DETECTION OF NEWCASTLE DISEASE VIRUS 
IN INFECTED CHICKEN EMBRYOS 
AND CHICKEN TISSUES BY RT-PCR 

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Abstract 

RT-PCR for the detection of Newcastle disease virus (NDV) in allantoic fluids of SPF embryonated eggs as well as in tissues of SPF chickens infected experimentally is described. The method proved to be specific as all tested NDVs were detected and no cross reaction with other RNA viruses was observed. Sensitivity of the method was established at $10^5$ ELD$_{50}$/0.1 ml. To detect NDV in chicken tissues, SPF chickens were inoculated with $10^6$ EID$_{50}$ of 3 NDV reference strains: La Sota (lentogenic), Roakin (mesogenic) and Italy (velogenic pigeon variant) and 5 d p.i. various tissue samples were aseptically collected followed by RT-PCR and virus isolation on SPF embryos. The results showed high concordance: 93% (La Sota and Italy) to 100% (Roakin) between both methods. 

Key words: chicken embryos, chickens, Newcastle disease virus, RT-PCR. 

Newcastle disease (ND) is a highly contagious infection of poultry caused by avian paramyxovirus serotype 1 (Newcastle disease virus, NDV). The disease is spread worldwide affecting various species of poultry and other birds (2, 3, 14). However, chickens appear to be the most susceptible to the disease whereas aquatic birds, including geese and ducks, are relatively resistant. NDV differs in virulence and has been grouped into 5 pathotypes: velogenic viscerotropic, velogenic neurotropic, mesogenic, lentogenic and asymptomatic. It was shown that NDV virulence is dependent on the amino acid sequence at the cleavage site of F0 gene. The presence of multiple basic amino acids at the cleavage site indicates that the virus isolate is pathogenic (2). In diagnosis of Newcastle disease, methods recommended by OIE Manual (16) and EU Council Directive (5) comprise isolation on SPF embryonated eggs and identification in the haemagglutination inhibition test. Recently, as an alternative, OIE regulations have proposed methods based on molecular biology (16). Reverse transcription and polymerase chain reaction (RT-PCR) methods are applied in many laboratories of the world for the detection and identification of NDV (1). 

The aim of the present study was to apply RT-PCR for rapid detection of Newcastle disease virus in experimentally infected chicken embryos and tissues of chickens. 

Material and Methods 

Embryonated eggs. Specific pathogen free (SPF) eggs were imported from Valo-Lohmann (Germany). 

Viruses. In the study 17 NDV strains were used: 3 reference strains representing different pathotypes: La Sota (lentogenic), Roakin (mesogenic) and Italy (velogenic, pigeon variant) and 14 field strains isolated in Poland from: chickens - Radom strain isolated at the beginning of the 70-ties and 3 strains isolated in 1990-2004, turkeys - 1 strain isolated in 2004, racing pigeons - 7 strains isolated at the late 80-ties and beginning of the 90-ties, kindly provided by the Department of Microbiology, Agricultural University in Lublin, and feral pigeons - 2 strains recovered in 2002. Prior to testing in RT-PCR, all the strains were propagated on SPF embryonated eggs and allantoic fluid was used for further studies. 


Experimental design. Three groups of four 4-week-old SPF chickens kept in isolation were inoculated intraocularly and intranasally with $10^6$ EID$_{50}$ of the following strains of NDV: La Sota, Roakin and Italy. Five days post inoculation (p.i.) tracheal and cloacal swabs as well as samples from the trachea, lungs, liver, spleen, heart, brain, kidneys, bursa of Fabricius, duodenum, caecal tonsils, and rectum were aseptically collected. Supernatants of the organs (used for viral isolation as well as for RT-PCR) were prepared according to the Annex III of the Council Directive
RT-PCR was established by the ten-fold diluting of the strand buffer, synthesized using 5 (UV) transillumination. Molecular mass marker and visualized by ultraviolet stained with ethidium bromide, compared with were separated in 1.5% agarose gel in 1 x TAE buffer (Fermentas, Lithuania) and 1 (Qiagen, USA) as recommended by the supplier. Allantoic fluids using commercial RNeasy Mini Kit inhibitor (20 U/µl), 1 µl of 10mM dNTP, 1 µl of MgCl₂ (25 mM), 0.5 µl of Taq polymerase (Fermentas, Lithuania) and 1 µl of each primer. The thermocycler conditions were as follows: 2 min at 94°C (initial denaturation), followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 48°C (annealing), 30 s at 72°C (elongation). The PCR ended with a final elongation for 7 min at 72°C.

