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Full Length Research Paper

An alternative method for the establishment of virulence of Newcastle disease virus isolates

Charlotte Enastacia Boucher*, Robert Richard Bragg and Jacobus Albertyn

Department of Microbial, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Science, University of the Free State, P. O. Box 339, Bloemfontein 9300, Republic of South Africa.

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Newcastle disease virus (NDV) causes major losses in the poultry industry and is regarded as endemic in many parts of Africa. Differences in virulence of the virus occur and during any disease outbreak, determining virulence is essential for effectively controlling the disease. The virulence of the virus is dependent on cleavage of the fusion site and is characterised by different sequences in the genome. Thus a reliable and rapid method to determine virulence is to sequence the fusion site. The alternative method is to do a conventional mean death time (MDT) study, which is time consuming. Furthermore, the molecular techniques required for the sequencing of the fusion glycoprotein are not within reach of many regional laboratories in developing countries, where NDV is a serious problem. Thus a simple method is described in which the virulence of a NDV field virus can be determined, using conventional MDT methods, more rapidly. In this study viral samples were treated with 0.25% trypsin free of EDTA, and the MDT was compared to untreated control samples. Results indicate that if the viral isolate is lentogenic in nature, and treated with 0.25% trypsin it caused mortalities in eggs within 70 h post inoculation, whereas control samples resulted in mortalities from 96 h. By using this technique, the time to identify lentogenic isolates is substantially reduced.

Key words: Newcastle disease virus, rapid diagnosis, mean death time, fusion site, trypsin, pathogenicity, virulence.

INTRODUCTION

Newcastle disease virus (NDV) exhibits a wide range of pathogenicity and virulence, which is directly related to the cleavability of a precursor protein (F_0) of the fusion glycoprotein by cellular proteases (Glickman et al., 1988). Cleavage of the precursor glycoprotein F_0 to F_1 and F_2, by host cell enzymes, is required for the progeny virus to become infective (Zorman et al., 2002; Glickman et al., 1988; Morrison, 2003). Dibasic amino acids, surrounding the glutamine residue at position 114 are present in the F protein cleavage site of mesogenic and velogenic isolates. Lentogenic NDV isolates lack this motif (Zorman Rojs et al., 2002; Glickman et al., 1988; Morrison, 2003). As a result, the lentogenic strains can only replicate in areas with trypsin-like enzymes such as the respiratory and intestinal tract, whereas the virulent viruses can replicate in a range of tissues and organs frequently resulting in a fatal systemic infection (Zorman Rojs et al., 2002; Seal et al., 2000; Jestin and Jestin, 1991; King and Seal, 1998; Morrison, 2003; Panda et al., 2004).

Newcastle disease virus is one of the most important diseases in poultry worldwide (Seal et al., 2000). Its importance is mainly due to the resulting economic losses that occur upon infection with virulent strains (Verwoerd, 1997).

The first recorded outbreak of Newcastle Disease in South Africa (SA) occurred in May 1945, Natal (now

*Corresponding author. E-mail: boucherce@ufs.ac.za. Tel: (+27 51)401 2274. Fax: (+27 51) 401 9376.

Abbreviations: NDV, Newcastle disease virus; **MDT,** mean death time**; F,** fusion; **RT- PCR,** reverse transcription polymerase chain reactionprotein; **ICPI,** intracerebral pathogenicity index; **IVPI,** intravenous pathogenicity index; **SPF,** specific pathogen free.

KwaZulu Natal Province) (Kaschula et al., 1946), and diagnosis was confirmed with Heamagluttination inhibition tests (HI) (Kaschula et al., 1946). There is no scientifically published literature on NDV outbreaks in SA hence the reliance on newspapers.

A subsequent outbreak also occurred in Durban (Kwazulu Natal Province) in 1978, killing thousands of birds (Natal Mercury: 2/10/1978), and in 1985, a 10 to 12% loss in an estimated 2 million chicken per month production rate due to ND was also reported in Durban/ Natal (The Citizen: 23/10/1985).

