibody responses were determined by the antibody detection ELISA described elsewhere in this volume (Morrissey et al., 2004). A sample was considered positive for ELISA antibodies if the optical density (OD<sub>450</sub>) of serum tested at 1:50 dilution was >0.2. Vaccinated and unvaccinated meat ducks were challenged by intramuscular injection of  $10^{5.5}$  50% lethal doses (LD<sub>50</sub>) of the standard NAVETCO challenge virus.

### Pilot trial

The trial was undertaken on meat ducks from 4 flocks in 3 wards (Nhan Duc, Phuoc Kieng and Hiep Phuoc) of the Nha Be district, Ho Chi Minh City. All 800 vaccinated ducks were identified with wing tags. Before vaccination, blood samples were collected at random from 10% of the flock to determine basal levels of antibody.

### Extensive regional trial

The regional trial involved 221 flocks and more than 170 000 vaccinated ducks in 13 districts of Tien Giang and Long An provinces. Details of the numbers of ducks vaccinated at various locations are shown in Table 1. Flock sizes varied from tens of ducks to thousands of ducks. Blood samples for antibody determination were obtained from ducks in 17 meat flocks before vaccination. From 15 of these flocks antibody levels were tested 3 weeks after vaccination and from the other 2 flocks, 8 weeks after vaccination.

Vaccinated and unvaccinated meat ducks were purchased from 2 flocks, 3 weeks after vaccination and from another 2 flocks, 8 weeks after vaccination. They were taken to NAVETCO for artificial challenge. All 221 flocks were part of the safety audit.

## Results

### Pilot trial

Ducks in the study population lacked detectable antibody against duck plague virus before vaccination. None of the 800 vaccinated ducklings developed clinical signs that could be attributed to the vaccine. The development of ELISA antibodies and the responses to challenge with virulent duck plague virus are shown in Table 2.

At 1 week after vaccination, there was poor correlation between production of ELISA antibody and protection against challenge. Ducks lacking antibody

were resistant to challenge. From 2 to 8 weeks after vaccination, the presence of antibody gave a good prediction of survival after challenge. The correlation was not absolute. At 6–8 weeks after vaccination, some ducks that had produced antibody succumbed to challenge, and some antibody-free ducks survived.

### Extensive regional trial

All vaccinated ducks were observed by farmers and veterinarians. There were only 3 reports of unusual observations following vaccination, which are detailed below.

**Table 1.** Locations and numbers of vaccinated ducks.

Province	District	No. of wards	No. of vaccinated duck flocks	No. of vaccinated ducks
Tien Giang	Cái Bè	2	13	3 453
	Cai Lậy	6	38	7 340
	Châu Thành	1	11	3 170
	Chợ Gạo	2	27	5 948
	Gò Công Đông	7	37	39 900
	Gò Công Tây	5	11	4 500
Total		23	137	64 311
Long An	Thủ Thừa	3	15	21 000
	Châu Thành	3	13	16 000
	Cần Đước	3	11	14 500
	Tân Trụ	3	6	11 000
	Bến Lức	4	12	12 500
	TX. Tân An	4	14	21 500
	Thạnh Hoá	2	13	10 500
Total		22	84	107 000
Total		45	221	171 311

**Table 2.** ELISA antibody response and protection against challenge of ducks after vaccination with cell culture vaccine.

Ward	Ducks	No.	Weeks post vaccination				
			1	2	3	6	8
Nhan Duc	vaccinated	180 <sup>d</sup>	3/20 <sup>a</sup>		14/19	14/19	
			16/20 <sup>b</sup>		14/19	13/19	
	control	60 <sup>d</sup>	(80) <sup>c</sup>		(73.7)	(68.4)	
			0/15		0/10	0/10	
Hiep Phuoc	vaccinated	320 <sup>d</sup>	0/15		0/10	0/10	
			(0)		(0)	(0)	
				16/18	15/16	13/16	
	control	30 <sup>d</sup>		18/18	14/16	13/16	
				(100)	(87.5)	(81.2)	
				0/9	0/8	0/8	
Phuoc Kieng	vaccinated	200 <sup>e</sup>			0/9	0/8	0/8
					(0)	(0)	(0)
				17/20	15/20		
	vaccinated	100 <sup>d</sup>		17/20	14/20		
				(85)	(70)		
				17/20	17/20		
control	20 <sup>d</sup>		18/20	15/20			
			(90)	(75)			
			0/10	0/9			
			0/10	0/9			
			(0)	(0)			

<sup>a</sup> positive ELISA antibody/samples tested; <sup>b</sup> no. ducks survived/no. challenged ducks; <sup>c</sup> percentage of protection; <sup>d</sup> 3-week-old ducks; <sup>e</sup> 10-day-old ducks.

One flock of 40 Muscovy ducklings vaccinated at 3 weeks of age, showed reduced appetite for 2 or 3 days, commencing 1 or 2 days after vaccination. Another flock of 200 ducks receiving vaccine from the same bottle showed no abnormal clinical signs.

In a flock of 200 layer ducks, a 15–20% drop in egg production occurred in the week following vaccination. The third incident involved a flock of 115 ducks which were 3 weeks old. The ducks showed clinical signs of illness 2 days after vaccination, and 63% died on the third day. The remainder survived after treatment with antibiotics. Laboratory diagnosis confirmed a diagnosis of pasteurellosis, not duck plague. If all these instances of morbidity and mortality are attributed to the vaccine, the percentage of adverse reactions was about 0.2% of ducks in 1.4% of flocks.

The detection of ELISA antibodies in ducks 3 weeks after vaccination (15 flocks) and 8 weeks after vaccination (2 flocks) is detailed in Table 3. All ducks were negative for ELISA antibodies prior to vaccination. Post vaccination responses were consistent throughout both provinces, with 85.7% of samples yielding detectable ELISA antibody at 3 weeks, and 100% at 8 weeks. The response to buy-back challenge is shown in Table 4. Antibody production and protection against challenge were well correlated.

**Table 3.** Detectable ELISA antibodies in ducks after vaccination under field conditions.

Province	Duck flock	No. ELISA antibody positive/ no. tested	
		3 weeks post vaccine	8 weeks post vaccine
Tien Giang	1	5/5	
	2	3/5	
	3	5/7	
	4	5/5	
	5	7/10	
	6	7/12	
	7	14/15	
	8	19/20	
	9	13/15	
	10	15/15	
Long An	11	35/40	
	12	4/5	
	13	5/5	
	14	8/10	
	15	17/20	
	16		10/10
	17		6/6
Total		162/189 (85.7%)	16/16 (100%)

**Table 4.** Protection of ducks challenged with virulent duck plague virus at 3 and 8 weeks after field vaccination.

Status	Weeks after vaccination	ELISA antibodies No. positive/No. tested		Protection No. survived/ No. challenged
		Pre- vaccination	Post- vaccination	
Vaccinated	3	0/10	10/10	10/10
Control	—	0/5	0/5	0/5
Vaccinated	8	—	16/18	15/18
Control	—	—	0/10	0/10

## Discussion

Although the new cell culture duck plague vaccine had proved safe and efficacious in laboratory trials, approval of the vaccine for registration required extensive field testing. These tests were undertaken by NAVETCO and DAH in provincial Vietnam.

