Rapid Detection of Newcastle Disease virus by LAMP

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Newcastle Disease (ND)

- ND is caused by Newcastle disease virus (NDV).
- A disease with high infectivity.
- Mortality rate may be extremely high depending on the virus strain responsible.
- Practically, all avian species can be affected.
Clinical symptoms of ND (1)

Respiratory symptoms such as oral breathing

Nervous signs such as torticollis and paralysis

Sudden death in 1 to 3 days after onset
Clinical symptoms of ND (2)

- Sudden death with few clinical signs
- Respiratory symptoms
- Nervous signs
- Loss of appetite, depression, and lethargy
- Dark greenish diarrhea
- Decreased egg production
Gross lesions

Hyperemias and hemorrhages in the trachea

Hemorrhages and ulcer in the duodenum mucosa membrane
Newcastle disease virus (NDV)

• also called as avian paramyxovirus type I.
• belongs to Mononegavirales, Paramyxoviridae, Paramyxovirinae, Genus Avulavirus.
• possess hemagglutininins (HA).
• is serologically monovalent.
Newcastle disease virus (NDV)
Newcastle disease virus (NDV)

- NDV strains can be classified into 3 pathotypes
  - Velogenic strains
  - Mesogenic strains
  - Lentogenic strains (vaccine)
- Wild or free-living birds (waterfowls) play a role in the spread of NDV
- Several outbreaks in many countries
Transmission of NDV from wild birds

Human (Conjunctivitis)

Waterfowls

Low virulent

highly virulent

Passage in chickens

Increased virulence
The NDV genome (1)

15 kb of the viral genome encoding 7-9 viral proteins

Lentogenic  G -- K/R -- Q -- G/E -- R -- L
Mesogenic    R --  R --  Q --  K/R --  R --  F
Verogenic   112  117
The NDV genome (2)

Can be digested by a few kinds of proteases

Replicate only in the trachea or lung

Lentogenic  G -- K/R -- Q -- G/S -- R -- L
Mesogenic
Verogenic  R -- R -- Q -- K/R -- R -- F

Can be digested by many kinds of proteases

Replicate in many organs
8 genotypes of NDV strains can be isolated from the field
Diagnosis

- Viurs isolation using embryonated eggs or tissue cultures (chicken kidney cells)
- HA test and HI test using the NDV-specific antiserum
- Pathogenicity test
  - 10-day-old embryo
  - 1-day-old chick
- Detection of the NDV genome
  - or nucleotide sequencing of the F gene
Detection methods of the NDV genome

- RT-PCR, RT-nested PCR
  (conventional, NP, F, HA, M)
- Real-time PCR
  (F, differentiation of genotypes)
- Multiplex PCR
  (NP, F, differentiation of genotypes)
- LAMP
LAMP for the viral genomes

- Easy to perform
- Very sensitive and specific
- Only water bath or heat block necessary
- Reverse transcription is required to detect RNA viruses
- Primers can be designed from the conserved regions
### DNA viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Target gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td></td>
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</tr>
</tbody>
</table>
# LAMP for the detection of viral genomes (2)

## RNA viruses

<table>
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<tr>
<th>Virus</th>
<th>Target gene</th>
<th>reference</th>
</tr>
</thead>
</table>
Reverse transcription
(1) BIP anneals to the template RNA, and cDNA is synthesized by reverse transcriptase.

(2) B3 primer anneals to the region outside of the BIP, and a new cDNA is being synthesized, while concurrently releasing the cDNA strand previously formed by the BIP.

(3) The single stranded cDNA synthesized from BIP is released. The FIP then anneals to this single stranded cDNA.

Steps for starting structure formation
(4) With strand displacement activity, the 3’ end of F2 region in FIP becomes the starting point to synthesize complementary DNA strand.

(5) F3 primer anneals to the region outside of FIP, and its 3’ end becomes the starting point to synthesize while concurrently releasing the DNA strand previously formed by FIP.

(6) The DNA strand synthesized by F3 primer together with the template DNA strand forms a double stranded DNA.

