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# Molecular detection of Newcastle disease virus using Flinders Tehnology Associates-PCR

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**ABSTRACT**

The feasibility of using Flinders Technology Associates (FTA) filter papers to store the Newcastle disease virus (NDV), infected allantoic fluid (AF) and tissue samples, for the molecular detection of NDV by reverse transcriptase - polymerase chain reaction (RT-PCR) - was investigated. An FTA card is a cotton based cellulose membrane, with lyophilized chemicals that lyse the viruses and bacteria. The viral RNA was detectable from FTA cards up to a concentration of $10^{7.6}$ $EID_{50}$/100 µL (a 100 times dilution of $10^{9.6}$ $EID_{50}$/100 µL of initial stock). The inactivated virus remained stable on the cards for up to 30 days, both at room temperature and 4 ºC. NDV was detected by RT-PCR from all the FTA imprints of the caecal tonsils, kidney, proventriculus, spleen, trachea, faecal swabs and intestinal lesions of NDV-suspected birds. NDV was inactivated upon contact with FTA, as shown by the inability of the virus to propagate in embryonated eggs and its inability to infect chicken embryo fibroblast culture. In conclusion, FTA cards are suitable for collecting and transporting NDV infected samples, without cold storage. The virus inactivated in FTA cards, however, is a suitable source of viral RNA for molecular detection and characterization.

**Key words:** Newcastle disease virus, filter paper, molecular detection

## Introduction

Newcastle disease (ND), popularly known as Ranikhet disease in India, is an economically important viral disease that poses a great threat to the poultry industry.

This disease is caused by a virus (NDV), which is a significant avian pathogen with worldwide distribution and is classified as a List A Virus by the Office International des Epizooties (ALEXANDER, 2000). The reverse transcription-polymerase chain reaction

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(RT-PCR) procedure has been established as a reliable tool for NDV detection in allantoic fluid (AF). However, RT-PCR and direct nucleotide sequencing are not available in all countries or regions, so samples need to be transported in a safe way to laboratories with those capabilities, following high standards of biosecurity during transport. Conditions for the importation of infectious agents by the United States Department of Agriculture require that these infectious organisms must be inactivated by chemicals, such as phenol or formalin, before being transported (SNYDER, 2002). But chemically inactivated samples might not prove very efficient in terms of detecting the virus, due to problems in nucleic acid extraction. An alternative, safe way of transporting inactivated organisms is represented by Flinders Technology Associates (FTA) filter paper, which is a chemically treated filter paper designed for the collection and room-temperature storage of biological samples for subsequent analysis (NATARAJAN et al., 2000). FTA cards have been used for multiple molecular studies, such as DNA processing from human or wildlife samples (RAINA and DOGRA, 2002). Very recently, use of this technique has become a very interesting approach for the detection of poultry microorganisms such as Mycoplasmas, infectious bronchitis virus (IBV) and infectious bursal disease virus (IBDV) (MOSCOSO et al., 2006). Hence, in the present study, the feasibility of using FTA cards for the sampling, detection and inactivation of NDV from AF and tissue samples by RT-PCR was assessed, with the outline bordering on the propagation of NDV in Chicken Embryonated Eggs (CEEs), collection of AF from NDV infected CEEs and organs of NDV infected chicks, and application on FTA filter paper, detecting the presence of NDV in FTA filter paper using RT-PCR, studying the sensitivity and stability of the FTA/RT-PCR system and assessing virus inactivation by means of FTA filter paper.

## Materials and methods

*Virus.* The virulent NDV strain used in this study was obtained from the NDV repository of the Department of Animal Biotechnology, Madras Veterinary College, Chennai, India. This strain was adapted in Chicken Embryo Fibroblast (CEF) culture and further used for FTA inactivation studies.

*RT-PCR detection of NDV.* Total RNA isolation was carried out using TRI Reagent® (MRC, INC, Ohio) as per the manufacturer's protocol. cDNA synthesis was carried out using a Revert Aid™ first strand cDNA synthesis kit (Fermentas, USA) as per the manufacturer's protocol. PCR was carried out as per the protocol of SEAL et al., (1995) with a slight modification in the reaction mix concentration.