In 1994, a countrywide enzootic outbreak of NDV was reported in the majority of the provinces in SA. In the Eastern Cape Province, a mortality rate of 90% was reported (Eastern Cape Herald: 25/08/1994). A total of three million chickens died during this outbreak, which led to a shortage of poultry and eggs with an accompanying increase in prices (Eastern Cape Herald: 13/09/1994). In 2006 an outbreak of NDV was again reported in the Eastern Cape in the villages of Frankfort and Peelton, killing off hundreds of chickens. A Spokesperson, representing the ARC said, “That these birds were infected the previous year and that the results can only be made available during this year” (Daily Dispatch Newspaper: 01/02/2006).

Outbreaks were also reported in the Ndanga district (The Southern Province of Masvingo) in Zimbabwe. It was the second outbreak to hit Zimbabwe in that year. It was feared that it would hamper the country’s multimillion-dollar poultry and ostrich industry (The Star: 10/06/1994).

Thus, we can conclude that a rapid and effective technique is required as a preliminary test to diagnose ND isolates, which gives results in a period of less than seven days.

One of the most characteristic properties of different strains of NDV has been their great variation in pathogenicity (Manual of Standards for Diagnostic Tests and Vaccines, 2000). Current conventional tests used to determine the pathogenicity of NDV isolates includes Mean death time (MDT) assays, intracerebral pathogenicity index (ICPI) and Intravenous pathogenicity index (IVPI) (Alexander, 2000). Based on the severity of the disease, NDV isolates can be grouped into five pathothypes (Zorman Rojs et al., 2002); lentogenic, mesogenic, velogenic, vicetropic velogenic, neurotropic velogenic and asymptomatic pathotypes (Alexander, 2000).

Reverse transcription coupled to polymerase chain reaction (RT-PCR) is the method of choice to amplify the F protein gene sequences (Glickman et al., 1988; Jestin and Jestin, 1991; King and Seal; 1998; Morrison, 2003; Seal et al., 2000; Panda et al., 2004). Although this method is efficient, it is expensive and is not always available in laboratories that are basically equipped.

Thus an effective, easy and relatively cheap method, which is aimed at laboratories in the developing communities that are fundamentally equipped, is required to rapidly classify the pathogenicity of field isolates.

MATERIALS AND METHODS

Virus isolates and molecular identification

NDV Field Isolates were obtained from the Poultry Reference Laboratory, University of Pretoria, South Africa. The NDV field strains were collected during outbreaks of the disease dated 1995 to 2005 in South Africa.

The isolates represent various pathogenicity groups; velogenic, mesogenic and lentogenic, with lentogenic and velogenic control strains LaSota® and Texas GB (Central Veterinary laboratories, Weybridge, UK). The virulence of these strains was determined by MDT and molecular sequencing. For this study Velogenic isolates are represented by field isolates 300/05, 411/04 and 1186/04a together with control strain Texas GB. Lentogenic isolates are represented by field isolate 1297/98 together with control strain LaSota®. The mesogenic isolates are represented by field isolates F1018/97 and F842/97.

These isolates were subjected to RT-PCR and sequencing to confirm identity and virulence. RNA extractions were performed using a one-step reagent, Tri-Reagent™ (Bio Basic Inc, SA). The fusion protein cleavage site amplification was accomplished by means of nested PCR. Firstly, a 1008 base pair (bp) fragment was amplified using primers K1 (R) 5’- GGG RAA GAR AGT GAC WTT TGA CA-3’ and K2 (F) 5’- TKG GAT AAW CCR YYR GTG ACC TC-3’ amplifying a 1008bp fragment. This product was used as template in a second round of amplification using primers MV1 (R): 5’- CCY RAA TCA YYR YGR YRC YRG ATA A-3’and B2 (F): 5’- KCR GCR TTY TGK KTG KCT KGT AT-3’ (Fermentas, SA), amplifying a 400bp fragment, indicative of the fusion protein. To enable sequencing of the PCR product, the amplicons were ligated into pGEM™ TEasy vector (Promega, SA) and transformed into competent *Escherichia coli (E. coli)* cells. The isolated clones were analyzed for the presence of the insert of interest by restriction enzyme analysis. Clones containing the inserts of interest were subjected to sequencing using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, SA).