The initial trial was on a pilot scale involving 800 vaccinated meat ducks. The results obtained were similar to those achieved in the laboratory. The vaccine produced no clinical signs. It protected, at 1–8 weeks after vaccination, against a very high dose ( $10^{5.5}$  LD<sub>50</sub>) of challenge virus. This is probably a more severe challenge than is encountered in the field. As had been found in the laboratory, a protective response could be demonstrated 1 week after vaccination, before most ducks had produced detectable ELISA antibody. Others (Tran Minh Chau, 1980; Leibovitz, 1971) have made similar observations. This early protection may be attributed to the production of interferon, or of a cell mediated immune response.

These results justified an extensive regional trial to validate safety and efficacy of the vaccine. This was undertaken in 2 provinces of Vietnam and utilised the combined resources of NAVETCO and DAH. The major emphasis was on indicating safety of the vaccine. Reactions possibly attributable to the vaccine were recorded in only 355 of 171 311 ducks and 3 of 221 flocks. Two incidences did not involve mortality; the first was the transient loss of appetite in young ducklings, and the second a transient drop in egg production in laying ducks. Both may have been attributed directly to the vaccine, or to the stress of handling for vaccination. A third incident was more serious, when about 70 of 115 ducklings died of pasteurellosis soon after vaccination. It is possible that vaccination, or the vaccine, had activated a latent pasteurella infection. The trials indicated a high level of safety of the vaccine.

Vaccine efficacy was indicated by the production of ELISA antibody in a high proportion of ducks

in all of the 17 flocks sampled. Only a limited challenge trial was warranted. This confirmed the results of the pilot trial, most ducks developing ELISA antibodies being resistant to challenge. Efficacy under field conditions was less than that achieved in the laboratory. Vaccine preservation, reconstitution in water of various grades and the health status of the flocks may all influence efficacy of the vaccine.

It will be necessary to establish the longevity of protection. Ducks were protected for at least 8 weeks after vaccination. This is adequate for meat ducks that are marketed at about 2 months of age in Vietnam. Breeding ducks will require a much longer duration of protection.

These results, and samples of the vaccine, were presented to the National Centre for Veterinary Medicine Quality Control, DAH. The vaccine was approved for use in Vietnam (Vietnam Certification, Standard TCN 161-92 and 183-93-promulgated in 1994 by the Ministry of Agriculture and Rural Development).

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# Antigenic relatedness of duck plague viruses isolated in Vietnam

## Mức độ tương đồng kháng nguyên của virút dịch tả vịt phân lập ở Việt nam

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### Abstract

Cross neutralisation tests were used to demonstrate antigenic differences among duck plague viruses in Vietnam. Seven field isolates, a vaccine strain and a virulent challenge strain could be divided into 2 antigenic groups. R values indicated that these groups could be considered as separate serotypes or major subtypes. Antigenic grouping did not correlate with virulence or geographic location.

The study also showed that adaptation to cell culture of the duck egg cultured vaccine had not substantially affected its neutralising characteristics. Sera raised against either of the vaccines had poor neutralising activity against the standard challenge strain used at NAVETCO and 3 field isolates from serogroup 2. However, neutralising activity does not appear to correlate with *in vivo* protection. Some of the field isolates examined in this study could be investigated further as possible vaccine candidates or alternative challenge viruses.

### Tóm tắt

Nghiệm pháp trung hòa chéo được sử dụng để chứng minh những sai khác kháng nguyên giữa các chủng virút dịch tả vịt ở Việt nam. Bảy phân lập thực địa, một chủng vắc xin và một chủng virút cường độc có thể được phân thành 2 nhóm kháng nguyên. Các giá trị R đã chỉ rõ các nhóm này có thể coi như những serotíp riêng biệt hay là các subtytíp chủ yếu. Phân nhóm kháng nguyên không tương quan với độc lực hay vị trí địa lý.

Nghiên cứu cũng chỉ rõ sự thích ứng vào tế bào của vắc xin sản xuất trên phôi trứng đã không ảnh hưởng đến đặc tính trung hòa của nó. Huyết thanh tạo được sau tiêm chủng vắc xin có hoạt tính trung hòa yếu đối với chủng cường độc đang được sử dụng ở Công ty Thuốc Thú y TW2 và 3 phân lập thực địa thuộc nhóm huyết thanh 2. Tuy nhiên, hoạt tính trung hòa hình như không tương ứng với sự bảo hộ trên cơ thể sống. Một vài phân lập thực địa khảo sát trong nghiên cứu này nên được nghiên cứu tiếp tục để có thể sử dụng như là chủng virút cường độc hoặc chủng vắc xin thay thế trong tương lai.

### Introduction

DUCK plague is a major disease of ducks in Vietnam. At the start of an ACIAR project to improve the vaccination and diagnosis of duck plague in Vietnam the only strains of duck plague virus available in Vietnam were a vaccine strain, which had been

obtained from China in the 1970s, and a virulent challenge strain. The vaccine was produced in embryonated duck eggs and was used for vaccination of local ducks. During the project, a total of 99 field isolates of duck plague virus were collected from 353 diseased ducks and the Chinese vaccine strain was adapted to growth in chicken cell cultures.

Little is known about the antigenic diversity of duck plague viruses and there is no information on the diversity of duck plague viruses in Vietnam. The objectives of the work described in this paper were to determine if there is antigenic variation among Vietnamese isolates of duck plague virus, to determine if antibodies induced by the vaccine strain currently used in Vietnam neutralise Vietnamese field isolates of duck plague virus and to investigate whether adaptation of the duck plague vaccine to cell culture had altered the antigenic state of the virus. In addition, it was hoped that from the field isolates obtained in the study, new virulent viruses that could be used in challenge experiments and new candidate vaccine viruses could be selected. Seven field isolates of duck plague virus from 6 provinces of Vietnam, the old vaccine, the cell culture-adapted vaccine and the old standard challenge virus were tested in a cross neutralisation test to determine their antigenic relatedness.

## Materials and methods

### Viruses

Detailed information about the viruses used in this work is shown in Table 1. The 7 field isolates of duck plague virus were isolated from brain, spleen or liver of dead or sick ducks sent to the laboratory by farmers in 1996–1997 (Tran Dinh Tu et al., 2004). These 7 isolates were selected from 99 isolates obtained in that study. The isolates were passaged several times in duck embryo fibroblast (DEF) cell cultures until their titres reached at least  $10^5$  50% tissue culture infectious doses per ml (TCID<sub>50</sub>/ml).

The cell culture-adapted duck plague vaccine (CC vaccine) was a derivative of the existing vaccine,

adapted to chicken embryo fibroblast (CEF) cell culture and stored as a master seed. The seed virus strain underwent 15 passages in embryonated chicken eggs and 12 passages in CEF cell cultures. The CEF cell culture fluid from the 13<sup>th</sup> passage was harvested and stored as vaccine master seed. The 14<sup>th</sup> passage served as the CC vaccine.

The standard challenge virus was cultured in embryonated duck eggs and stored as duck embryo allantoic fluid. The virus had been kept virulent by regular passages in ducks. The livers of these ducks were collected and the virus re-isolated in embryonated duck eggs.

The identity of the field isolates, the challenge strain and the 2 vaccine strains was confirmed by PCR test (Kim Van Phuc, 2004).