*Sensitivity and stability of FTA / RT-PCR system.* Serial ten-fold dilutions up to 10-12 were made from the initial AF stock ($EID_{50}$ $10^{9.6}$/100 µL). Then, 75 µL of serially diluted samples were applied to the four matrix circles present in the FTA cards. The cards were labeled properly with appropriate sample identification. After 24 hours, 10 punches were

taken from one matrix circle of each loaded card, using a 2 mm puncher. The loaded sample was purified using FTA purification reagent (Whatman International Ltd., U.K.) as per the manufacturer's protocol, prior to RT-PCR amplification. The 2 mm discs were placed in a PCR tube and 200 µL of FTA purification reagent added. It was incubated for 5 minutes at room temperature, after thorough mixing.

The FTA reagent was removed and discarded using a pipette, and washing was repeated twice. Then, 200 µL of TE buffer was added and incubated for 5 minutes at room temperature. The TE buffer was removed and discarded using a pipette, and the discs were allowed to dry at room temperature for an hour, and then used for down streaming.

In order to assess sensitivity, RT-PCR was done for each dilution applied to the cards to determine the highest dilution at which the viral RNA was detectable. To evaluate the stability of the viral RNA on FTA cards, 75 µL of the undiluted AF was applied on the matrix circles present on the cards, and the cards were stored at room temperature and 4 ºC. Virus identification by RT-PCR was attempted at days 0, 5, 10, 15, 20 and 30.

*Organ selection for FTA/RT-PCR detection of NDV.* Tissue samples from the caecal tonsils, kidney, proventriculus, spleen and trachea, and faecal swabs and swabs from intestinal lesions were collected from NDV-suspected birds from Namakkal, Tamil Nadu, India. An imprint was made by gently pressing the tissue against the provided matrix area on the FTA cards. The cards were transported to Chennai by mail, and after 24 hours, RT-PCR was performed to check for the presence of NDV.

*Virus inactivation by FTA cards.* Allantoic fluid and organ imprints of NDV positive samples were allowed to elute using FTA purification reagent. Then, 100 µL of this elute was inoculated into allantoic route from 9 days old CEEs and CEF culture. Three days post-infection, the AF and culture fluid were checked for the presence of the virus by Haemagglutination (HA) test and RT-PCR using F-gene specific primers.

## Results

*Newcastle disease virus isolation and identification.* The NDV isolate used in this study was propagated in 9 days old CEEs. The $EID_{50}$ was calculated and found to be $10^{9.6}$/100 µL. The infected embryos, when compared with the normal embryos, showed stunted growth and haemorrhaging.

*Haemagglutination test.* The initial HA titre of the virus was found to be 512, and HI testing was done using NDV serum to confirm the presence of the virus in AF.

*Reverse transcription - polymerase chain reaction and nucleotide sequencing.* The total RNA samples extracted from NDV-infected AF were subjected to RT-PCR using F gene specific primers, and the expected amplicon size of 254 bp was observed in 2% agarose gel electrophoresis. The purified PCR products were sequenced and on BLAST

analysis, 98% homology was obtained with other NDV isolates from other parts of the world available in GenBank. The specificity of primers used for RT-PCR was tested with other viral RNA (IBV) and no amplification was observed.

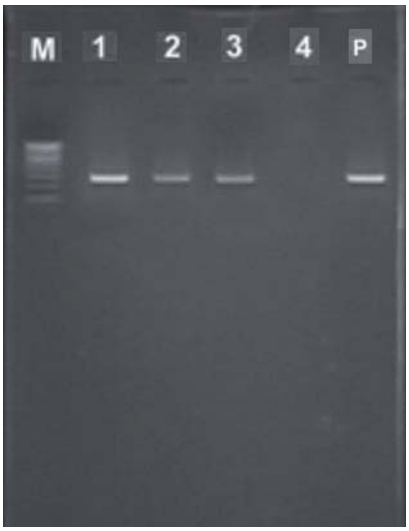


Fig. 1. Plate 1. Agarose gel electrophoresis (2%) showing sensitivity of FTA/RT-PCR system. Lane M: 100 bp. Ladder; Lane(s) 1-4: Diluted $10^{9.6}$ EID50 /100 µl - 10, $10^1$, $10^2$, $10^3$ respectively; Lane P: Positive control.

*Sensitivity of FTA/RT-PCR system.* The lowest concentration at which nucleic acid amplification from the FTA cards occurred was $10^{7.6}$ $EID_{50}$/100 µL (Plate 1). The detection level of NDV nucleic acids was always higher when AF, prior to its inactivation, was used as study material, in comparison with the level of detection observed for the FTA cards (one $\log_{10}$ difference shown in Table 1).

