การศึกษาลักษณะของไวรัสโรคนิวคาสเซิลที่เพิ่มจำนวนได้จากการเพาะเลี้ยงเซลล์ LLC MK2

รติยา คูเขตพิทักษ์วงศ์[1*], ปรีชา หอมจำปา[2], จารุณี สาตรา[3], ฐิตวัฒน์ จันทวร[3], สาริสา ตระการรังสี[3],
จริยา หาญวจนวงศ์[4], วิชัย ลีลาวัชรมาศ[5], วัชรี คุณกิตติ[1]

บทคัดย่อ

    บทนำ: ไวรัสโรคนิวคาสเซิลสายพันธุ์ลาโซต้านิยมใช้เป็นสายพันธุ์วัคซีนสำหรับวัคซีนเชื้อเป็น ซึ่งมักได้มาจาก
การเตรียมในไข่ไก่ฟักปลอดเชื้อจำเพาะ การเตรียมไวรัสจากเซลล์เพาะเลี้ยงก็เป็นทางเลือกหนึ่งในการผลิตวัคซีน วัตถุประสงค์
ของการศึกษานี้เพื่อที่จะตรวจสอบการเปลี่ยนแปลงของไวรัสโรคนิวคาสเซิลที่ได้จากการเพาะเลี้ยงเซลล์ LLC MK2 **วิธีการ:**
ใช้ปฏิกิริยาลูกโซ่พอลเมอเรสแบบย้อนกลับ (RT-PCR) ในการเตรียมยีนฟิวชันของไวรัส และศึกษาการเพิ่มจำนวนของเซลล์
ม้ามและการตอบสนองของแอนติบอดี้จำเพาะ ในหนูถีบจักร BALB/c ที่ได้รับภูมิคุ้มกัน **ผลการวิจัยและอภิปราย:** ยีนฟิวชัน
ของไวรัสที่ได้จากการเพาะเลี้ยงเซลล์ยังคงเป็นของสายพันธุ์ที่ไม่ก่อโรค พบการกลายพันธุ์แบบเป็นจุด 2 แห่งบนสาย DNA
ของไวรัสโดยไม่มีการเปลี่ยนแปลงชนิดของกรดอะมิโน ไวรัสสามารถกระตุ้นการเพิ่มจำนวนของเซลล์ม้ามและเหนี่ยวนำให้
ระดับ IgG2a และ IgG2b ในซีรั่มเพิ่มขึ้นเหมือนกับที่พบในไวรัสจากไข่ไก่ฟัก **สรุป:** ไวรัสที่ได้จากการเพาะเลี้ยงเซลล์ LLC
MK2 สามารถใช้เป็นแอนติเจนสำหรับวัคซีนเชื้อเป็น

คำสำคัญ: ไวรัสโรคนิวคาสเซิล, ไม่รุนแรง, โปรตีนฟิวชั่น, การเพาะเลี้ยงเซลล์, ภูมิคุ้มกัน

Abstract

Characterization of Newcastle Disease Virus Propagated from LLC MK2 Cell Culture

Ratiya Kukhetpitakwong[1*], Preecha Homchampa[2], Jarunee Satra[3], Thitawat Chantaworn[3], Sarisa Trakarnrungsee[3],
Chariya Hahnvajanawong[4], Vichai Leelavacharamas[5], Watcharee Khunkitti[1]

IJPS, 2012; 8(3) : 91-99

    Introduction: Newcastle disease virus (NDV) strain LaSota is commonly used as a vaccine strain for
live vaccines. The virus is commonly cultivated from specific pathogen free embryonated eggs. Propagation of

[1]   Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002 THAILAND

[2]   Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002 THAILAND

[3]   Veterinary Biologics Assay Center, Bureau of Veterinary Biologics, Department of Livestock Development, Ministry of Agriculture
and Cooperatives, Pakchong, Nakornratchasima 30310 THAILAND