### Indirect antibody ELISA

An indirect ELISA developed by the Australian Animal Health Laboratory as part of the ACIAR duck plague project was used to quantify duck plague virus antibodies. Briefly, sera were diluted 1 in 50 in phosphate-buffered saline containing 0.05% Tween 20 (PBST) and 1% skim milk powder, followed by serial twofold dilutions. Fifty  $\mu$ L of each serum dilution was added to wells of a microtitre plate coated with duck plague virus antigen, incubated with shaking for 30 min at 37°C then washed with PBST. Goat anti-duck IgG-horse radish peroxidase conjugate was diluted 1:2000 in the same dilution buffer as above and added in a volume of 50  $\mu$ L, the plates were incubated as above, then 50  $\mu$ L of freshly prepared substrate (TMB) was added. The reaction was stopped after 5 min by addition of 50  $\mu$ L 1M H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) at 450 nm was determined. The antibody titre was regarded as the reciprocal of the highest dilution with an OD<sub>450</sub>  $\geq$  0.2.

**Table 1.** Duck plague virus strains and isolates used in the study.

Virus	Area of origin	Culture	No. survived/ no. challenged <sup>a</sup>	Virulence
Duck egg vaccine	China	Embryonated duck eggs	3/3	avirulent
CC vaccine	China	Chick embryo fibroblasts	3/3	avirulent
Challenge strain	Hanoi	Embryonated duck eggs	1/4	virulent
Field isolate 25	Dong Thap	DEF (7)	3/4	mild
Field isolate 47	Dong Thap	DEF (7)	4/4	avirulent
Field isolate 57	Dong Nai	DEF (6)	4/4	avirulent
Field isolate 63	Bien Thuan	DEF (7)	4/4	avirulent
Field isolate 65	Dong Nai	DEF (7)	4/4	avirulent
Field isolate 203	Dong Thap	DEF (3)	3/4	mild
Field isolate 252	Tien Giang	DEF (7)	1/4	virulent

CC: Cell culture adapted; DEF: duck embryo fibroblast cell cultures (Numerals indicate passage level).

<sup>a</sup>Ducks were inoculated intramuscularly with  $10^5$  TCID<sub>50</sub> or  $10^5$  EID<sub>50</sub> of each virus.

### Production of serum

The field isolates, challenge strain and vaccines were inoculated into 3 or 4 local Vietnamese ducklings, which were 3 weeks old, at a dose of  $10^5$  TCID<sub>50</sub> or  $10^5$  50% egg infectious doses (EID<sub>50</sub>) per duck by the intramuscular route. The numbers of ducks surviving after the inoculation were recorded.

Two weeks after inoculation, blood samples were collected from surviving ducks. Serum was separated and tested for duck plague virus antibodies by indirect ELISA. If the ELISA antibody titre was <3200, the duck was re-inoculated once or twice at 2-weekly intervals and blood samples collected 2 weeks after each inoculation. When the ELISA antibody titre was  $\geq 3200$ , the duck was bled by cardiac puncture. Sera were separated and inactivated at 56°C for 30 mins. Sera from ducks from the same group were pooled by mixing equal volumes of each serum and the pooled samples were stored in 1 ml volumes at -20°C.

### Neutralisation test

A beta virus neutralisation method was used, using 2-fold serum dilutions against constant virus (20–1000 TCID<sub>50</sub> per well). Sera were diluted in Earles minimum essential medium (EMEM) from 1/2 to 1/1024. Pooled sera from Pekin ducks in Australia was used as a negative control. The serum-virus mixtures were incubated at 37°C for 1 hour, then added to a 24-hour second passage CEF monolayer in wells of a 96-well microtitre plate or to 100  $\mu$ L of  $1 \times 10^6$  second passage CEF cells in EMEM with 10% foetal bovine serum, penicillin and streptomycin and then the mixture was added to wells of a microtitre plate. After 4 days of incubation at 37°C, all wells were examined microscopically for cytopathic effects (CPE). The highest dilution of serum at which the viral CPE was inhibited was recorded as the neutralising antibody titre. The test was run in duplicate and geometric mean titres were calculated.

### Calculation of R values

Cross-reactivity (R) values were calculated according to the formula described (Archetti and Horsfall, 1950) and applied (Gravendyck et al., 1996; Giambrone and Solano, 1988) previously. The R% value is  $100 \times$  the square root of  $r_1 \times r_2$  where

$$r_1 = \frac{\text{titre of antiviral 2} - \text{serum against virus 1}}{\text{titre of antiviral 1} - \text{serum against virus 1}}$$

$$r_2 = \frac{\text{titre of antiviral 1} - \text{serum against virus 2}}{\text{titre of antiviral 2} - \text{serum against virus 2}}$$

Where the neutralising antibody titre was >1024, a value of 1024 was used in the calculations. R values between 0 and 10% are considered as a serotype dif-

ference, 11 and 32% a major subtype difference, between 33 and 70% a minor subtype difference, and values greater than 70% are considered to have little or no difference (Giambrone and Solano, 1988).

### Results

The results of *in vivo* challenge with the vaccines, challenge strain and the 7 field isolates are shown in Table 1. The viruses could be divided into those which caused no deaths following challenge (avirulent), those causing some deaths (mild) and those causing death in 3 of 4 inoculated birds (virulent).

Detailed information on the ELISA titres of individual duck and pooled sera is shown in Table 2. The ELISA titre of the pooled sera used in the neutralisation test ranged from 1600 to 102 400.

The geometric mean titres of the virus neutralisation tests are shown in Table 3. Neutralisation titres ranged from 0 to >1024. With most, but not all, sera, the highest neutralising titre was obtained against the homologous virus. The neutralisation titre of the cell culture adapted vaccine antiserum against each of the viruses was similar to that obtained with the original vaccine produced in embryonated eggs. Antisera raised against the 2 vaccines had low or negative neutralisation titres against some of the field isolates (47, 63, 203) and the challenge strain. The negative control serum from Australia had some neutralising activity against field isolates 47, 57, 63 and 203.

The R-values are recorded in Table 4. The viruses could be divided into 2 main groups based on their antigenic relatedness. The first group comprised field isolates 25, 65, 252 and the vaccine strain, with R values ranging from 58–100% among the group. Serogroup 2 comprised field isolates 47, 57, 63 and 203, with R values from 35–100%. Although the data for the challenge strain were not complete, its R values against the 7 field isolates indicated a close relationship with serogroup 1. The R values between viruses in the 2 groups ranged from 0 to 20%.

Field isolates from the same area belonged to different antigenic groups. For example, isolates 25 and 47 were both from Dong Thap province but were antigenically distinct. Viruses within each antigenic group were of varying virulence.

### Discussion

Cross neutralisation tests demonstrated serologic differences among duck plague viruses isolated in Vietnam. The 7 field isolates examined in this study could be divided into 2 antigenic groups, with the current vaccine strain belonging to serogroup 1. The R values between groups were sufficiently low to

**Table 2.** ELISA titres of individual and pooled sera collected from ducks inoculated with duck plague vaccines, challenge strain and field isolates.