*Stability of NDV RNA on FTA cards.* The stability of viral RNA on FTA cards was measured by performing RT-PCR on initial AF stock at days 5, 10, 15, 20 and 30 after collection, for samples stored both at room temperature and at 4 ºC. The viral RNA was stable at both temperatures until day 20, but the intensity of the PCR band was faint by day 30 (Plate 2).

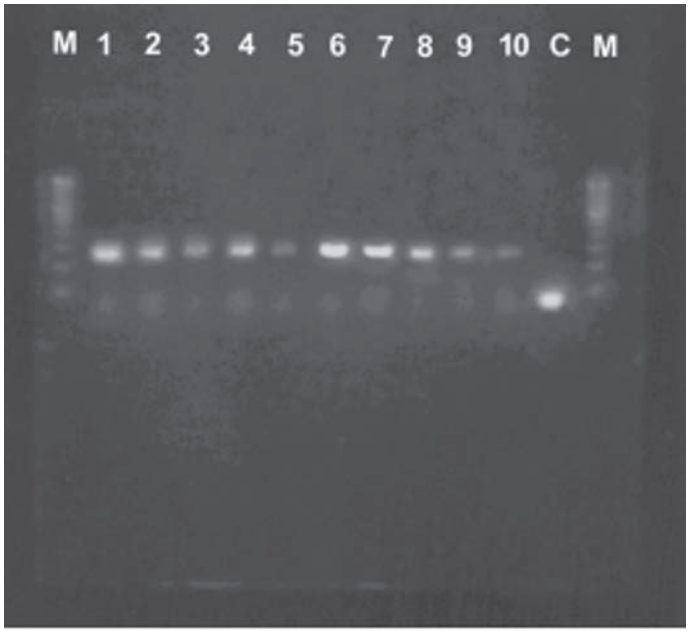


Fig. 2. Plate 2. Agarose gel electrophoresis (2%) showing stability of FTA/RT-PCR system. Lane M: 100 bp. Ladder; Lane(s) 1-5: 4 °C storage at days 5, 10, 15, 20 and 30 respectively; Lane(s) 6-10: Room temperature storage at days 5, 10, 15, 20 and 30 respectively; Lane C: Negative control.

Table 1. Sensitivity of FTA/RT-PCR system

| RT - PCR amplifications (Initial allantoic fluid concentration - $10^{9.6}$ $EID_{50}$/100 µL) | | |
|---|---|---|
| Virus Dilution | Allantoic Fluid | FTA Card |
| $10^0$ | + | + |
| $10^1$ | + | + |
| $10^2$ | + | + |
| $10^3$ | + | - |
| $10^4$ | + | - |
| $10^5$ | - | - |
| $10^6$ | - | - |

+: 254 bp amplicon; -: No amplicon

*Organ selection for FTA/RT-PCR detection of NDV.* RT-PCR positive results were obtained from all the organs, i.e. the caecal tonsils, kidney, proventriculus, spleen, trachea, faecal swabs and intestinal lesions, imprinted on FTA cards (Plate 3 and Plate 4). No amplification was obtained with the organs collected from control birds.

*Inactivation of NDV on FTA cards.* Both embryo and cell culture systems were used to study viral inactivation by the FTA cards. The AF obtained from embryos inoculated with FTA card elute failed to haemagglutinate chicken red blood cells. No amplicons were observed from the FTA card inactivated fluid after RT-PCR analysis. Similarly, no cytopathic effects were observed after 72 hours, when the FTA elute was inoculated into CEF culture. Also, the culture fluid failed to haemagglutinate chicken red blood cells and no amplification was obtained by RT-PCR.