[4]   Faculty of Medicine, Khon Kaen University, Khon Kaen 40002 THAILAND

[5]   Faculty of Technology, Khon Kaen University, Khon Kaen 40002 THAILAND

*   Corresponding author: e-mail: ratkom@kku.ac.th

the virus in cell culture is an alternative for vaccine production. The objective of this study was to investigate the differentiation of NDV propagated from LLC MK2 cell culture. **Methods:** Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to prepare the fusion (F) gene of the adapted virus. Splenocyte proliferation and specific antibody response in immunized BALB/c mice were studied. **Results and Discussion:** The F gene of the adapted virus was still of lentogenic strain. Two point mutations on the DNA sequences of the virus were observed without change in amino acids. The virus could stimulate splenocyte proliferation and induce increase of serum IgG2a and IgG2b levels similar to those of the NDV from embryonated eggs. **Conclusion:** The adapted virus from LLC MK2 cells could be used as antigen for live vaccine.

Keywords: Newcastle disease virus, avirulent, fusion protein, cell culture, immune

Introduction

Newcastle disease is an endemic disease in birds and poultry. It is a highly contagious and fatal viral disease affecting domestic poultry and wild birds, characterized by gastro-intestinal, respiratory and nervous signs. Within a flock, transmission is caused by inhalation of Newcastle disease virus in the air and ingestion of water or feed contaminated with secretion of infected birds (OIE, 2009).

Newcastle disease virus (NDV) is an avian paramyxovirus classified as a member of the genus Avulavirus belonging to the family *Paramyxoviridae.* NDV is an enveloped virus with a single stranded, non-segmented RNA genome (15,186 nucleotides long) which encodes six proteins including nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) (Park *et al.,* 2003). Additional proteins (designated V and W protein) may be produced by an RNA-editing event that occurs during transcription of the P gene (Steward *et al.,* 1993; Leeuw and Peeters, 1999). The virulence of NDV strains is related to the cleavability of the F protein. The F protein is produced as a precursor (F0) which could be proteolytically cleaved to obtain two disulfide-linked polypeptides, F_1 and F_2 (Nagai *et al.,* 1976; Tiwari *et al.,* 2004). The F protein of virulent strains is cleaved into by host proteases found in most tissues, whereas the F protein of avirulent strains

can be cleaved only in cells containing trypsin-like enzymes (Kant *et al.,* 1997). NDV strains are divided into five pathotypes on the basis of clinical signs: subclinical enteric, lentogenic or respiratory, mesogenic, neuro-tropic velogenic and viscerotropic velogenic categories (OIE, 2009). Avirulent strains of NDV are commonly used as vaccine strains especially for live vaccine to protect poultry against the disease. The LLC MK2 cell line has been used for cultivation, adaptation and attenuation of parainfluenza viruses, which belongs to the Paramyxoviridae family (Henrickson, 2003). They have been used in a NDV plaque assay (Ishida *et al.,* 1985; Kournikakis and Fildes, 1988). Therefore in this present work, LLC MK2 cells were considered for propagation of NDV strain LaSota from the parent vaccine virus.

The virulence of NDV had to be characterized by determining intracerebral pathogenicity index (ICPI), the mean death time of chicken embryo (MDT) or the intravenous pathogenicity index (IVPI) (Mishra *et al.,* 2001; Orsi *et al.,* 2009). However, differentiation of pathotypes of the virus strains is required. The present study was conducted in order to investigate NDV strain LaSota propagated in LLC MK2 cells.

Methods

1.   Adaptation of Newcastle disease virus

Rhesus monkey kidney epithelial (LLC MK2) cell (ATCC®

No.CCL-7) was kindly provided by the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand. Newcastle disease virus (NDV) strain LaSota from lyophilized live vaccine (Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand) with an infectivity of 10^8 mean egg infectious doses (EID_{50}) was used as NDV inoculums. LLC MK2 cells were grown in culture medium in 25 cm^2 TC flasks and incubated at $37^{\circ}C$ for 48 hr to prepare a confluent monolayer. At this stage, 0.5 mL of NDV inoculum was added to the center of monolayer and spread over the surface of cells by tilting the flask. The inoculated flasks were placed at $37^{\circ}C$ for 1 hr. Maintenance medium (4.5 mL/flask) was added and the flasks were then incubated at $37^{\circ}C$ for 5 days. The virus was harvested and freezed-thawed three times before separation by centrifugation at 2000 rpm for 10 min. Harvested virus was infected again to LLC MK2 cells using same media and techniques. Virus in each passage was kept at $-20^{\circ}C$.