Virus	No. of ducks	ELISA titre of individual sera		ELISA titre of pooled sera
		Post 2nd inoculation	Post 3rd inoculation	
Duck egg vaccine	335	12 800		1 600
	337	3 200	6 400	
	311	3 200		
CC vaccine	345	800	6 400	1 600
	303	3 200		
	302	800	3 200	
Challenge strain	330	3 200		6 400 <sup>a</sup>
Field isolate 25	307	3 200	12 800	6 400
	314	12 800	25 600	
	321	25 600	51 200	
Field isolate 47	390	3 200	6 400	6 400
	663	12 800	6 400	
	384	6 400	12 800	
	392	6 400	6 400	
Field isolate 57	399	3 200	12 800	6 400
	656	3 200	12 800	
	497	25 600		
	387	51 200		
Field isolate 63	398	1 600	3 200	6 400
	382	6 400	6 400	
	657	3 200	6 400	
	386	25 600	12 800	
Field isolate 65	317	12 800	6 400	12 800
	350	12 800	102 400	
	319	6 400	12 800	
	326	6 400	6 400	
Field isolate 203	372	3 200	3 200	6 400
	664	6 400	12 800	
	380	6 400	12 800	
Field isolate 252	322	25 600		102 400 <sup>a</sup>

CC: Cell culture adapted; <sup>a</sup>Serum from 1 duck only.

**Table 3.** Results of cross neutralisation test between sera from ducks inoculated with vaccines, challenge strains or field isolates against homologous and heterologous viruses.

Serum	Virus								
	CC vaccine (20) <sup>a</sup>	Challenge strain (>1000)	25 (200)	47 (400)	57 (100)	63 (100)	65 (20)	203 (>1000)	252 (200)
Negative control	0 <sup>b</sup>	0	0	2	32	8	0	2	0
Duck egg vaccine	384	0	64	0	4	4	192	4	192
CC vaccine	512	1	96	3	32	8	256	4	192
Challenge strain	nd	2	128	0	4	12	384	12	96
25	256	2	48	4	12	16	512	8	64
47	768	288	192	>1024	>1024	>1024	384	640	128
57	512	0	128	>1024	>1024	>1024	384	512	96
63	>1024	3	128	>1024	>1024	>1024	256	128	64
65	256	2	128	0	16	16	384	16	96
203	768	64	192	>1024	>1024	>1024	768	>1024	128
252	192	1	192	0	8	6	640	16	128

CC: Cell culture adapted; <sup>a</sup> Amount of virus added to the serum dilutions (TCID<sub>50</sub>);

<sup>b</sup> Tests were conducted in duplicate; results are expressed as the geometric mean titre; nd: Not done.

**Table 4.** R values (%) of the duck plague vaccine, challenge strain and field isolates.

Virus serum	CC vaccine	25	47	57	63	65	203	252	Challenge strain
CC vaccine	100								
25	100	100							
47	4.7	12.5	100						
57	17.7	17.7	100	100					
63	8.8	20.4	100	100	100				
65	57.7	188.5	0	12.5	10.2	100			
203	7.6	17.7	79.1	70.7	35.4	17.3	100		
252	75	141.4	0	7.6	5.4	111.8	12.5	100	
Challenge strain	nd <sup>b</sup>	163.3	0	0	13.2	100	61.2	61.2	100

CC: Cell culture adapted.

<sup>a</sup> R values of 0–10% indicate serotype difference, 11–32% major subtype difference, 33–70% minor subtype difference, >70% means little or no difference.

<sup>b</sup>nd: Not done.

indicate either a serotype or major subtype difference between the 2 groups.

Within each group there was a range in virulence, indicating that antigenic type does not correlate with pathotype. Viruses from both antigenic groups were isolated from ducks in the same region. This suggests that one particular antigenic type does not circulate exclusively in a region and that both antigenic types can coexist.

Only 7 field isolates were examined in this study. It is possible that with investigation of further isolates, more than 2 antigenic groups of duck plague viruses would be found to exist in Vietnam.

Evidence of antigenic diversity using cross neutralisation tests has been shown in many viruses, e.g. avian reoviruses and psittacine herpesviruses (Giambrone and Solano, 1988; Gravendyck et al., 1996). It has been reported that no differences in antigenicity occurred between duck plague virus isolates from The Netherlands, India and the USA using a plaque-reduction test (Richter and Horzinek, 1993). No details of the isolates tested or the methodology used were provided, so it is difficult to determine why the results in the current study differed from that previous report. However, it appears from this study that at least 2 different serotypes or subtypes of duck plague virus occur in Vietnam.

The adaptation of the duck plague vaccine to cell culture does not appear to have affected the antigenic state of the virus, since neutralisation titres of the 2 antisera against each of the field isolates were very similar. Three of the field isolates from serogroup 2 were poorly neutralised by sera raised against either the duck egg vaccine or the cell culture adapted vaccine. However, neutralising antibody titre does not appear to correlate with protection *in vivo* because the current virulent challenge strain was also poorly neutralised by the vaccine antisera, yet both

vaccines give good protection against this challenge strain (Nguyen Thi Kim Dinh et al., 2004; Nguyen Thi Thu Hong et al., 2004). Presumably, cell mediated immunity plays a major role in the protection induced by vaccination against duck plague. To investigate this further, challenge experiments of vaccinated ducks could be performed using isolates from serogroup 2. This would clarify whether the current vaccine strain (from serogroup 1) is protective against viruses from the other antigenic group.

Some of the field isolates examined were shown to produce mild or no clinical signs and no deaths in inoculated ducks. Of these avirulent viruses, some belonged to the same antigenic group as the vaccine strain while others belonged to the other group. These viruses could be investigated further as possible vaccine candidates.

One of the field isolates (252) caused the same mortality rate as the virulent challenge strain and belonged to the same antigenic group. This isolate could be investigated as an alternative challenge virus. One limitation of the current challenge strain is the fact that it must be administered by an unnatural route and does not transmit to in-contact ducks. Further studies could be undertaken to determine if isolate 252 is able to infect ducks by routes other than the intramuscular one and whether it can be spread by contact to other ducks.

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# Duck plague in Muscovy ducks in Can Tho province

## Bệnh dịch tả vịt trên vịt xiêm ở tỉnh Cần thơ

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### Abstract

Muscovy ducks are widely raised all over Can Tho province in relatively small flocks that are allowed to roam around in the backyards and gardens.

A retrospective survey showed that 14.5% of Muscovy ducks in Can Tho had experienced a disease resembling duck plague. The prevalence of suspected duck plague varied among different locations within the province, with the highest rates occurring in Can Tho City and Thot Not district. The prevalence also varied slightly at different times of the year, with peaks from March to May and November to December. The prevalence was highest in birds over 12 weeks of age.

Of 882 serum samples taken from Muscovy ducks not vaccinated with duck plague vaccine and raised in 9 districts and towns of the province, 15.40% had antibodies detected by Ab ELISA. This percentage ranges from 5% to 36% depending on location within the province.

Duck plague was considered to be a serious constraint to the raising of Muscovy ducks in the area.

### Tóm tắt

Vịt xiêm được nuôi khá rộng rãi khắp tỉnh Cần thơ trong các đàn tương đối nhỏ được thả tự do trong sân vườn.

Kết quả một cuộc điều tra hồi cứu cho thấy 14.5% vịt xiêm nuôi ở tỉnh Cần thơ bị nhiễm một bệnh giống như dịch tả vịt. Sự lưu hành của bệnh nghi là dịch tả vịt thay đổi theo địa bàn trong tỉnh, trong đó ở TP Cần thơ và huyện Thốt nốt có tỷ lệ nhiễm cao nhất. Tỷ lệ nhiễm cũng thay đổi theo các tháng trong năm, cao nhất trong khoảng từ tháng 3 đến tháng 5 và từ tháng 11 đến tháng 12. Tỷ lệ nhiễm bệnh ở vịt xiêm trên 12 tuần tuổi là cao nhất.