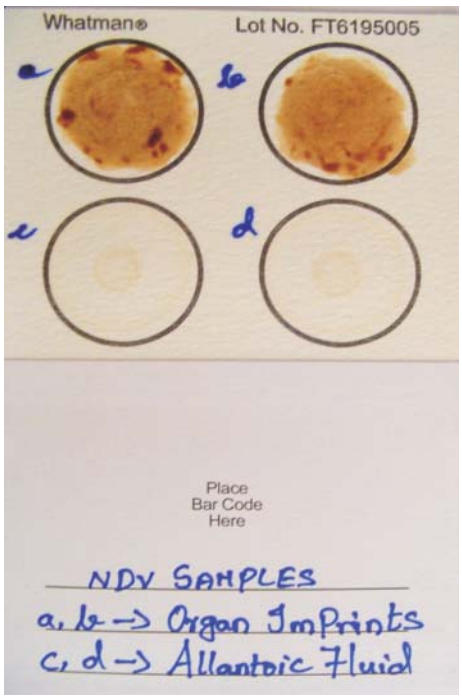


Fig. 3. FTA card showing organ imprints of NDV infected birds

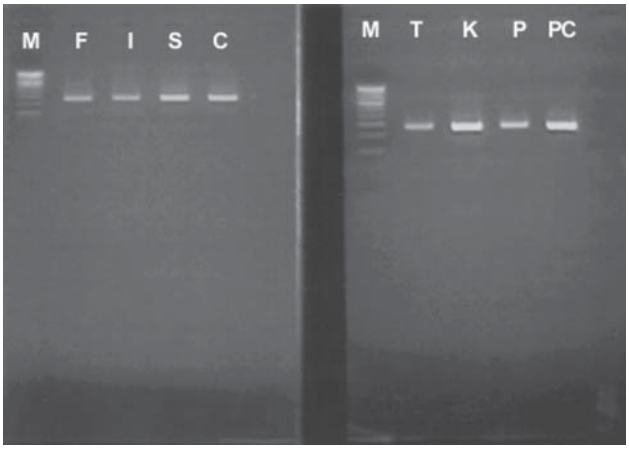


Fig. 4. Plate 4. Agarose gel electrophoresis (2%) for molecular detection of NDV from organ imprints on FTA cards. Lane M: 100 bp ladder; Lane F: Faecal swabs; Lane I: Intestinal Lesions; Lane S: Spleen; Lane C: Ceacal Tonsils; Lane T: Trachea; Lane K: Kidney; Lane P: Proventriculus; Lane PC: Positive control.

## Discussion

Ever since its reported outbreak in Ranikhet, Uttar Pradesh in 1927 (EDWARDS, 1928), ND has been reported in all parts of India. The disease causes considerable loss to the poultry industry in terms of mortality and loss of egg production. Thus, NDV has become a constant threat to most birds reared, and represents a major limiting factor for increasing poultry production in many countries.

The RT-PCR procedure has been established as a reliable tool for NDV detection (STAUBER et al., 1995). However, molecular detection and characterization of NDV is not commonly performed on chemically inactivated samples, for example, using phenol or formalin (SNYDER, 2002), due to reports of RNA modifications and problems in nucleic acid extraction, which compromise the yield of high quality DNA or RNA (COOMBS et al.,1999; MASUDA et al., 1999). A virus inactivation process ensuring high quality RNA for molecular pathotyping would be an improvement in field sampling and the shipping of NDV for diagnosis. FTA filter paper, which is a chemically treated filter paper designed for the collection and room-temperature storage of biological specimens for subsequent analysis, is used to ensure the safe transportation of infectious agents (NATARAJAN et al., 2000). In the current study, the feasibility of using FTA filter papers for virus detection from AF and tissue samples by RT-PCR and virus inactivation was assessed. The Egg Infectivity $Dose_{50}$ reflects directly on the titre of the virus present in the samples to be

used for subsequent analysis. PEROZO et al., (2006) used an initial AF stock with an $EID_{50}$ of $10^{8.8}$/mL to assess the sensitivity of FTA. In the present study, an AF sample with $10^{9.6}$ $EID_{50}$/100 µL was used for the detection of NDV in the FTA/RT-PCR system. Reverse transcription coupled to PCR was widely used to amplify the gene sequences of various RNA viruses. In the present study, RT-PCR for the FPCS region of NDV was performed using degenerate oligonucleotide primers and the expected 254 bp amplicon was obtained as reported by SEAL et al. (1995). Even though PCR is a highly sensitive method, non-specific reaction may occur (PEROZO et al., 2006). Hence, sequencing of PCR products and subsequent sequence analysis is necessary to confirm the F gene of NDV. Homology analysis of the F gene sequence of NDV showed 98% homology with other isolates from different regions of the world.

In the present study, the FTA cards for NDV sampling and inactivation, coupled with RT-PCR, allowed the detection of the virus from AF with a titre of $10^{7.6}$ $EID_{50}$/100 µL (a 100 times dilution of the $10^{9.6}$ $EID_{50}$/100 µL viral stock). However, PEROZO et al. (2006) reported a $10^{-3}$ dilution of the initial AF stock with the use of FTA cards. Differences in sensitivity observed in RT-PCR for the FTA-inactivated fluids when compared with the control samples may be due to the detrimental effect of FTA inactivation on the viral RNA, as previously reported for other chemically inactivated samples (COOMBS et al., 1999). The FTA/RT-PCR system for NDV was able to detect as little as 0.5 ng of RNA from 75 µL of AF spotted onto the FTA matrix.