Culture medium consisted of Eagle's minimum essential medium with Hank's salts supplemented (Gibco®, Invitrogen™) with 10%v/v heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate.

Maintenance medium was modified from over-lay medium for plaque assay (Kournikakis and Fildes, 1988). The medium was composed of 25%v/v Medium 199 with Hank's salts (Gibco®, Invitrogen™), 2.5 μg/mL Trypsin-TPCK, 0.225%w/v sodium bicarbonate, 0.25μg/mL L-glutamine, 3%v/v MEM vitamin (100x), 0.1%w/v glucose, 0.64%w/v sodium chloride, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate.

2. Investigation of Newcastle disease virus

2.1 Haemagglutination (HA) test

Red blood cells (RBC), obtained by bleeding the wing vein of unvaccinated chicken and kept in Alsevier's solution, were kindly provided by Upper Northeastern Veterinary Research and Development Center, Department of Livestock Development, Ministry of Agriculture and Cooperatives, Khon Kaen, Thailand. The RBC suspension was washed with phosphate buffered saline (pH 7.4) three times using centrifuga-tion technique. The stock RBC suspension (10%) was achieved by a haemacytometer count ($8x10^8$ cells/mL) (Hierholzer and Killington, 1996). To perform the HA test, serial two-fold dilutions of virus suspension were made in equal volume of PBS (50 μL). Then, 50 μL of 0.5% chicken RBCs were dispensed to each well and mixed gently. The RBCs were allowed to settle for about 40 min at room temperature. The agglutination was assessed by tilting the plates and observing the presence or absence of tear-shape streaming of the RBCs. The HA titer of virus suspension is defined as being the reciprocal of the highest dilution which shows complete agglutination (no streaming) and is expressed as the number of HA units (HAU).

2.2 Preparation of viral genome

RNA were extracted from NDV in each passage and from live vaccine by using viral RNA/DNA kits (Purelink®, Invitrogen™), as described by the manufacturer. Briefly, 0.2 mL of virus suspension were mixed with 250 μL of a mixture (proteinase K and carrier RNA in lysis buffer; 1:8, v/v). The reaction mixture was incubated at $56^{\circ}C$ for 15 min. Lysate was precipitated with 250 μL of absolute alcohol and then purified using a viral spin column. Purified RNA was finally eluted with 50 μL of RNase-free water.

Reverse transcription reaction was then performed to synthesize cDNA by using a first-strand synthesis kit (SuperScript® III, Invitrogen). Briefly, RNA (5 μL) was incubated with 1 μL of random hexamer primer (50 ng/μL), 1 μL of 10 mM dNTP mix, 3 μL of DEPC-treated water at $65^{\circ}C$ for 5 min. The mixture was placed on ice for 1 min before adding 10 μL of cDNA synthesis mix. Then, the reaction was carried out at 25°C for 10 min followed by incubation at 50°C for 50 min. The enzyme was heat inactivated at 85°C for 5 min.

DNA templates were incubated with 1 μL of RNase H at 37°C for 20 min before storage at -20°C.

Polymerase chain reaction was performed to amplify the cDNA templates. Two pairs of primers were used to amplify a 255 bp sequence (Tiwari *et al.,* 2004). The amplification was carried out in a 50 μL reaction volume containing 5 μL of cDNA, 5 μL of 10X PCR buffer, 2 μL of each 10 μM primer combination (A+B or A+C; A-5$'$-TTGATGGCAGGCCTCTTGC-3$'$, nucleotide position 141-159; B-5$'$-AGCGT(C/T)TCTGTCTCCT-3$'$, nucleotide position 395–380; C-5$'$-G(A/G)CG(A/T)CCCTGT(C/T) TCCC-3$'$, nucleotide position 395-380, Invitrogen™), 1 μL of 10 mM dNTPs mix, 1.5 μL of 50 mM MgCl2 and 0.2 μL of Taq DNA polymerase (Platinum®, Invitrogen™). The reaction was carried out at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, annealed at 55°C for 30 sec and extended at 72°C for 1 min before final extension at 72°C for 5 min by using thermal cycler (Applied Biosystems, version 2.08). The reaction mixtures were then cooled down to 4°C before storage at -20°C. The amplicons of F gene were sequenced by First BASE Laboratories Sdn Bhd, Malaysia.

