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*Full Length Research Paper*

# Impregnation and storage of *Newcastle disease virus* on to filter papers and detection of viral RNA by a single tube RT-PCR assay

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**Suitability of storing infected allantoic fluid (AF) and cell culture supernatants (CCS) with strain I-2 of *Newcastle disease virus* (NDV) on Whatman filter papers at room temperature (22-25°C) and 37°C was determined. RNA was extracted from filter papers or liquid aliquots and subjected to reverse transcriptase-polymerase chain reactions (RT-PCR). The results showed that filter papers soaked with NDV infected AF or CCS stored at 37°C yielded amplicons with intensity similar to that kept at room temperature for up to 150 days. The study demonstrates that NDV infected samples can be soaked onto filter papers, stored and subsequently detected by RT-PCR. This method might be safely used for storage and transportation of NDV samples to the designated laboratories for molecular studies without the need for cooling.**

**Key words:** Allantoic fluid, chicken embryo fibroblast, Newcastle diseases virus, polymerase chain reaction, RNA storage onto filter paper, strain I-2.

## INTRODUCTION

Newcastle disease (ND) is a major cause of mortality in chickens and hence the most economically important disease in poultry industry. Epidemiological surveys or outbreak investigation on ND usually involves collection of samples for laboratory diagnosis and confirmation of the disease. Conventional transport of the infected materials for diagnosis or virus isolation of disease causative agents has been by use of a cold chain environment or by adding protective agents such as glycerol. However, these methods have limitations especially in remote areas where cold chain systems are unreliable or unavailable.

Because of poor infrastructures, transport of those samples usually takes a long time to reach the intended laboratories. On the other hand, more often, some laboratory studies are required to be done by use of specialised facilities, which are lacking in some countries with the result that the laboratory work must be undertaken in other parts of the world. Therefore a safe, cheap and effective method is required for successful transfer of infected materials from the remote areas especially in the tropical countries to the designated laboratories without dependant on cold chain system yet prevent degradation of viral nucleic acids. In recent years, several workers have tried to store samples on filter papers (Pitcovski et al., 1999; Yamamoto et al., 2001; Vilček et al., 2001) and successfully extracted nucleic acids (RNA or DNA) for molecular studies of viruses.

So far no studies have been undertaken to store NDV especially strain I-2 on filter papers. The objective of the present study was therefore to evaluate the usefulness of the storage of infected allantoic fluid (AF) and cell culture supernatant (CCS) with strain I-2 on Whatman filter paper no. 1 under different storage temperatures and detecting viral RNA by RT-PCR techniques.

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**Table 1.** Storage of allantoic fluid and cell culture supernatant infected with strain I-2 of *Newcastle disease virus* onto filter papers at 22-25ºC.

| Storage duration (days) | Type of sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Allantoic fluid | | | | Cell culture | | | |
| | Filter paper | | Liquid | | Filter paper | | Supernatant | |
| | Infected | Non-infected | Infected | No-infected | Infected | Non-infected | Infected | Non-infected |
| 7 | + | - | + | - | + | - | + | - |
| 14 | + | - | + | - | + | - | + | - |
| 30 | + | - | + | - | + | - | + | - |
| 60 | + | - | + | - | + | - | + | - |
| 90 | + | - | + | - | + | - | + | - |
| 120 | + | - | + | - | + | - | + | - |
| 150 | + | - | + | - | + | - | + | - |

## MATERIALS AND METHODS

The strain I-2 of ND vaccine was propagated in 10-day-old embryonated chicken eggs from a working seed that had been produced by one passage from the vaccine master seed, as described by Spradbrow et al. (1995) and Alexander (1998). Preparation and propagation of I-2 virus in chicken embryo fibroblast (CEF) cells was done as previously described by Uruakpa (1997). The 50% tissue culture infectious doses ($TCID_{50}$) and 50% embryo infectious doses ($EID_{50}$) were calculated as described by Reed and Muench (1938).