(7) Since the FIP linked DNA strand, released (5), contains complementary sequences at both ends, it self-anneals and forms a dumbbell-like structure. This structure becomes the starting structure of the LAMP cycling amplification.
LAMP for the NDV genome

- Detection of all pathotypes of NDV strains
- Reverse transcription using random hexamers
- 4 primers targeting the conserved region in the F gene (F1)
RT-LAMP of the NDV genome (1)

**Samples**

- Virus isolation

  - samples from infected chickens
  - organs (100mg)
  - (100µl of allantoic fluid)

**RNA extraction from samples**

- TRIZOL reagent
RT-LAMP of the NDV genome (2)

Reverse transcription (RAV2 42°C for 1 hour)

Water bath

Heat block

LAMP (65°C C for 2 hour)

(Electrophoresis)

(80°C C for 10 min)
NDV-specific primers used for NDV

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2-S</td>
<td>5’-TTATCGGCAGTGTAGCTCTTT-3’</td>
</tr>
<tr>
<td>F2-AS</td>
<td>5’-TCAGTTAGGTACAAAGTTGGGAG-3’</td>
</tr>
<tr>
<td>F1-loop-S</td>
<td>5’-TCCTTAAGCCGGAGGATGTTGGTTTTTTT-GCAACAGCTGACAGATAACA-3’</td>
</tr>
<tr>
<td>F1-loop-AS</td>
<td>5’-ACTGACGGATTATCACAAGCTTTTTT-GGTCCATTAACAAACTGCTGCA-3’</td>
</tr>
</tbody>
</table>

Primers were designed using a software called “PrimerExplorer V3” (http://primerexplorer.jp/elamp3.0.0/index.html).
Locations of LAMP primers for NDV

- **F2-S**: 362-382
- **F1-loop-S**: 387-408
- **F2**: 1-1,662
- **F1**: 445-467, 505-527, 545-565
- **F1-loop-AS**: 627-648
- **F1-loop-S**: 505-527

## Comparison of the specificity of LAMP and nested PCR

<table>
<thead>
<tr>
<th>NDV strains</th>
<th>virulence</th>
<th>LAMP</th>
<th>Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDV/chicken/Japan/Sato/30</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Komarov/40</td>
<td>M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Hitchner B1/48</td>
<td>L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/Miyadera/51</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/Ishii/62</td>
<td>L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/sparrow-hawks/Japan/Taka/73</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/Chiba/81</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/pheasant/Japan/Gunma/85</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/pigeon/Japan/ Niigata/88</td>
<td>M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Kagoshima/91</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/Tokyo/96</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/parakeet/Japan/ Chiba/97</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/ Chiba-222/99</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/chicken/Japan/Ibaraki/2000</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/pigeon/Japan/Kumamoto/2000</td>
<td>M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDV/quail/Japan/ Chiba/2001</td>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APMV2/chicken/California/Yucaipa/56</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APMV3/turkey/Wisconsin/68</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APMV3/dove/Tennessee/75</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Sensitivities of the LAMP and nested PCR methods
Restriction enzyme analysis of the LAMP products

NDV strains

1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  M

1,000 bp

300 bp
262 bp
248 bp
Sensitivities of visual inspection of the LAMP products

![Image of UV light effects on LAMP products]

- N
- 5X
- 10X
- 15X
- 20X

UV

1,000 bp

500 bp
Detection of the NDV genome by the LAMP method in chickens experimentally infected with a lentogenic NDV strain

<table>
<thead>
<tr>
<th>Specimen</th>
<th>3 day after inoculation</th>
<th>6 day after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAMP</td>
<td>nested PCR</td>
</tr>
<tr>
<td>Brain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intestine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cecal tonsil</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Lentogenic B1-infected chickens
LAMP for the detection of NDV (summary)

- Very specific to NDV (no cross reaction with APMV2, 3 or 6)
- Can not determine pathotypes of the NDV strains
- Needs some amplification of NDV in eggs
- Feces can not be directly used for RNA extraction
Rapid detection and differentiation of NDV by real-time PCR with melting-curve analysis

Pham et al., *Arch. Virol.* 150: 2429-2438, 2005
Locations of primers for real-time PCR of NDV

3'OH → NP → P/V/W → M → F → SH → HN → L → 5'

3'OH → F2 → F1 → 5'

Cleavage site

F1-S (253-270)

F2-AS (428-448)
Melting curve analysis of a 196 bp fragment containing the F cleavage site.

Verogenic: $89.23 \pm 0.27^\circ C$

Mesogenic: $90.17 \pm 0.35^\circ C$

Lentogenic: $91.25 \pm 0.14^\circ C$
## Detection of the NDV genome

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Time (hr)</th>
<th>Identification of pathotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMP</td>
<td>F2</td>
<td>2-3</td>
<td>No</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>F1-F2</td>
<td>2-3</td>
<td>Yes</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>NP</td>
<td>3-6</td>
<td>Yes (exceptions)</td>
</tr>
</tbody>
</table>
references

- **LAMP**

- **Real-time PCR**
  Pham et al., *Arch. Virol.* 150: 2429-2438, 2005

- **PCR-RFLP**
  Pham et al., *Arch. Virol.* 149: 1559-1569, 2004
Thank you

Summer

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Winter

Teaching Hospital