2.3 Restriction enzyme analysis

The PCR products were verified by digestion with StyI restriction enzyme (New England Biolabs). Briefly, 5 μL of PCR products were added into 1 μL of StyI, 2 μL of 10X NEbuffer3, 2 μL of 10x purified BSA and 10 μL of water. The reaction mixtures were incubated at 37°C for 16 hr before heat inactivation at 65°C for 20 min.

2.4 Agarose gel electrophoresis

PCR products were analyzed by electrophoresis in 2% agarose gel in Tris-Borate-EDTA (TBE) buffer, pH 8.0. 50bp DNA ladder (Invitrogen™) was used as marker. Gels were then stained with SYBR® Gold dye (Invitrogen™) and photographed using gel documentation system (Syngene®, Synoptics Ltd, England).

3.   Immunization of Mice

Inbred female BALB/c mice, 6-8 weeks old, were purchased from the National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand. The animals were housed under standard conditions at 25 ± 2°C and were provided with chow pellets and tap water ad libitum. The procedures applied to the mice were approved by the Animal Ethics Committee of Khon Kaen University, in accordance with the requirements of the Ethics of Animal Experimentation of the National Research Council of Thailand (AEKKU008/04).

Each group of five mice was injected subcutaneously at the base of the neck with either 0.1 mL of virus suspension or control. A group of mice injected with phosphate-buffered saline (PBS) was used as a negative control. Immunizations were performed twice at 10-day intervals and mice were euthanized 10 days or 20 days after the last immunization (Estrada *et al.,* 2000). Serum collected from animals on days 10, 20 and 30 were used for measurement of anti-specific antigen IgG, IgG1, IgG2a, IgG2b and IgM by ELISA. Spleens were collected from immunized mice on day 30 for proliferation assay.

3.1 Mitogen- and specific antigen-stimulated splenocyte proliferation

Splenocyte suspensions were prepared at a concentration of 1×10^{6} cells/mL (for mitogen stimulation) and at the concentration of 2×10^{6} cells/mL (for antigen stimulation) in complete medium. The complete medium was composed of RPMI medium 1640 (Gibco®, Invitrogen™) supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. Fifty μL of splenocyte suspensions were mixed with either 50 μL of concanavalin A (Con A) or pokeweed mitogen (PWN) to make the final concentration of 1 μg/mL. The specific antigen, NDV was then added to make the final concentration of 1×10^{8} EID_{50}/mL. Specific antigen- and Con A-stimulated cultures were incubated at 37°C for 72 hr whereas PMW-stimulated

cultures were incubated at the same temperature for 96 hr. The cell proliferation ELISA, Bromo-2-deoxyuridine (BrdU) colorimetric kit (Roche Diagnostics, Germany) was used (Porstmann *et al.,* 1985). After incubation, 10 μL of BrdU labeling reagent were added into each culture. The cultures were re-incubated for further 24 hr before centrifugation at 2,000 rpm at 4°C for 15 min. Splenocytes in the cultures were fixed and denatured as described by the manufacturer. Mouse monoclonal antibodies conjugated with peroxidase (0.1 mL of anti-BrdU) were added and incubated at 37°C for 90 min before washing. Substrate solution (0.1 mL) was added and allowed to stand at room temperature for 30 min. The reaction was stopped by adding 25 μL of 1M H$_2$SO$_4$. The absorbance (Abs) at 450 nm was measured using ELISA reader (Bio-Rad$^®$, BioRad Laboratories, USA). Stimulation index (SI) was calculated as the following equation;

$$SI = \frac{(Abs_{Antigen-or\ mitogen-stimulated\ cultures} - Abs_{Background})}{(Abs_{Nonstimulated\ culture} - Abs_{Blank})}$$