The clarified virus from AF and CCS at the volume of 200 µL each was loaded into Whatman filter paper number 1 which was cut into triangular disks of about 4.5 cm base and 11 cm each side. The base was used for labelling and the sample was loaded from the tip and migrated upwards toward the base. The area impregnated with the sample was marked with a pencil. The filter paper disks loaded with I-2 virus were sealed in plastic containers and left at room temperature or at 37°C for 150 days. As a positive control, I-2 virus in AF and CCS were left in microfuge tubes at room temperature or at 37°C. Non-infected AF and CCS were used as negative controls and were treated the same way as described above.

The filter papers loaded with or without I-2 virus were cut into small pieces of about 1 $cm^2$ and put into microfuge tubes and 800 µL of sterile double distilled water was added and eluted for 1 h at 4°C. Thereafter were centrifuged at 8000 rpm for 10 min. The supernatants were transferred to new microfuge tubes and labelled. The samples were used immediately for RNA extraction.

The QIAamp Viral RNA Mini Kit (Qiagen, prod. no 52904) was used to extract RNA. The procedure was done essentially according to the manufacturer's instructions. Pure concentrated RNA was eluted in RNase-free water. The eluate was aliquoted into 5 µL portions and stored frozen at –20°C for subsequent use for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Two degenerate primers, forward 5′-GTYAAYATATACACCTCATCYCAGACWGG-3′ (Y=C or T, W=A or T) and reverse 5′-CTGCCACTGCTAGTTGBGATAATCC-3′ (B=G, C or T) that recognize sequences surrounding the cleavage site for the NDV $F_0$ protein (Jorgensen et al., 1999) were used in this study. GeneWorks Pty Ltd (SA) synthesized the oligonucleotide primers. The expected product from these primers was 387 bp.

The single tube RT-PCR reaction was carried out according to the manufacturer's instructions for the Access RT-PCR system Kit (Promega, prod. no A1250). The amplication was carried out in a thermocycler (PCR Sprint, Hybaid Ltd, UK). The thermocycling profile for single tube RT-PCR included cycle 1: 48°C for 45 min (RT reaction); cycle 2: 95°C for 2 min; cycles 3-42 (40 cycles): 94°C for 30s, 60°C for 1 min, 68°C for 2 min and cycle 43: 68°C for 7 min (final extension). The amplified products were analysed on 1.5% agarose gel.

The experiment was repeated three times under the same conditions using above protocol. Attempts were also made to re-isolate the I-2 virus from filter papers by using embryonated eggs as described by Alexander (1998); however, the results were negative.

## RESULTS AND DISCUSSION

Tables 1 and 2 summarise the results of storage of AF and CCS infected and non-infected with strain I-2 of NDV on filter papers.

The results showed that filter papers infected with the virus from AF or CCS stored at 37°C yielded an amplicon with intensity similar to that kept at room temperature for the period of 150 days. The electrophoretic bands of the amplified product from I-2 virus stored in liquid aliquots from AF or CCS had higher intensity than the dried samples on filter papers stored at room temperature and 37°C (Figure 1).

All samples from dried or liquid aliquots of the same non-infected AF or CCS showed no electrophoretic band (negative results).

In order to evaluate the sensitivity of the RT-PCRT the I-2 virus from AF and CCS from both liquid aliquots and filter papers were 10-fold serially diluted, respectively and used in a 50-µL reaction using the protocol described above. The RNA was amplified from the virus from AF and CCS for liquid and dried samples with titres of 1 x $10^4$ $EID_{50}$/mL and 1 x $10^5$ $TCID_{50}$/mL, respectively. These results suggest that the detection limit of RT-PCR assay in different storage conditions could be as few as $10^4$ infected cells based on serial titrations.