Detection of PCR products. PCR products were separated in 1.5% agarose gel in 1 x TAE buffer stained with ethidium bromide, compared with molecular mass marker and visualized by ultraviolet (UV) transillumination.

Sensitivity of RT-PCR. The sensitivity of the RT-PCR was established by the ten-fold diluting of the allantoic fluid containing lentogenic La Sota strain (10⁵ ELD₅₀/0.1 ml). Subsequently, RNA was isolated and RT-PCR was performed according to the procedure described above. The highest dilution with positive RT-PCR signal was determined.

Specificity of RT-PCR. To evaluate specificity of the method, cDNA of the following RNA viruses was used: paramyxovirus serotype 3, avian influenza virus (H5N2 and H7N1), infectious bursal disease virus (vaccinal 228E strain and very virulent 99/150 Polish field strain), and avian infectious bronchitis virus (strains M-41 and 4/91). PCR was performed according to the protocol described above.

Results

All strains previously identified serologically as NDV also tested positive in RT-PCR test with NDV specific primers (Fig 1). No cross reaction was found with other RNA viruses used in the study (data not shown). Sensitivity of the RT-PCR has been established at 10² ELD₅₀/0.1 ml (Fig. 2).

Table 1 shows the results of RT-PCR and virus isolation performed on tissue samples collected from SPF chickens 5 days p.i. By RT-PCR method the positive results were obtained in all 13 tested samples (13/13) p.i. with mesogenic Roakin strain, 11 samples p.i. with velogenic Italy strain and 8 samples p.i. with lentogenic LaSota strain. Positive result of virological examination was noted in all samples (Roakin strain), 12 samples (Italy strain) and 10 samples (La Sota strain). Both pooled samples (number 1 and 2) were positive in RT-PCR and virus isolation (La Sota, Roakin and Italy strains). Italy strain was isolated from all samples after the first embryo passage. Isolation of Roakin strain required one passage from all samples except for cloacal and tracheal swabs. La Sota strain was isolated during the first embryo passage only from the samples collected from the respiratory tract, brain, bursa of Fabricius and pooled sample 1. Concordance between RT-PCR and virus isolation was: 93% (La Sota and Italy strains) and 100% (Roakin strain).

Fig. 1. Electrophoresis of RT-PCR products: M: marker Gene Ruler 100 bp DNA Ladder (Fermentas, Lithuania), Lane 1: NDV/Radom/chicken/70; Lane 2: NDV/chicken/89/90; Lane 3: NDV/chicken/18/91; Lane 4: NDV/chicken/548/04; Lane 5: NDV/pigeon/AR1; Lane 6: NDV/pigeon/AR2; Lane 7: NDV/pigeon/AR3; Lane 8: NDV/pigeon/AR4; Lane 9: NDV/pigeon/AR5; Lane 10: NDV/pigeon/AR6; Lane 11: NDV/pigeon/AR7; Lane 12: NDV/pigeon/PW/46/02; Lane 13: NDV/pigeon/PW/166/02; Lane 14: NDV/turkey/549/04; Lane 15: NDV/LaSota; Lane 16: NDV/Roakin; Lane 17: NDV/Italy; Lane 18: negative control.
**Fig. 2.** Sensitivity of RT-PCR: M: marker Gene Ruler 100 bp DNA Ladder (Fermentas, Lithuania); Lane 1: positive control; Lane 2: 10^7 ELD\(_{50}\); Lane 3: 10^6 ELD\(_{50}\); Lane 4: 10^5 ELD\(_{50}\); Lane 5: 10^4 ELD\(_{50}\); Lane 6: 10^3 ELD\(_{50}\); Lane 7: 10^2 ELD\(_{50}\).