Trong số 882 mẫu huyết thanh lấy từ vịt xiêm chưa được tiêm chủng vắc xin dịch tả vịt và được nuôi ở 9 huyện thị của tỉnh có 15.4% số mẫu hiện diện kháng thể dịch tả vịt được phát hiện bằng kỹ thuật Ab-ELISA. Tỷ lệ này giao động trong khoảng từ 5 đến 36% tùy thuộc vào vị trí lấy mẫu.

Dịch tả vịt được xem là một hạn chế quan trọng đối với chăn nuôi vịt xiêm của tỉnh Cần thơ.

### Introduction

MUSCOVY ducks (*Cairina moschata*) are widely raised by farmer households in the Mekong River Delta with a total population of about 4 500 000 birds, accounting for 16% of the total population of waterfowl being raised in the region. Muscovy ducks are commonly allowed to roam around in backyards and gardens or confined in simple enclosures around farmer households. The size of flocks varies from 10–30 birds, with mixed ages. These ducks are fed with rice or bran, or remains from pig feed. They can feed themselves with fish, snails and earthworms, in the gardens of households or on the canals.

Duck plague or duck virus enteritis is an important contagious herpesvirus infection in domesticated and wild waterfowl of the *Anatidae* family, the *Anseriformes* order. The disease has been recognised as the most serious in the Mekong River Delta (Tran Dinh Tu, 1995; Tran Dinh Tu and Kim Van Phuc, 1998, 1999; Nguyen Duc Hien, 1997), causing great losses to many domestic duck producers, but no official report on the disease in Muscovy ducks has been recorded.

The yearly rates of morbidity and mortality in Muscovy ducks are quite high and most are suspected to be induced by duck plague virus. In order to provide more specific evidence, we have

conducted surveys on duck plague infection in Muscovy ducks and investigated the prevalence of duck plague virus antibody in the serum of Muscovy ducks which have not been vaccinated with duck plague vaccine. This report is a collection of preliminary results of a survey on duck plague in Muscovy ducks in Can Tho province.

### Materials and methods

A survey was carried out by the Department of Animal Health of Can Tho province to determine the numbers and distribution of domestic waterfowl in the province, including the distribution of Muscovy ducks.

A disease survey by retrospective study with questionnaire forms given to farmer households was carried out by local veterinarians and students of the Veterinary Medicine School, Can Tho University. The total number of forms was 12 500, accounting for 8–10% of farmer households raising Muscovy ducks in Can Tho province.

A disease survey was carried out by a cross-sectional design with serum samples taken at one time point from flocks of Muscovy ducks which were not vaccinated with duck plague vaccine. Blood samples were collected from 882 Muscovy ducks from 27 villages in 9 districts of Can Tho province. The presence of duck plague virus antibody in the serum was demonstrated by indirect ELISA.

### ELISA techniques

The ELISA technique applied for the survey at the laboratory of the Department of Animal Health of Can Tho Province was provided by Australian Animal Health Laboratory (AAHL). AAHL developed the test and transferred the technology through training courses at NAVETCO under the Ministry of Agriculture and Rural Development.

The duck plague indirect antibody ELISA and duck plague herpesvirus antigen capture ELISA were

applied following the procedures of AAHL and as standardised to comply with Vietnamese conditions by the Virology Laboratory, Research Center of NAVETCO.

## Results and discussion

### Muscovy duck raising situation in Can Tho Province

The results of the survey on the situation of waterfowl raising in Can Tho province carried out in 2002 by the Department of Animal Health of Can Tho Province are summarised in Table 1.

The total population of waterfowl in Can Tho province is large (over 3 200 000 birds), 90% of which are Pekin ducks. Muscovy ducks account for 7% of the total population, with a small percentage of geese. However, the distribution of Muscovy ducks is not uniform. The most densely populated areas are Phung Hiep and Long My districts, which account for nearly 50% of the Muscovy ducks of the whole province.

Muscovy ducks are raised at farmer households in small flocks (10–30 birds/farmer household) compared with the larger flocks of Pekin ducks. These Muscovy ducks are raised all the year round, following the traditional habits of the farmers and fed with self-processed bran of local feed grains. Small flocks of muscovy ducks are usually of mixed ages. There are just a few large flocks of 100–500 Muscovy ducks, concentrated mainly around Can Tho City. These larger flocks of Muscovy ducks are usually of the same age and fed with industrially-processed feed.

### The duck plague situation in Muscovy ducks in Can Tho Province

The outbreaks suspected to be caused by duck plague in Muscovy ducks are characterised by diarrhoea, death in 2–3 days and no response to treatment.

**Table 1.** The distribution of waterfowl in Can Tho Province.

No.	Location	Common (Pekin) ducks	Muscovy ducks	Geese
1	Can Tho City	134 120	11 373	391
2	O Mon District	665 170	24 877	1 184
3	Thot Not District	389 380	15 653	1 080
4	Chau Thanh District	204 910	24 856	741
5	Chau Thanh A Dist.	225 090	24 220	758
6	Phung Hiep District	390 550	49 092	1 355
7	Long My District	573 590	55 409	3 402
8	Vi Thuy District	310 820	19 115	994
9	Vi Thanh Town	117 720	6 063	261
10	Collective farms	28 770	1 842	56
	Whole province	3 040 120	232 500	10 222

Results from the interviews, conducted with more than 4000 farmer households in the province by local veterinarians and students of Can Tho University in 2002, are presented in Tables 2, 3 and 4.

**Table 2.** Geographical distribution of suspected duck plague in Muscovy ducks in Can Tho Province during 2000–2002.

No.	Location	Total number of birds surveyed	Diseased Birds	Affected Percentage (%)
1	Can Tho City	3 312	769	23.20
2	O Mon District	6 320	894	14.10
3	Thot Not District	5 206	1 157	22.20
4	Chau Thanh District	6 036	647	10.70
5	Chau Thanh A Dist.	5 900	1 217	20.60
6	Phung Hiep District	7 413	666	9.00
7	Long My District	8 478	699	8.20
8	Vi Thuy District	5 543	711	12.80
9	Vi Thanh Town	3 176	670	21.10
	Whole province	51 384	7 431	14.50

**Table 3.** Distribution of suspected duck plague by month of the year.

Month	No. of Muscovy ducks surveyed	No. of Muscovy ducks with suspected duck plague	Percentage (%)
1	4 560	547	12.00
2	5 201	674	12.70
3	4 681	756	16.10
4	5 228	1 005	19.20
5	3 672	566	15.40
6	3 116	406	13.00
7	3 200	483	15.10
8	4 192	505	12.00
9	4 150	455	11.00
10	5 120	493	9.60
11	4 374	980	22.40
12	3 890	595	15.30
Total	51 384	7 431	14.50

**Table 4.** Distribution of suspected duck plague in Muscovy ducks by age.

Weeks of age	Infected birds	Percentage (%)
0–4	625	8.40
5–12	1976	26.60
>12	4831	65.00
Total	7431	100.00

The suspected rate for duck plague in Can Tho Province is only 14.5%. However, this rate is not equally distributed in the province, being highest in

Can Tho City at 23.2% and lowest in Long My District at 8.2%.

The level of probable duck plague infection in Muscovy ducks is not as high as in common ducks. This is probably due to the small size of flocks, and their sparse distribution, with limited contact with other types of ducks which reduces opportunities for the spread of duck plague virus from flock to flock. The infection resembling duck plague occurred repeatedly among larger-size flocks of over 50 birds that had not been vaccinated with duck plague vaccine.