3.2 Measurement of antigen-specific antibody

Antigen-specific IgG, IgG1, IgG2a, IgG2b and IgM antibodies in sera were determined according to the method previously described by Sjolander *et al.* (1997) with some modifications. Briefly, 96-well immunoplates (Maxisorp^{TM}, Nunc A/S, Denmark) were coated with 0.1 mL of antigen in 50 mM carbonate-bicarbonate buffer, pH 9.6 at 4°C for 24 hr and then blocked with 0.2 mL of Tris-buffered saline, pH 7.4 (TBS) containing 10 mg/mL of bovine serum albumin (BSA) at 37°C for 2 hr. The plates were washed with TBS containing 0.05% Tween$^®$ 20 before adding 0.1 mL of diluted serum samples (IgG, 1:1000; IgG1, 1:1000; IgG2a, 1:50 or IgG2b 1:50) or TBS/BSA as a control. The plates were then incubated at 37°C for 2 hr, followed by washing. An aliquot (0.1 mL) of horseradish peroxidase conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgM (Southern

Biotechnologies) diluted 1:40000 in TBS containing 1 mg/mL of BSA was added. The mixtures were incubated at 37°C for 1 hr. After washing, 0.1 mL of 3,3$'$,5,5$'$-tetramethylbenzidine liquid substrate (Sigma$^®$) was added. The reaction mixture was incubated at room temperature for 15 min and the reaction was terminated by adding 0.1 mL of 1N H$_2$SO$_4$. The absorbance at 450 nm was measured using the ELISA reader. Anti-specific antigen antibody titers were reported as the mean absorbance of the tested sera (after subtraction of the mean absorbance of the control) multiplied by the dilution factor of the serum.

Results

Adaptation of Newcastle disease virus on LLC MK2 cells

Lentogenic NDV from the parent vaccine virus was serially passed 7 times in a LLC MK2 cell culture. During the first passage, NDV did not produce clear evidence of cytopathic effect except that LLC MK2 cells were detached from the surface and rounded at day 2 post-infection. Syncytium formation was observed in the fifth passage (data not shown). It was found that virus titer of NDV increased gradually from each passage. NDV obtained from the first passage (P1 virus) had the titer of 2^1 HAU and P2 virus had the titer of 2^2 HAU. The maximum titer of 2^5 HAU was observed in P5 virus to P7 virus.

Characterization of Newcastle disease virus

RT-PCR was applied on all passages of NDV from LLC MK2 cell culture using the degenerate primers designed from F gene encoding for the cleavage site as the method of Tiwari *et al.* (2004). In Figure 1, DNA bands with 255 bp (lane 1-7) could be amplified only from primer A+C. The amplified DNA could be cleaved by StyI to be 118 bp and 72 bp as DNA from NDV from the live vaccine (lane 8). Thus, DNA from each passage was sequenced in comparison with the parent vaccine virus.
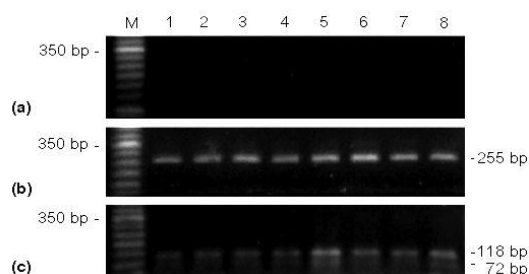
Figure 1 Agarose gel electrophoresis of PCR products generated from Newcastle disease virus strain LaSota amplified by (a) primer combination A+B, (b) primer combination A+C, (c) primer combination A+C and then digested with StyI. **Lane M,** 0.5 μg of 50bp DNA ladder; **Lane 1-7,** PCR products of NDV adapted in LLC MK2 cells passage no. 1-7, respectively; **Lane 8,** PCR products of NDV from live vaccine.

To identify the precise mutation of NDV propagated from LLC MK2 cells, DNA F genes were sequenced as shown in Figure 2. Sequence analysis of the F gene demonstrated that there was a point mutation of DNA from the adapted NDV which was different from the parent vaccine virus. However, the presence of a T at position 373 in the DNA from the adapted NDV was similar to the DNA from NDV strain LaSota (GenBank: AF077761.1). When compared with the reference NDV (GenBank: AF077761.1), a nucleotide change observed in DNA sequences from both the adapted NDV and the parent vaccine virus was G-to-A change at position 391.