It has been demonstrated that other poultry viruses such as infectious bursa disease and haemorrhagic enteritis viruses can be immobilized on nitrocellulose paper and thereafter-viral RNA is efficiently amplified using RT-PCR (Pitcovski et al., 1999). In the present study NDV was chosen as a model virus under Paramyxo

**Table 2.** Storage of allantoic fluid and cell culture supernatant infected with strain I-2 of *Newcastle disease virus* onto filter papers at 37ºC.

| Storage duration (days) | Type of sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Allantoic fluid | | | | Cell culture | | | |
| | Filter paper | | Liquid | | Filter paper | | Supernatant | |
| | Infected | Non-infected | Infected | Non-infected | Infected | Non-infected | Infected | Non-infected |
| 7 | + | - | + | - | + | - | + | - |
| 14 | + | - | + | - | + | - | + | - |
| 30 | + | - | + | - | + | - | + | - |
| 60 | + | - | + | - | + | - | + | - |
| 90 | + | - | + | - | + | - | + | - |
| 120 | + | - | + | - | + | - | + | - |
| 150 | + | - | + | - | + | - | + | - |

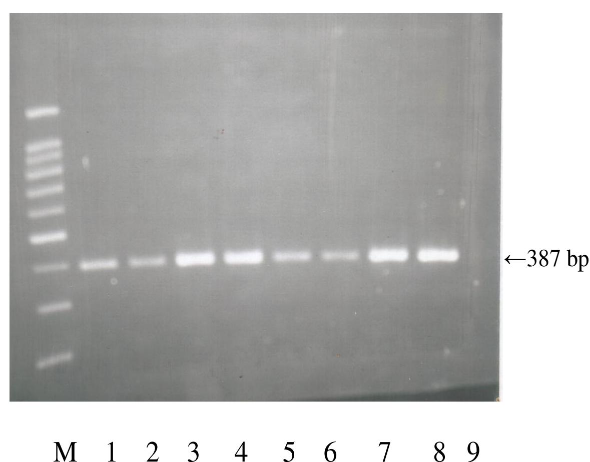


**Figure 1.** Electrophoretic profiles of PCR products from different storage conditions and temperatures. M = DNA ladder (100 bp), lane 1 = Allantoic fluid (AF) onto filter paper at room temperature, lane 2 = AF onto filter paper at 37ºC, lane 3 = AF in liquid at room temperature, lane 4 = AF in liquid at 37ºC, lane 5 = Chicken embryo fibroblast (CEF) cell culture onto filter paper at room temperature, lane 6 = CEF cell culture onto filter paper at 37ºC, lane 7 = CEF cell culture supernatant at room temperature, lane 8 = CEF cell culture supernatant at 37ºC, lane 9 = negative control.

viridae family. NDV is an enveloped virus. A previous study was successfully undertaken using no- enveloped virus (Pitcovski et al., 1999) for impregnation on filter paper and later extracted RNA was subjected to RT-PCR.

RT-PCR has been found to be very sensitive method for the detection of NDV (Jestin and Jestin, 1991). The ability to differentiate between isolates of NDV by RT-PCR was first demonstrated by Kant et al. (1997) and recently by Wang et al. (2001). Since then several studies have been carried out and have been detailed reviewed by Aldous and Alexander (2001), Yusoff and Tan (2001) and Cavanagh (2001). Virus isolation, the classical method for diagnosis of NDV presents technical limitations to its large-scale use. RT-PCR assays provide some advantages, being particularly fast and allowing the use of stored samples retrospectively. The present study demonstrates that NDV in infected AF or CCS can be immobilized onto a filter paper and subsequently detected by RT-PCR. Although NDV is relatively labile RNA virus, stabilization of viral RNA could be achieved by immobilization on a solid carrier.

Sample of 200 µL was sufficient for the detection of the I-2 virus on the filter paper after storage either at room temperature or at 37°C for a period of up to 150 days. It may be possible to store the samples for much longer periods. Immobilization and storage of NDV onto filter paper enables large scale and worldwide screening of viruses and undertaking of molecular epidemiology studies.

Filter paper was used in this study because of its rapid and efficient immobilization of I-2 virus and broad availability and low price of the filter. Other reasons include high stability of immobilized NDV RNA, ease of transport and storage without need for cooling (Vilček et al., 2001). Furthermore, it enables storage of samples for long periods without need for any special equipment or storage conditions. The loss of viral infectivity in samples spotted on to filter paper can be advantage during handling in a laboratory.

If this technique is used appropriately and necessary precautions are taken, it can be applied in laboratories from developing countries to transport samples to other parts of the world with specialised diagnostic facilities.

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