In the present study, RT-PCR detection of NDV in AF loaded on FTA cards was stable for at least 20 days at both room temperature and 4 °C. However, on the 30$^{th}$ day, a decrease in stability was observed, as indicated by the low intensity of the DNA band in the agarose gel in samples stored at both temperatures. This decrease in stability over time has been previously explained as a consequence of RNA denaturation by the formation of nicks on the RNA strands (DOBBS et al., 2002). The nucleic acids of NDV inactivated on FTA cards from organ imprints showed positive results by RT-PCR.

All the organs used for the study namely the kidney, spleen, caecal tonsils, trachea, proventriculus, faecal swabs and intestinal lesions, confirmed the presence of NDV when tested by RT-PCR. PEROZO et al. (2006) reported a negative result with trachea swabs. Failure to identify the virus in the trachea swabs might be related to the amount of viral RNA present in the swabs, when compared with the amount of virus obtained from a tissue imprint, in which epithelial cells actively targeted by replication remain over the FTA card matrix. However, in the present study, not only trachea but also faecal swabs and intestinal lesions showed positive results by RT-PCR. Moreover, even at extreme or high temperatures during mid-May, the imprints remained stable on FTA when transported from Namakkal to Chennai distance by mail. Thus the use of FTA for the

safe transportation of NDV for molecular detection, even at high temperatures, can be recommended.

The NDV used in the study from infected AF and organ imprints was rendered non-infectious or inactive after being in contact with the FTA, within a short period. The AF obtained by inoculating CEEs with FTA elute caused no haemagglutination with chicken erythrocytes. Also, the RT-PCR assay failed to identify the viral RNA from AF of embryos inoculated with FTA elute, which means virus re-isolation from the cards was not possible. Likewise, the FTA elute, when inoculated into CEF, resulted in no cytopathic effects such as clumping of cells, granulation of cytoplasm, syncytia, etc. (KUMANAN and VENKATESAN, 1994) that are otherwise expected due to NDV infection. Similar inactivation has been reported for avian mycoplasma (MOSCOSO et al., 2004). From perusal of the data, we conclude that the FTA inactivation of NDV samples is useful for the safe transportation of the viruses.

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**NARAYANAN, M. S., M. PARTHIBAN, P. SATHIYA, K. KUMANAN: Dokaz virusa newcastleske bolesti molekularnim postupkom FTA-PCR. Vet. arhiv 80, 51-60, 2010.**

**SAŽETAK**

Istražena je mogućnost uporabe Flinders tehnologije s filtrirnim papirom za pohranjivanje alantoisne tekućine zaražene virusom newcastleske bolesti i uzoraka tkiva radi molekularnog dokazivanja toga virusa lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju (RT-PCR). Flinders kartica celulozna je membrana pripravljena na osnovi pamuka s liofiliziranim kemikalijama koje liziraju viruse i bakterije. Virusna RNA mogla se na takvoj kartici dokazati u količini od $10^{7,6}$ $EID_{50}$/100 µL (100 puta manje razrjeđenje od $10^{9,6}$ $EID_{50}$/100 µL osnovne suspenzije). Inaktivirani virus bio je stabilan na karticama tijekom 30 dana pri sobnoj temperaturi i 4 ºC. Virus je bio dokazan RT-PCR-om u svima Flinders tehnologijom pripravljenim otiscima cekalnih tonzila, bubrega, voljke, slezene, dušnika, obriscima fecesa i crijevnih lezija ptica sumnjivih na newcastlesku bolest. Virus je bio inaktiviran nakon dodira s FT filtrirnim papirom što je bilo potvrđeno činjenicom da se nije više mogao uzgojiti u kokošjim embrijima ni na kulturi fibroblasta podrijetlom od kokošjih embrija. Zaključuje se da su FT papirići prikladni za uzimanje i prijenos uzoraka koji sadrže virus newcastleske bolesti bez potrebe pohranjivanja u hladnom prostoru. Virus inaktiviran na FT papiriću (kartici) odgovarajući je izvor virusne RNA za njegov molekularni dokaz i identifikaciju.

**Ključne riječi:** virus newcastleske bolesti, filtrirni papir, molekularni dokaz