Probable duck plague outbreaks in Muscovy ducks occurred fairly uniformly through the year. The severe outbreaks seen in scavenging Pekin ducks (ducks feeding themselves with fallen grain, snails and fish in paddy fields) occurred rarely. Minor peaks occurred from March to May and November to December as occurs with duck plague in Pekin ducks (Nguyen Duc Hien, 1997). This may be due to the changing weather during this period that partly affects the resistance of Muscovy ducks, making them more susceptible to the disease.

The suspected duck plague affected Muscovy ducks of all ages, but the rate of infection increased with age. Like other types of ducks, adult Muscovy ducks are quite susceptible to duck plague. Muscovy ducks over 12 weeks of age suffered a morbidity rate of 65%, while young Muscovy ducks (under 4 weeks of age) accounted for only 8.4% of the total infected birds during the survey period. Young Muscovy ducks of local species are reputed to enjoy a higher survival rate than other types of ducks.

#### Serological survey on the prevalence of duck plague virus

In parallel with the retrospective survey by questionnaire forms, we took 882 serum samples from flocks of Muscovy ducks that had not been vaccinated with duck plague vaccine to detect duck plague virus specific antibody. The results of the serological survey are shown in Table 5.

The rate of positively reacting serum samples against specific duck plague antigen for the whole province was 15.4%. The rate of positive serum samples varied greatly from place to place. The highest rate of positive samples was from Chau Thanh A District (36%) and Can Tho City (31.2%) and the lowest in Phung Hiep District (5%) and Chau Thanh District (5.7%).

Because of the unavailability of enzyme-labelled anti-Muscovy duck antibody, we used goat anti-duck HRP conjugate in the reaction. The following trial was performed to validate this approach. We used

goat anti-duck HRP conjugate to detect the presence

**Table 5.** Antibody survey of Muscovy ducks not vaccinated with duck plague vaccine by duck plague indirect Ab-ELISA.

No.	Location where samples were taken	No. of samples tested	No. of positive samples	Percentage (%)
1	Can Tho City	96	30	31.20
2	O Mon District	93	9	9.70
3	Thot Not District	118	23	19.50
4	Chau Thanh District	106	6	5.70
5	Chau Thanh A Dist.	89	32	36.00
6	Phung Hiep District	101	5	5.00
7	Long My District	90	9	10.00
8	Vi Thuy District	91	7	7.70
9	Vi Thanh Town	98	15	15.30
	Whole province	882	136	15.40

of anti-duck plague virus antibody present in the sera of ducks, Muscovy ducks and geese vaccinated with duck plague vaccine and challenged with a virulent duck plague virus 2 weeks post vaccination. All of the duck and Muscovy duck serum samples became positive in the ELISA reaction. In contrast, the percentage of ELISA positive goose sera was much lower (about 30%). This may be due to the fact that the genetic relationship between ducks and Muscovy ducks is closer than between ducks and geese,

resulting in cross-reaction between the anti-duck antibody and Muscovy duck immunoglobulin but not with goose immunoglobulin. We can conclude that the results in the survey above are acceptable. Furthermore, in the process of disease diagnosis at the laboratory, we received duck plague suspected specimens from Muscovy ducks which were positive by antigen capture ELISA.

Duck plague in Muscovy ducks needs further intensive study. The disease survey and the antibody survey convince us that the problem in Muscovy ducks is real.

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# Isolation of reoviruses from Pekin ducks in Australia

## Phân lập reovirus từ vịt Bắc kinh ở Úc

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### Abstract

With the aim of isolating herpesviruses, virus isolation was attempted from tissue samples from 73 ducks in Australia, where duck plague is considered an exotic disease. No herpesviruses were isolated, but reoviruses were isolated from 9 ducks. Most of the isolates were obtained from samples of intestines and all of the 9 ducks that yielded reoviruses were considered to be healthy. The viruses were isolated in duck embryo liver or kidney cells and caused cytopathic effect and cell death. The viruses were passaged in embryonated duck eggs and the chorioallantoic membrane of the eggs showed a pock-like thickening. The isolates were identified as reoviruses by their characteristic electron microscopic appearance. The study highlights the importance of confirming the identity of viruses isolated from ducks. In countries where duck plague is common, reoviruses could easily be misidentified as duck plague virus.

### Tóm tắt

Với mục đích phân lập herpesvirus, phương pháp phân lập virút đã được cố gắng thực hiện từ các mẫu mô bào lấy ở 73 vịt nuôi ở Úc nơi mà bệnh dịch tả vịt (DTV) được coi là một bệnh ngoại lai. Không có herpesvirus nhưng reovirus đã được phân lập từ 9 vịt. Đa số các phân lập virút nhận được từ mẫu ruột và tất cả 9 vịt mang reovirus đều khỏe mạnh. Virút được phân lập trên tế bào gan hoặc tế bào thận phôi vịt và tạo bệnh lý tế bào (CPE) và gây chết các tế bào này. Các phân lập này được cấy chuyển vào phôi vịt và CAM có biểu hiện sưng dày lên giống như nốt loét. Các phân lập đều được giám định là reovirus dựa vào hình thái đặc trưng của chúng trên kính hiển vi điện tử. Nghiên cứu đã làm sáng tỏ tầm quan trọng của sự nhận dạng các virút được phân lập từ vịt. Ở những nước đang có bệnh dịch tả vịt, reovirus có thể bị nhận dạng lầm là virút DTV.

### Introduction

THE Australian Centre for International Agricultural Research (ACIAR) provided funding for a project on the control and diagnosis of duck plague in Vietnam. The project involved collaboration between scientists at the National Veterinary Company (NAVETCO) in Vietnam, and the Australian Animal Health Laboratory and The University of Queensland in Australia. One of the objectives of the project was to develop an improved vaccine for the control of duck plague. As part of this objective, virus isolation from ducks in Australia was attempted. The aim was to identify non-pathogenic herpesviruses that might be suitable vaccine candidates for use in Vietnam.

### Materials and methods

#### Ducks

Samples of liver, kidney, spleen, intestines and trachea were collected from 73 ducks from farms in New South Wales, Australia. Of these ducks, 68 were considered healthy and the remaining 5 were suffering from bacterial infections.

#### Virus isolation

The tissue samples were homogenised using sterile sand, phosphate buffered saline (PBS) and antibiotics and a mortar and pestle, to make a 20% suspension. The homogenate was stored for 1 hour at 22°C, then centrifuged at 1000 g for 10 minutes.

Primary duck embryo fibroblast, liver and kidney cell cultures were prepared from 14-, 19-, and 24-day-old duck embryos respectively. The supernatant was inoculated into these 3 types of cell culture and 3 blind passages were performed with a freeze-thaw step between each passage. The cell cultures were observed daily for cytopathic effect (CPE). The sample was discarded if no CPE was observed in the cell cultures. If CPE was observed, the freeze-thawed cell culture material was inoculated onto the chorioallantoic membrane (CAM) of 14-day-old embryonated duck eggs and 3 passages were performed. After 5–7 days incubation, all embryonated eggs were examined for gross lesions.

