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SCIENCE DIRECT
Journal of Virological Methods 136 (2006) 224–229
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Detection of virulent Newcastle disease virus using a phage-capturing dot blot assay

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Received 22 February 2006; received in revised form 15 May 2006; accepted 16 May 2006
Available online 23 June 2006

Abstract

Newcastle disease virus (NDV) strains can be classified as virulent or avirulent based upon the severity of the disease. Differentiation of the virus into virulent and avirulent is necessary for effective control of the disease. Biopanning experiments were performed using a disulfide constrained phage displayed heptapeptide library against three pathotypes of NDV strains: velogenic (highly virulent), mesogenic (moderately virulent) and lentogenic (avirulent). A phage clone bearing the peptide sequence SWGEYDM capable of distinguishing virulent from avirulent NDV strains was isolated. This phage clone was employed as a diagnostic reagent in a dot blot assay and it successfully detected only virulent NDV strains.

Keywords: Filamentous phage; Dot blot; NDV; Pathotypes; Phage display

1. Introduction

Newcastle disease virus (NDV) poses a major threat to the avian species worldwide particularly to domestic poultry. NDV can be grouped into three pathotypes based on the severity of disease; the viscerotropic or neurotropic velogenic strains are highly contagious, causing severe intestinal lesions or neurological disease, resulting in high mortality of flocks; the mesogenic strains cause respiratory and nervous signs with moderate mortality; the lentogenic strains cause only mild and imperceptible respiratory infection (Yusoff and Tan, 2001). The velogenic and mesogenic strains are both virulent and have been frequently identified as the causative agent of outbreaks in many countries. On the other hand, the lentogenic strains are avirulent and have been used as live vaccines to control the disease. However, current diagnosis of NDV is unable to differentiate virulent NDV in a vaccinated flock of chicken. The ability to distinguish the virulent NDV strains in flocks is important because international veterinary regulatory bodies require a definitive diagnosis of virulent NDV to enable effective prevention of an outbreak by strict control measures and trade embargo restrictions (Aldous and Alexander, 2001).

Conventional methods used for pathotyping NDV strains involve the use of inoculated embryonated chicken eggs or chicks from which parameters such as mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) are determined (Alexander, 2001). These methods are laborious and time consuming. Over the past decade, numerous molecular techniques have been employed to identify and delineate NDV pathotypes: polymerase chain reaction (PCR) coupled to restriction enzyme digestion (Stauber et al., 1995); PCR-sequencing (Seal et al., 1995); a triple one-step PCR (Wang et al., 2001), reverse transcription (RT)-nested PCR coupled with ELISA detection (Kho et al., 2000) and real-time PCR (Aldous and Alexander, 2001; Tan et al., 2004). In addition, monoclonal antibodies against the haemagglutinin-neuraminadase (HN) and fusion (F) proteins have been produced via hybridoma technology for pathotyping NDV strains (Alexander and Manvell, 1997).

Most recently, we have shown that a filamentous M13 bacteriophage displaying a peptide bearing the TLTTKLY sequence isolated from a phage displayed peptide library against the velogenic NDV strain AF2240 could be used to differentiate

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doi:10.1016/j.jviromet.2006.05.017

velogenic NDV strains from mesogenic and lentogenic strains via a newly established indirect phage ELISA (Ramanujam et al., 2004). In the present study, we employed a new biopanning strategy for the selection of a phage displayed heptapeptide capable of distinguishing virulent (velogenic and mesogenic) from avirulent (lentogenic) NDV strains. The phage clone was further developed as a capture reagent in a phage dot blot assay to differentiate virulent from avirulent NDV strains.

2. Materials and methods

2.1. Avian viruses

The pathotypes of the NDV strains used in this study were determined by MDT test and nucleotide sequence analysis of the F cleavage site. The velogenic strains were AF2240, Ijok, 986/91 P1, 3410/91 P2, 6270/92 P3, 4059/91 P2, 3147/89 P2. The mesogenic strains comprised 2641/91 P2 and 01/C, and the lentogenic strains were S, 00/IKS, 4989/92 P3, 5953/89 P3, Ulster 2C, V4 Queensland, Hitchner B1, La Sota, F, V4 UPM, 5270/89 P3, 8820/92 P3, 1266/89 P3, 5147/91 P2, 4083/92 P3 and 5731/88 P4. Other avian viruses such as chicken anaemia virus (CAV), fowl pox virus (FPV) and avian influenza virus (AIV) were included as negative controls.

