Rapid detection and differentiation of Newcastle disease virus isolates by a triple one-step RT-PCR

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ABSTRACT

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A triple one-step RT-PCR was developed to screen and differentiate virulent from avirulent Newcastle disease virus (NDV) isolates. Three sets of oligonucleotides were designed, each specific for amplifying NDV fusion protein gene-specific RNA from virulent, avirulent or all isolates respectively. The sensitivity of one-step RT-PCR was determined using viral RNA extracted from serially diluted NDV-infected allantoic fluid and found to be 10^{-5} HA units. Application of one-step RT-PCR to various NDV samples, including wild-type virulent isolates and avirulent vaccine strains, demonstrated the potential for rapid identification (3–4 h) of NDV isolates as well as the differentiation of virulent from avirulent strains.

Keywords: Detection, identification, Newcastle disease virus, NDV, RT-PCR, Triton X-100 differentiation of NDV isolates

INTRODUCTION

Newcastle disease is a highly contagious and fatal disease affecting numerous species of birds (Alexander 1997a). It was first identified early in the 20th century, but remains one of the most important diseases threatening the poultry industry. Newcastle disease virus (NDV), a negative-stranded RNA virus in the family *Paramyxoviridae,* has been categorized into three main pathotypes based on the severity of the disease produced in chickens: lentogenic (avirulent), mesogenic and velogenic (virulent). Most live vaccine strains, such as LaSota and V4, are lentogenic. Mesogenic and velogenic strains are classified as virulent, causing respiratory or neurological clinical signs, with mortalities ranging from low to high depending on the causative isolate. In order to confirm that a disease outbreak is caused by a virulent strain of NDV, the intracerebral pathogenicity index (ICPI) of the isolates must be assessed in 24–40 hour-old chicks (Alexander 1997b). This laborious test requires at least 5 days and 10 specific pathogen free (SPF) chicks per dilution (OIE Manual of Standards 2000). Mean death times (MDT) in embryonated eggs or intravenous pathogenicity indices (IVPI) in 6-week-old chicks are also used for this purpose.

In recent years, the genetic differences between the virulent and the avirulent genotypes of NDV have been determined (Toyoda, Sakaguchi, Imai, Inocencio, Gotoh, Hamaguchi & Nagai 1987; Glickman, Syddall, Iorio, Sheehan & Bratt 1988; Rott & Klenk 1988; Collins, Bashiruddin & Alexander 1993). Virulence is defined by the amino acid sequence of the post-translational cleavage site of the precursor fusion protein (F_0), one of the major protective viral anti-

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gens. The consensus cleavage site of virulent NDV F_0 protein was characterized as being R/K-R-Q-R-R/K$^-$F, while that of avirulent NDV was characterized as G/E-K/R-Q-G/E-R$^-$L. The former contains more basic amino acids and can be efficiently cleaved (F_0 into F_1 and F_2) by the proteases present in a wide range of cells and tissues, resulting in a fatal systemic infection. F_0 molecules in viruses of low virulence are restricted in their sensitivity to proteases and these viruses can grow only in certain host cell types (Alexander 1997a). Oligonucleotide primers and DNA probes have been synthesized and used to identify and distinguish virulent isolates of NDV via a two-step reverse transcription—polymerase chain reaction (RT-PCR; Kant, Koch, Van Roozelaar, Balk & Ter Huurne 1997; Oberdörfer & Werner 1998) or DNA hybridization (Jarecki-Black & King 1993). The RT-PCR has certain advantages: it is rapid and labour friendly combined with high specificity and sensitivity. However, the previously described two-step RT-PCR, in which the RT and PCR steps are conducted separately, is inconvenient and prone to contamination. A one-step RT-PCR format on tissue and faecal material was recently described by Kho, Mohd-Azmi, Arshad & Yusoff (2000) and Gohm, Thür & Hofmann (2000) in which only the detection of NDV was performed.

This report describes the development of a triple one-step RT-PCR on allantoic fluid that can not only identify NDV, but also differentiate virulent from avirulent strains.

MATERIALS AND METHODS

Viruses

The mesogenic Onderstepoort NDV strain was obtained from Dr H. Jaeger (Onderstepoort Biological Products, South Africa). Wild-type isolates were obtained from Dr D. Verwoerd (Virology Division, Onderstepoort Veterinary Institute, South Africa). All virus isolates (including commercial vaccine strains) were propagated in embryonated SPF chicken eggs prior to extraction of RNA from the infected allantoic fluid samples. Haemagglutination (HA) titres of samples were tested before RT-PCR was executed (Alexander 1997b).