(a) NDV strain LaSota (GenBank: AF077761.1)

TT GAT GGC AGG CCT CTT GCA GCT GCA GGA ATT GTG GTT ACA GGA GAC AAA GCC GTC AAC ATA TAC ACC TCA TCC CAG ACA GGA TCA ATC ATA GTT AAG CTC CTC CCG AAT CTG CCC AAG GAT AAG GAG GCA TGT GCG AAA GCC CCC TTG GAT GCA TAC AAC AGG ACA TTG ACC ACT TTG CTC ACC CCC CTT GGT GAC TCT ATC CGT AGG ATA CAA GAG TCT GTG ACT ACA TCT GGA GGG GGG AGA CAG GGG CGC C

(b) NDV strain LaSota from live vaccine

TT GAT GGC AGG CCT CTT GCA GCT GCA GGA ATT GTG GTT ACA GGA GAC AAA GCA GTC AAC ATA TAC ACC TCA TCC CAG ACA GGA TCA ATC ATA GTT AAG CTC CTC CCG AAT CTG CCC AAG GAT AAG GAG GCA TGT GCG AAA GCC CCC TTG GAT GCA TAC AAC AGG ACA TTG ACC ACT TTG CTC ACC CCC CTT GGT GAC TCT ATC CGT AGG ATA CAA GAG TCT GTG ACT ACA TC**G** GGA GGG GGG AGA CAG GG**A** CGC C

(c) NDV adapted from LLC-MK2 cell culture

TT GAT GGC AGG CCT CTT GCA GCT GCA GGA ATT GTG GTT ACA GGA GAC AAA GCA GTC AAC ATA TAC ACC TCA TCC CAG ACA GGA TCA ATC ATA GTT AAG CTC CTC CCG AAT CTG CCC AAG GAT AAG GAG GCA TGT GCG AAA GCC CCC TTG GAT GCA TAC AAC AGG ACA TTG ACC ACT TTG CTC ACC CCC CTT GGT GAC TCT ATC CGT AGG ATA CAA GAG TCT GTG ACT ACA TC**T** GGA GGG GGG AGA CAG GG**A** CGC C

Figure 2 Nucleotide sequences of F gene obtained by NDV strain LaSota (a) GenBank: AF077761.1; (b) RT-PCR product of NDV from live vaccine; (c) RT-PCR products of the adapted NDV. The sequences are presented as cDNA in the $5'$–$3'$ direction.

Effect of Newcastle disease virus on the immune system

NDV from live vaccine (titer of 10^8 EID$_{50}$/mL) and the third passage of NDV adapted in LLC MK2 cell culture (NDP) (titer of $10^{3.1}$ EID50/mL) were used as antigen. Effect of the antigens on mitogen- and NDV-stimulated splenocyte proliferation in immunized mice after 10 days of the second immunization is shown in Figure 3. It was found that the splenocyte stimulation index of NDP significantly increased after exposed to PWM ($p<0.05$). However, all treatment groups except the negative control group (PBS) showed significant increase in splenocyte proliferation after exposure to NDV and there was no significant differences of splenocyte stimulation index among them.
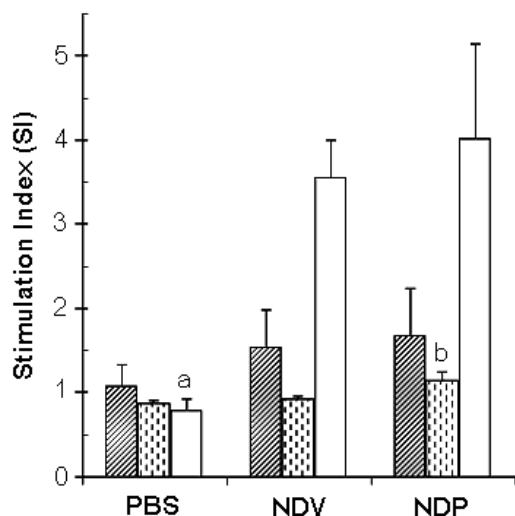
Figure 3 Effect of antigen on mitogen- and NDV-stimulated splenocyte proliferation ex vivo. Mice were immunized subcutaneously with phosphate-buffered saline (PBS), pH 7.4, NDV obtained from live vaccine (NDV) or adapted from LLC MK2 cell culture (NDP) on day 0 and day 10. Splenocytes were prepared 20 days after the last immunization and cultured with (▨) Con A, (▣) PWM, or (▢) NDV. Splenocyte proliferation was measured by BrdU ELISA. Stimulation index are presented as mean+S.E. (n=5). Significant differences with NDV groups were designed [a] (p<0.05) and [b] (p<0.001) analyzed by t-test.