### Virus identification

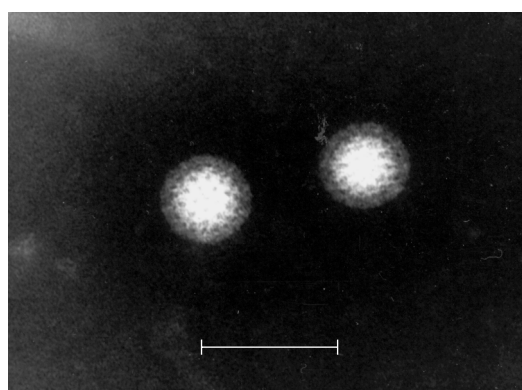
A chloroform sensitivity test according to the method of Feldman and Wang (1961) was performed on virus isolates from cell cultures that showed CPE. Briefly, 50 µL of chloroform was added to 1.0 mL of the CPE-positive cell culture supernatant and vortexed for 10 minutes at room temperature. The mixture was centrifuged for 5 minutes at 500 g and the upper layer removed and inoculated into cell cultures. Cell culture supernatant alone and mixed with PBS were used as controls. The 3 samples were then titrated in cell culture. If the titres of the 3 samples were similar, it was concluded that the virus was non-enveloped.

Electron microscopy (EM) was performed on cell cultures that showed CPE. When CPE was evident, the cell culture medium was removed, centrifuged lightly and the supernatant used directly in negatively stained EM. This procedure was performed at the Animal Research Institute, Yeroongpilly.

### Results

Viruses were isolated from 9 of 73 ducks. All of the 9 ducks were healthy. In cell cultures, a CPE of

syncytium formation and cell rounding followed by cell death was observed. The lesion observed in embryonated duck eggs was a pock-like thickening of the CAM. All viruses were non-enveloped (resistant to chloroform treatment) and 6 were identified as reoviruses by EM (Figure 1). The remaining 3 were also considered to be reoviruses based on their CPE and the lesions on CAM. Most of the viruses were isolated from the intestines and most were isolated in duck embryo kidney cells. However, reoviruses were also isolated from the trachea of one duck and from the spleen, liver and trachea of another duck. No herpesviruses were isolated. Details are shown in Table 1.



**Figure 1.** Electron micrograph of reovirus particles isolated in duck embryo liver cell culture from the spleen of a 3-week-old duck. Negatively stained with 1% phosphotungstate acid. Bar is 100 nm.

### Discussion

Although herpesviruses were not isolated from the ducks in this study, we isolated reoviruses from 9 birds. This demonstrates the importance of confirming the identity of virus isolates. In countries

**Table 1.** The isolation of reoviruses from Australian Pekin ducks.

Age of duck (weeks)	Organ	Cell culture with first appearance of CPE (passage no.)	Virus identified by
3	Spleen, liver, trachea	Duck embryo liver (p3)	EM
1	Intestine	Duck embryo kidney (p2)	EM
5	Intestine	Duck embryo kidney (p2)	EM
5	Intestine	Duck embryo kidney (p2)	EM
5	Intestine	Duck embryo kidney (p2)	EM
5	Intestine	Duck embryo kidney (p2)	EM
5	Intestine	Duck embryo liver (p2)	CPE & pocks on CAM
1	Intestine	Duck embryo liver (p1)	CPE & pocks on CAM
3	Trachea	Duck embryo kidney (p2)	CPE & pocks on CAM

CPE, cytopathic effect; EM, electron microscopy; CAM, chorioallantoic membrane.

where duck plague is common, agents causing CPE in cell cultures or embryonated eggs could easily be misidentified as duck plague virus by inexperienced staff. The confirmatory test used in this study (EM) is not always available in countries where duck plague occurs. Thus, more accessible and affordable tests are required for identification of duck plague virus. During the ACIAR project on duck plague, a number of diagnostic tests were developed for the detection of duck plague virus antigen or nucleic acid (Morrissey et al., 2004). Some of these tests can be performed in laboratories with basic facilities, allowing a rapid and definitive diagnosis of duck plague to be made. This study shows the importance of performing such confirmatory tests.

Reoviruses are ubiquitous in many avian species and, in general, do not appear to be associated with severe disease (McNulty, 1993). All of the reoviruses demonstrated in this study were isolated from apparently healthy ducks. McFerran et al. (1976) isolated a reovirus from the faeces of a healthy mallard duck in Ireland. However, some avian reoviruses have been shown to cause disease in their hosts. Reoviruses are a primary cause of viral arthritis/tenosynovitis in chickens (reviewed by Kibenge and Wilcox, 1983). Reoviruses have been associated with outbreaks of disease in Muscovy ducks (Malkinson et al., 1981; Ziedler et al., 1988). The most common features of the disease were pericarditis, and hepatic and splenic necrosis, with mortality rates of 10–35% reported. Researchers in a number of countries were able to reproduce the disease in Muscovy ducks following inoculation with the reovirus (Malkinson et al., 1981; Marius-Jestin et al., 1988; Ziedler et al., 1988). A reovirus was isolated from a similar disease syndrome in geese in Hungary (Palya et al., 2003) and a reovirus was considered to be involved in disease outbreaks in common eiders (*Somateria mollissima*) in coastal Finland (Hollmen et al., 2002). In both of these reports, the diseases were reproduced in experimental infections with the reovirus. Finally, reoviruses have been shown to play a potentiating role in coccidial and cryptosporidial infections in chickens and quails (Guy et al., 1988; Ruff and Rosenberger, 1985). The pathogenicity of the 9 isolates obtained in this study is unknown. Inoculation trials could be conducted using ducks of various ages to determine if these isolates are capable of causing disease.

## Acknowledgments

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# The occurrence of duck plague in duck flocks scavenging rice fields in Can Tho province and field vaccination trials

Nguyen Duc Hien (1997), M.Sc. Thesis. Can Tho University, Vietnam.

## Abstract

Can Tho is a province on the Mekong delta where farmers produce large numbers of ducks. Infectious disease, especially duck plague, threatens rural livelihoods. During 1997 studies were undertaken in the field, at the Agricultural Breeding Centre of Can Tho, at the Can Tho Veterinary Service and at NAVETCO. The studies aimed to confirm the importance of duck plague in the area and to establish the efficacy of vaccination.

Samples were collected from 76 ducks suspected of having duck plague at 8 different sites in Can Tho. Of these samples 26 (34%) were positive by antigen capture ELISA and 17 (65%) of these yielded isolates of duck plague virus. Duck plague was most prevalent in districts with the highest densities of duck populations. Peak prevalence was in March.

Methods of vaccination were compared. Ducklings were vaccinated twice, at 1 and 21 days of age. They received either 2 intramuscular injections of vaccine, 2 vaccinations by eye drop, or eye drop vaccination followed by intramuscular injection.

ELISA antibody titres, measured 1 and 2 months after the last vaccination were similar in all groups. Protection against artificial challenge 2 months after the last vaccination was greatest (85%) in the group receiving 2 intramuscular injections. Protection in the other 2 groups was 70%. Control ducks produced no antibody and all died on challenge.

In a further experiment, the age at vaccination was varied — at either 1 and 21 days, 7 and 28 days or 14 and 35 days. In all groups the first vaccination was by eye drop and the second by intramuscular injection. ELISA antibodies and protection against challenge were established 2 months after the last vaccination. Antibody titres and protection (95%) were highest in the group receiving the final vaccine at 35 days. Protection levels were 80% and 85% in the other groups.

Statistical analysis indicated that intramuscular vaccination was more effective, and that the optimal response was obtained if the first vaccination was delayed until 14 days of age.