Enzymes and primers

AMV reverse transcriptase (RTase, Promega) and thermostable DNA polymerase (DyNaZYME, Finnzymes) were used for cDNA synthesis and PCR amplification respectively. Three sets of NDV specific primers (Table 1) were synthesized (Gibco BRL) according to the data of Kant *et al.* (1997): ALLs and ALLe were designed to hybridize to all NDV strains (Newcastle disease virus specific) and VLTe and AVLe (in combination with ALLs) were designed to hybridize to and amplify virulent and avirulent strains respectively.

Isolation of viral RNA

Infected allantoic fluid was centrifuged at 4 000 g for 5 min to remove cells. Viral RNA was isolated from 140 µℓ of the clarified supernatant using the QIAamp viral RNA purification kit (QIAGEN, Germany) according to the manufacturer's instructions and dissolved in 50 µℓ RNase-free ultra high quality (UHQ) water.

RT-PCR

Either Promega RT buffer (1X; 50 mM Tris-HCl pH 8.3 at 25 °C, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT) or DyNaZYME reaction buffer (1X; 10 mM Tris-HCl pH8.8 at 25 °C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100) were added to reaction mixtures comprising 1 µℓ AMV RTase (8 units/µℓ), 1 µℓ DyNaZYME polymerase (5 units/µℓ), 0.5 µℓ dNTP (10 mM nucleotide mix, Gibco BRL), 3 units RNase inhibitor (HPRI, Amersham), 20 pmol of each primer ALLs and ALLe together with 5 µℓ isolated viral RNA. The reaction was carried out in a Perkin Elmer GeneAmp PCR System 2400 as follows. RT was performed at 42 °C for 30 min, followed by inactivation of the RTase at 94 °C for 2 min. PCR amplification was performed at 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, with a 5 min 72 °C final elongation step. The PCR products were separated by 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining.

HA test

Haemagglutination (HA) tests were performed as specified in the OIE Manual of Standards (2000).

RESULTS

Conditions were sought wherein it would be possible to establish a single tube RT-PCR for NDV diagnosis. In the one-step approach, the presence of Triton X-100 in the buffer was found to be necessary for the activity of both RTase and *Taq* polymerase.

Subsequently, RNA extracted from allantoic fluid either mock-infected or infected with the LaSota vaccine strain, the Hitchner B1 vaccine strain, the Onderstepoort (Komarov) mesogenic strain or a field-isolated virulent NDV strain was used as a template for amplification with the ALLs primer in combination with the ALLe, VLTe or AVLe primers. The anticipated results were obtained (Fig. 1), namely amplification of a 362 bp fragment from all infected samples using the ALLs and ALLe primer set, and amplification of a 254 bp fragment from virulent or avirulent NDV-infected samples with ALLs and VLTe

TABLE 1 NDV-specific primers (Kant *et al.* 1997)

Primer	Sequence (5'–3')	Hybridization site on F gene	Amplicon length (bp)[1]: ALLs &
ALLs	TTGATGGCAGGCCTCTTGC	141–159	
ALLe	GGAGGATGTTGGCAGCATT	503–485	ALLe: 362
VLTe	AGCGT(C/T)TCTGTCTCCT	395–380	VLTe: 254
AVLe	G(A/G)CG(A/T)CCCTGT(C/T)TCCC	395–380	AVLe: 254

[1] Positive RT-PCR amplicons indicate the presence of NDV (using the ALLs & ALLe primer set), and whether it is a virulent (ALLs & VLTe primer set) or an avirulent strain (ALLs & AVLe primer set)