Ability of antigens to induce antibody response was investigated in 10-day interval immunized mice. Adapted virus on passage No.5 from LLC MK2 cell culture (NDP) was studied in comparison with virus obtained from live vaccine (NDV). In Figure 4, the serum IgM and IgG1 levels in NDP-immunized mice on day 10 were significantly higher than those of NDV-immunized mice whereas the serum IgG2a levels in the former was significantly less than those in the latter on days 10 and 20 (p<0.05). There were no significant differences between the serum IgG2b levels in both groups.
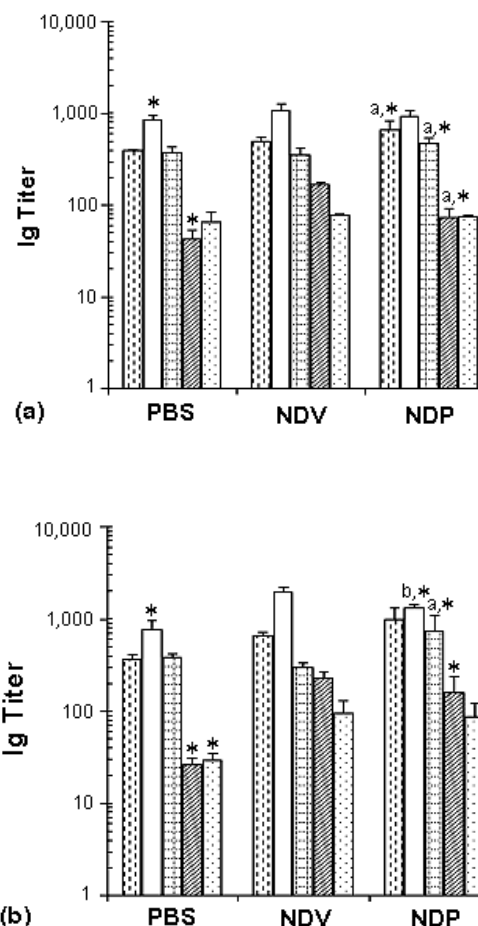

Figure 4 Effect of antigen on NDV-specific antibody responses. Mice were immunized subcutaneously on day 0 and day 10 with PBS, NDV or NDV adapted in LLC MK2 cell culture (NDP). NDV-specific (▣) IgM, (▢) IgG, (▤) IgG1, (▨) IgG2a, and (▢) IgG2b antibodies in sera were measured by an ELISA 10-day after (a) the first immunization, and 10-day after (b) the second immunization. The values are presented as mean+S.D. (n=4). Significant differences from the NDV-treated group are designated as a (p<0.05) and b (p<0.001) analyzed by t-test; those with the NDV-treated group in each antibody subclass were designated as * (p<0.001) tested by one-way ANOVA using pairwise comparison with LSD procedure.

Characterization of Newcastle Disease Virus Propagated
from LLC MK2 Cell Culture
Kukhetpitakwong R.*et al*.

IJPS

Vol. 8 No. 3 September-December 2012

Discussion and Conclusion

NDV in all passages were lentogenic strain because the F gene of the virus could be amplified by primer A+C and cleaved by StyI. Although two point mutations were observed in the F gene from the adapted NDV, amino acids translated from both codons were serine and glycine as found in the parent vaccine virus and the reference NDV (GenBank: AF077761.1).