2.2. Mean death time (MDT) assay

The MDT assay was performed as previously described (Alexander, 1988). Briefly, 0.1 ml of 10-fold dilution series between 10^{-6} and 10^{-9} infected allantoic fluid was inoculated into the allantoic cavity of five 9–10 days-old embryonated chicken eggs and then incubated at 37 °C. These steps were repeated once more with five eggs 8 h later and incubated at 37 °C. Each egg was examined twice daily for 7 days and the time of embryo deaths were recorded. NDV strains were classified into the following groups: velogenic (taking under 60 h to kill); mesogenic (taking between 60 and 90 h to kill); lentogenic (taking more than 90 h to kill).

2.3. PCR and nucleotide sequence analysis

Viral RNA was extracted from NDV infected-allantoic fluid (500 μl) using the Trizol® reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The RNA-pellet was resuspended in diethylpyrocarbonate (DEPC: 0.1% v/v) treated distilled water (15 μl). cDNA generation and reverse transcription-polymerase chain reaction (RT-PCR) of the F cleavage site were performed as previously described (Kho et al., 2000). The F gene cDNA fragment and PCR product were amplified using the avian murine virus reverse transcriptase (AMV-RT; Promega, Madison, WI, USA) and *Taq* polymerase (Promega, USA), respectively. Sequencing of the PCR products was performed using the CEQ dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) and sequencing products were analyzed on the CEQ8000 automated sequencer (Beckman Coulter, USA).

2.4. Propagation and purification of NDV strains

All of the NDV strains were propagated in allantoic fluid of 9–11 days-old embryonated chicken eggs at 37 °C for 3 days. The allantoic fluid was harvested and the presence of virus was confirmed by the haemaglutination test (Alexander, 1988). The allantoic fluid was directly used in the phage dot blot assay. NDV strains AF2240, 2641/91 P2 and V4 Queensland were purified as previously described (Yusoff et al., 1996) and used in the biopanning.

2.5. Biopanning of displayed peptide library against NDV

Purified NDV strains AF2240, 2641 and V4 Queensland (1.5 μg) in TBS (50 mM Tris–HCl; 150 mM NaCl; pH 7.4; 100 μl) buffer were coated onto three different microtiter plate wells and incubated overnight at 4 °C. The wells were washed three times with TBST (TBS supplemented with 0.1% Tween-20) and incubated for 2 h at room temperature with 5 mg/ml bovine serum albumin (BSA: 300 μl). After three washes with TBST, approximately 10^{11} pfu of phage particles (7-*mer* disulfide-constraint phage display library diluted in TBS-buffer; 100 μl; New England Biolabs, Ipswich, MA, USA) were added to each well. After 1 h incubation at room temperature, the wells were washed vigorously with TBST six times. Bound phages were eluted by incubating with elution buffer [0.2 M HCl–glycine (pH 2.2), 1 mg/ml BSA; 120 μl] for 5 min at room temperature and immediately neutralized with 1 M Tris–HCl, pH 9.1 (15 μl). Log phase *Escherichia coli* ER2738 (New England Biolabs, USA) cells were infected with the phage eluate and titered using a standard plaque assay as described (Smith, 1985). The amplified phage particles were subjected to biopanning for a further two rounds. Binding of phage clones to NDV coated on microtitre wells was determined by indirect phage ELISA as described below.

2.6. Indirect phage ELISA

Binding of phage clones to purified or unpurified NDV coated on microtiter wells was determined by indirect phage ELISA as described by Ramanujam et al. (2004) with some modifications. Briefly, NDV (1.5 μg in TBS; 100 μl) was coated onto microtiter plate wells and incubated overnight at 4 °C. The wells were washed three times with TBST and blocked with BSA (5 mg/ml; 300 μl) by incubating at 4 °C for 2 h. After six washes with TBST, phage suspension (10^{10} pfu/ml; 100 μl) was added and incubated for 1 h at room temperature. The wells were then washed vigorously with TBST six times and anti-M13 monoclonal antibody conjugated to horseradish peroxidase (HRP, Amersham Pharmacia, Sweden; 1:2500 dilution; 200 μl) was added. After 1 h incubation at room temperature, the wells were washed vigorously with TBST six times and then added with 2,2-azino-di(3-ethylbenthiazoline) sulfonic acid diammonium salt (ABTS: 0.2 mg/ml) in citrate–phosphate buffer (28.6 mM anhydrous citric acid, 41.2 mM $Na_2HPO_4{\cdot}7H_2O$; 200 μl). Absorbance was measured at 405 nm with a spectrophotometer (Model 550; Bio-Rad, USA).