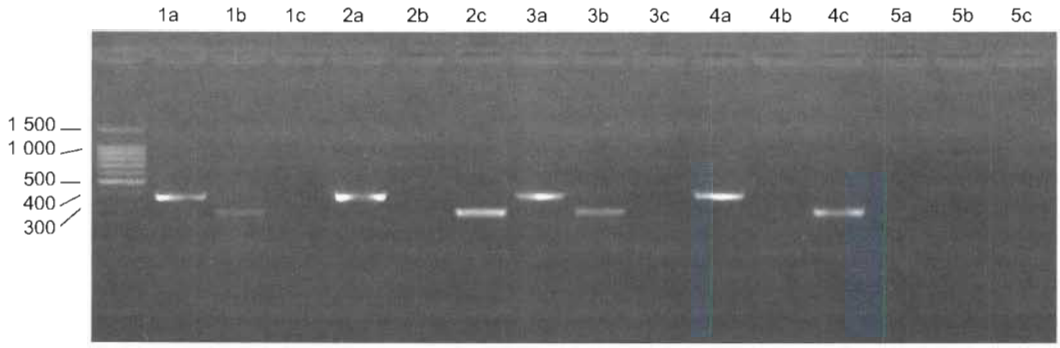

FIG. 1 One-step NDV RT-PCR detection (a lanes, using the ALLs & ALLe primer set) and NDV virulent (b lanes, using the ALLs & VLTe primer set) *versus* NDV avirulent (c lanes, using the ALLs & AVLe primer set) differentiation of RNA extracted from allantoic fluid infected with Onderstepoort (lanes 1), LaSota (lanes 2), and virulent (lanes 3) or avirulent (lanes 4) field-strains of NDV. PCR negative water controls (lanes 5) were included. The left-hand lane represents a 100 bp DNA ladder, with selected bands labeled

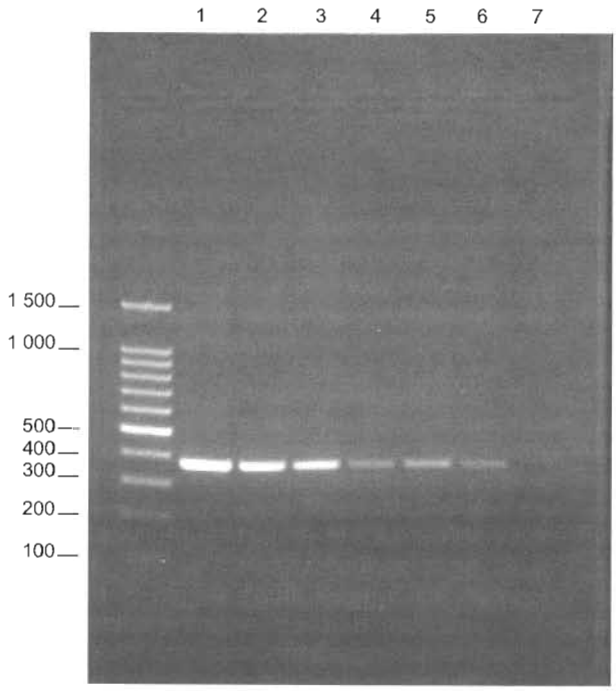

FIG. 2 Determination of the sensitivity of the one-step NDV RT-PCR. One-step RT-PCR was performed on RNA extracted from each 10-fold dilution (10^{-1} to 10^{-7}; lanes 1–7) of Onderstepoort NDV strain-infected allantoic fluid with an HA titre of 1:256. A DNA 100 bp ladder is represented in the left hand lane, with selected bands labeled

primer set and ALLs and AVLe primer set respectively.

The sensitivity of the one step RT-PCR in DyNAZYME buffer was determined by 10-fold serial dilutions of virulent NDV-infected allantoic fluid with a titre of 1:256 HA units.

RNA extracted from a 10^7 dilution and equivalent to 10^{-5} HA units, related to the amount of viral particles present, was found to be the minimum level detectable by this protocol (Fig. 2).

DISCUSSION

The conventional method used to distinguish NDV isolates from other haemagglutinating viruses such as the avian influenza virus, is the haemagglutination inhibition (HI) test for which a monospecific antiserum directed against NDV is required (Alexander 1997b). Furthermore, subsequent tests (MDT, ICPI, IVPI) are necessary to determine the virulent phenotype of the isolates in order to identify and confirm an outbreak of Newcastle disease. These require large numbers of SPF eggs and chicks and are extremely labour-intensive and time-consuming. In order to address these limitations, a two-step multireaction RT-PCR, in which a reverse transcription is followed by the polymerase chain reaction, was

developed (Kant *et al.* 1997, Oberdorfer & Werner 1998). This PCR format, however, is prone to contamination and is also labour intensive when a large number of samples are handled.

In this study, we report a rapid triple one-step RT-PCR in which reactions are carried out in a single buffer solution in a single tube and that is specific, rapid and an economical method for detecting NDV. This RT-PCR format can identify NDV as well as characterize the pathotype as being either virulent or avirulent. A multiplex format of this RT-PCR could be envisaged to simplify this procedure.

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