The potential of the adapted NDV to induce splenocyte proliferation and immune responses in mice was similar to mice immunized with the parent vaccine virus although the parent virus is prepared from specific pathogen free embryonated eggs. It is generally known that Con A stimulates T cells and PWM stimulates T cell-dependent B cell proliferation (Manosroi *et al.,* 2003; Meydani and Ha, 2000). When splenocytes separated from immunized mice were exposed to either Con A, PWM or NDV, it is possible that lymphocytes recognize and receive second signals and the adapted NDV could stimulate NDV-specific lymphocytes to proliferate and differentiate into effector cells and memory cells (Abbas and Lichtman, 2004). In addition, NDV from both sources exhibited high production of IgG1, IgG2a and IgG2b in immunized mice. IgG1 is associated with Th2-like response, while a Th1 response is associated with the induction of IgG2a, IgG2b and IgG3 antibodies (German *et al.,* 1995). These results suggested that the adapted NDV could be used as antigen against Newcastle disease.

Acknowledgements

References

Abbas AK, Lichtman AH. Basic Immunology; Functions and disorders of the immune system. 2nd ed. Elsevier: Philadelphia, 2004.

Estrada A, Katselis GS, Laarveld B, Barl B. Isolation and evaluation of immunological adjuvant activities of saponins from Polygala senega L. Comp Immunol Microbiol Infect Dis 2000; 23(1): 27-43.

German TM, Bongartz H, Dlugonska H. *et al.* Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. *Eur J Immunol* 1995; 25: 823-829.

Henrickson KJ. Parainfluenza viruses. *Clin Microbiol Rev* 2003; 16(2): 242-264.

Hierholzer JC., Killington RA. Virus isolation and quantitation. In Mahy BWJ, Kangro HO. editors. Virology methods manual. London: Academic Press; 1996. 205-255.

Ishida M, Nerome K, Matsumoto M, Mikami T, Oya A. Characterization of different tissue cultures for isolation and quantitation of influenza and parafluenza viruses. *J Clin Microbiol* 1985; 10: 32-36.

Kant A, Koch G, van Roozelaar F, Balk F, Huurne AT. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. *Avian Pathol* 1997; 26: 837-849.

Kournikakis B, Fildes J. Titration of avirulent Newcastle disease virus by the plaque assay method. *J Virol Methods* 1988; 20: 285-293.

Leeuw O, Peeters B. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. *J Gen Virol* 1999; 80: 131-136.

Manosroi A, Saraphanchotiwitthaya A, Manosroi J. Immunomodulatory activities of Clausena excavate Burm. F. wood extracts. *J Ethnopharmacol* 2003; 89(1):155-160.

Meydani SN, Ha W. Immunologic effect of yogurt. *Am J Clin Nutr* 2000; 71(4): 861-872.

Mishra S, Kataria JM, Sah RL, Verma KC, Mishra JP. Studies on the pathogenicity of Newcastle disease virus isolates in guinea fowl. *Trop Anim Health Prod* 2001; 33: 313-320.

Nagai Y, Klenk H-D, Rott R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virol* 1976; 72(2): 494-508.

OIE-World Organisation for Animal Health. Newcastle disease [Online]. 2009 Oct [cited 2011 Sept 14]. Available from http://www.oie.int/fileadmin/ Home/ eng/Animal_Health_in_the_World/docs/ pdf/ NEWCASTLE_DISEASE__FINAL.pdf.

Orsi MA, Doretto Júnior L, Reischak D, *et al.* Newcastle disease virus vaccine strains: immunogenicity is not incluenced by ICPI. *Braz J Poultry Sci* 2009; 11(2): 129-133.

Park M-S, García-Sastre A, Cros JF, Basler CF, Palese P. Newcastle disease virus V protein is a determinant of host range restriction. *J Virol* 2003; 77(17): 9522-9532.

Porstmann T, Ternynck T, Avrameas S. Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: an enzyme immunoassay for the assessment of the lymphoid cell proliferative response. *J Immunol Methods* 1985; 82(1): 169-179.

Sjolander A, van't Land B, Lovgren Bengtsson K. ISCOMc containing purified Quillaja saponins upregulate both Th1-like and Th2-like immune response. *Cell Immunol* 1997; 177(1): 69-76.

Steward M, Vipond IB, Millar NS, Emmerson PT. RNA editing in Newcastle disease virus. J Gen Virol 1993; 74: 2539-2547.

Tiwari AK, Kataria RS, Nanthakumar T, Dash BB, Desai G. Differential detection of Newcastle disease virus strains by degenerate primers based RT-PCR. Comp Immunol Microbiol Infect Dis 2004; 27(3): 163-169.