**Table 1**

Comparison of RT-PCR and virus isolation for the detection of NDV in tissues of infected chickens

<table>
<thead>
<tr>
<th>Samples</th>
<th>Virus isolation</th>
<th>Passage</th>
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<tr>
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<td>Roakin strain</td>
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<td>Italy strain</td>
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Discussion

As Newcastle disease is one of the most important infectious diseases of poultry, rapid detection and identification of the virus is crucial for the effective control of the disease. Conventional diagnostic methods such as virus isolation on SPF embryonated eggs followed by serological identification in haemagglutination-inhibition test is laborious and time-consuming. The speed of the diagnosis can be considerably increased by using methods based on molecular biology e.g. reverse transcription – polymerase chain reaction (1, 6, 7, 10-13).

RT-PCR for the detection of NDV was first described by Jestin & Jestin in 1991 (10) and to date it has been successfully developed in different modifications (1) e.g. using universal primers to detect all NDVs (6, 7), pathotype specific primers that enable rapid differentiation of the pathotype (12) or nested PCR (11, 13). In our study we have applied RT-PCR method at first for the detection and identification of NDV in allantoic fluids of infected embryonated eggs and then in tissues of experimentally infected chickens with NDV strains of different virulence: lento-, meso- and velogenic.

Our study on the sensitivity of the test indicates that at least 10^5 ELD50 should be present in 0.1 ml of the allantoic fluid to obtain a positive result. The sensitivity can be improved by the development of a modification of RT-PCR e.g. “nested” RT-PCR (11, 13). On the other hand, during infection with NDV strains, even with lentogenic ones, the ELD50 value of the virus in infected tissues is usually higher that the sensitivity threshold established in our studies (8, 9, 15). Indeed, applied RT-PCR method proved to be useful for the detection of NDV directly in chicken tissues and a high degree of correlation with virus isolation test was observed. Collection of samples was carried out 5 days post infection because the high amount of the virus was expected to be found in a variety of organs at that very time. Velogenic and to some extent mesogenic NDV strains are pantropic and can be found in many organs, what was confirmed by our studies (Roakin: 13/13 samples by RT-PCR and virus isolation, Italy: 11/13 samples by RT-PCR and 12/13 by virus isolation). Lentogenic strains are generally less invasive and can be found usually in the respiratory and digestive tracts (2, 4, 14, 17) but some of them spread to internal organs, especially during early phase of infection (7-9, 15). It is presumed that their multiplication rate in parenchymatous organs is lower than for meso- or velogenic strains (9). In our study La Sota strain was detected in 8/13 and 9/13 samples by RT-PCR and virus isolation, respectively. The need to perform 2 passages to obtain a positive result in case of samples collected from the liver, spleen, kidneys and duodenum suggests the LaSota strain multiplies in lower titres in these organs.

Regardless of the virulence of the strains, pooled samples of different organs were always positive in either test. Due to the fact that NDV strains of different virulence show diversity in tissue predilection in different time post infection, it is recommended to pool samples of various organs rather than examine selected tissues. It should also be taken into account that the viral titres decline relatively quickly in certain organs.

A significant reduction of time required to complete the RT-PCR test (8-12 h) in comparison with the standard virus isolation (up to 2 weeks) seems to be the greatest advantage of the RT-PCR method. Moreover, the use of primers encompassing cleavage site of F0 gene enables to establish virulence of an isolate, either by direct sequencing or by restriction enzyme analysis and can be used alternatively to in vivo method (1 day-old chicks inoculation) of the virulence assessment.

References

12. Kant A., Koch G., Van Roozelaar D., Balk F., Ter Huurne A.: Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by...


17. Singh K.V., El-Zein A.: Viral proliferation patterns of a velogenic (VLT), a mesogenic (Komarov), and a lentogenic (F) strain of Newcastle disease virus. Poult Sci 1978, 57, 1563–1566.