## Bệnh dịch tả vịt ở đàn vịt chạy đồng ở tỉnh Cần Thơ và các thử nghiệm qui trình tiêm chủng vắc xin trong điều kiện sản xuất

Nguyễn Đức Hiền (1997). Luận án Thạc sĩ. Trường Đại học Cần Thơ, Việt nam

### Tóm tắt

Cần Thơ là một tỉnh ở châu thổ sông Mêkông nơi người nông dân nuôi rất nhiều vịt chạy đồng. Bệnh truyền nhiễm, đặc biệt là dịch tả vịt (DTV) thường xuyên đe dọa cuộc sống của người nông dân. Các thí nghiệm được thực hiện trong năm 1996-1997 ở Trung tâm giống Nông nghiệp, Chi cục Thú y tỉnh Cần Thơ và Công ty Thuốc Thú y TW2. Mục đích nghiên cứu nhằm khẳng định tầm quan trọng của bệnh dịch tả vịt trong tỉnh và để xây dựng quy trình tiêm chủng phòng bệnh đạt hiệu quả cao.

Mẫu bệnh phẩm được thu thập từ 76 vịt nghi mắc bệnh dịch tả vịt ở 8 địa điểm khác nhau của tỉnh Cần Thơ. Trong số những mẫu này có 26 mẫu dương tính được phát hiện bằng kỹ thuật Ag-ELISA và 17/26 mẫu phân lập được virút DTV. Dịch tả vịt lưu hành nhiều nhất ở các huyện nuôi vịt chạy đồng với mật độ cao. Bệnh xảy ra nhiều nhất vào tháng Ba.

Các phương pháp tiêm chủng đã được so sánh. Vịt con được tiêm chủng 2 lần vào 1 và 21 ngày tuổi. Vịt con được chủng vắc xin 2 lần bằng phương pháp tiêm bắp thịt hoặc 2 lần nhỏ mắt hoặc lần đầu nhỏ mắt lần hai được tiêm bắp thịt.

Hiệu giá kháng thể ELISA đo vào lúc một tháng và 2 tháng sau khi tiêm chủng vắc xin lần 2 tương tự ở cả 3 nhóm. Tỷ lệ bảo hộ khi công cường độc vào tháng thứ hai sau lần tiêm chủng cuối cùng cao nhất ở nhóm tiêm bắp thịt (85%). Tỷ lệ bảo hộ ở 2 nhóm còn lại là 70%. Nhóm đối chứng không tạo kháng thể và tất cả vịt đều chết khi công cường độc.

Ở một thí nghiệm tiếp theo, tuổi vịt con được tiêm chủng thay đổi từ 1-21 ngày tuổi đến 7-28 và 14-35 ngày tuổi. Tất cả các nhóm vịt thí nghiệm được chủng vắc xin bằng phương pháp nhỏ mắt vào lần đầu và tiêm bắp vào lần thứ hai. Hiệu giá kháng thể ELISA và tỷ lệ bảo hộ khi thử thách cường độc được đánh giá vào tháng thứ 2 sau lần tiêm chủng cuối cùng. Hiệu giá kháng thể và tỷ lệ bảo hộ cao nhất (95%) ở lô vịt được tiêm chủng vắc xin vào 14 và 35 ngày tuổi. Tỷ lệ bảo hộ ở 2 lô khác là 80 và 85%.

Kết quả phân tích thống kê cho thấy phương pháp tiêm bắp thịt có hiệu quả hơn và đáp ứng tối ưu nhận được khi tiêm chủng lần đầu thực hiện vào lúc 14 ngày tuổi.

# Improving capacity to control Newcastle disease and duck plague in village poultry

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From a report prepared for NAVETCO, the University of Queensland and the Australian Agency for International Development (AusAID); December 2001.*

## Executive summary

The present report is part of the project 'Improving capacity to control Newcastle disease and duck plague in village poultry' financed by the Australian Agency for International Development (AusAID) and implemented in collaboration with NAVETCO, a state commercial company that produces veterinary pharmaceuticals in Ho Chi Minh City. During 5 days of field work in Dong Thap Province, in the Mekong Delta area, interviews were held with Women's Unions, Farmers' Associations, veterinary and extension services, local veterinarians as well as female and male farmers. Information was collected in order to describe the current situation with regard to poultry production by small farmers and to contribute to the improvement of extension methodologies and the sustainable usage of the Newcastle disease and duck plague vaccines.

In Dong Thap, there are approximately 2 million chickens and 2 million ducks, most of them (80%) kept by small farmers. Poultry are found everywhere in rural areas and are an integral part of the local farming systems. Each family has an average flock of about 10 chickens and 10 ducks. The daily management of small flocks of poultry is usually the responsibility of women and children. Women are responsible for poultry management because they stay at home most of the time while men go to the field, to fish or to the market. When the birds are sick and die, people often eat them and bury the feathers and other unused parts. Poultry meat and eggs are quite popular in the Mekong Delta and one-third of the household production is used for home consumption. The largest problem in poultry raising is the losses from Newcastle disease and duck plague with a mortality rate up to 100% in village flocks. The Australian Centre for International Agricultural Research (ACIAR) projects implemented by NAVETCO and

the University of Queensland have led to the development of appropriate vaccines to protect both chickens and ducks from these diseases.

Most of the veterinary and extension activity is concentrated on large and medium poultry production — for which there is a program of vaccination and extension material (booklets and leaflets) — and is male dominated. Extension services in each district are limited to one veterinarian who works in coordination with the local veterinarians and local people's organisations (Farmers' Associations and Women's Unions). There are 210 veterinary pharmacies, of which 31 are allowed to sell vaccine because they have cold storage facilities. A wide network of 373 local veterinarians (17 women and 356 men) provides assistance to the farmers in Dong Thap province. Small poultry producers receive veterinary information through the Farmers' Associations and Women's Unions. Regular meetings are carried out by these organisations in communities where leaders deliver information relating to political, social, agricultural and veterinary issues.

Unlike intensive poultry producers, small poultry producers have no access to vaccine. As yet no vaccination program has been established for small poultry producers, and no adequate training or organisation is in place to carry out regular vaccination campaigns. The commercialisation, since 1995, of the thermostable I-2 vaccine by NAVETCO allowed the development of initiatives by local veterinarians and Farmers' Association leaders, to vaccinate small flocks. However, when vaccination is carried out it is not performed on a regular basis. This vaccine, because it is heat tolerant and simple to administer (via eye drop, food or drinking water), can be easily used by small farmers in remote areas.

The current poultry production situation offers a very interesting opportunity to develop, on a pilot basis, a vaccination campaign for small poultry producers in 2 or 3 districts of Dong Thap province. Specific recommendations for small-scale female farmers and appropriate, gender sensitive extension material (eg radio programs) should be developed. The need to target female farmers lies mainly in the fact that women are the main actors in poultry raising and not merely the wives of male farmers. A shift has to be encouraged for researchers, veterinarians and extension agents to recognise that female farmers are farmers in their own right with specific characteristics and needs. Information that is to be delivered

has to be tailored to women's needs. A training manual for vaccinators (local veterinarians, Farmers' Associations and the Women's Union leaders) should be developed to guarantee adequate training and replicability of the project. A vaccinator's manual with a system of monitoring field activities should also be prepared. Adequate evaluation of the pilot project should be carried out with a preliminary baseline survey in a small but representative number of villages. To guarantee that the I-2 vaccine is used with success, the accompanying instruction sheet should be clearer in relation to the dilution of the vaccine and the use of appropriately tested and calibrated eye-droppers.