The sequence data was assembled using AutoAssembler and reverse complimented and aligned using DNAssist V2.2 (http://www.dnassist.org/dnassist.html). Analyzed sequences were compared to known NDV fusion protein sequences using nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov).

Mean death time (MDT) assays

MDT[1] assays were performed for all isolates as described by Hanson (1975). A dilution range of 10^{-1} to 10^{-9} was prepared. Egg mortalities were observed within the dilution range of 10^{-1} up until 10^{-6}, with no mortalities observed at higher dilutions. All other test was thus performed at a dilution value of 10^{-6}, as this is the minimum lethal dose to kill the embryos. The MDT assay was slightly adapted for this study by adding exogenous trypsin in a 1:1 v/v ratio to the original virus suspension; as described below: The infective allantoic fluid obtained when the virus samples were propagated in SPF embryonated eggs (ARC, Glen, Bloemfontein, SA) was incubated for 30 min at room temperature with 0.25% trypsin free of EDTA. A dilution of 10^{-6}, of the infective allantoic fluid, containing trypsin, was made. A total of 5 SPF embryonated eggs per isolate was inoculated for viral cultivation and incubated at

[1] The MDT is the average time in hours for the minimum lethal dose (the highest dilution at which all the eggs die; EID_{50}) to kill the embryos (Alexander, 1991).

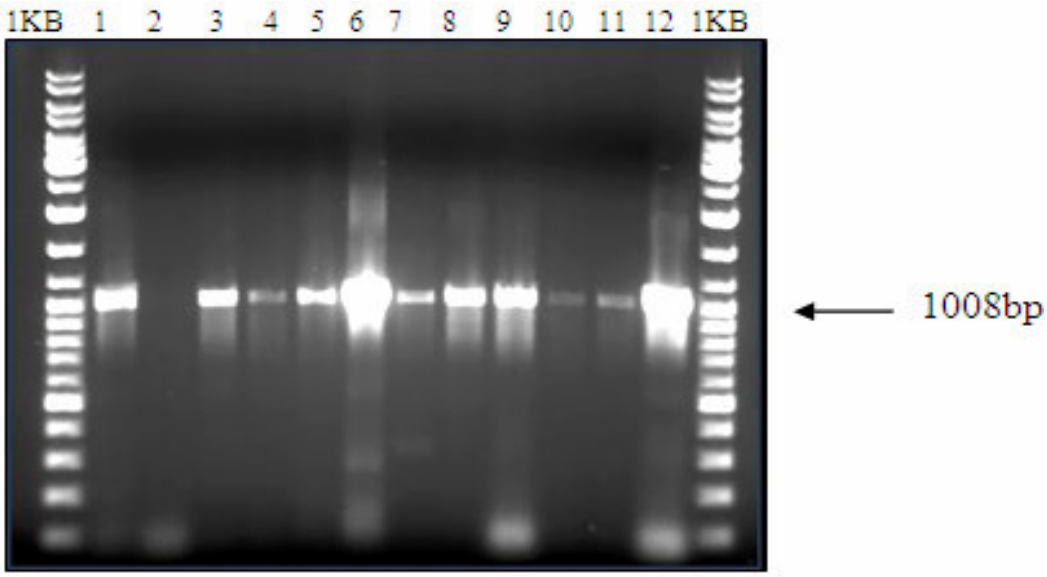

Figure 1. RT- PCR products observed after amplification using primers K1 and K2. 1 KB-O'GeneRuler ladder™ (fermentas); Lane 1: LaSota® (control); Lane 2: D1446/95; Lane 3: 411/04; Lane 4: 300/05; Lane 5: F1018/97; Lane 6:1297/98; Lane 7: 995/98; Lane 8: 118/04; Lane 9: F842/97; Lane 10 and 11: 834/05; Lane 12: Texas GB (control)

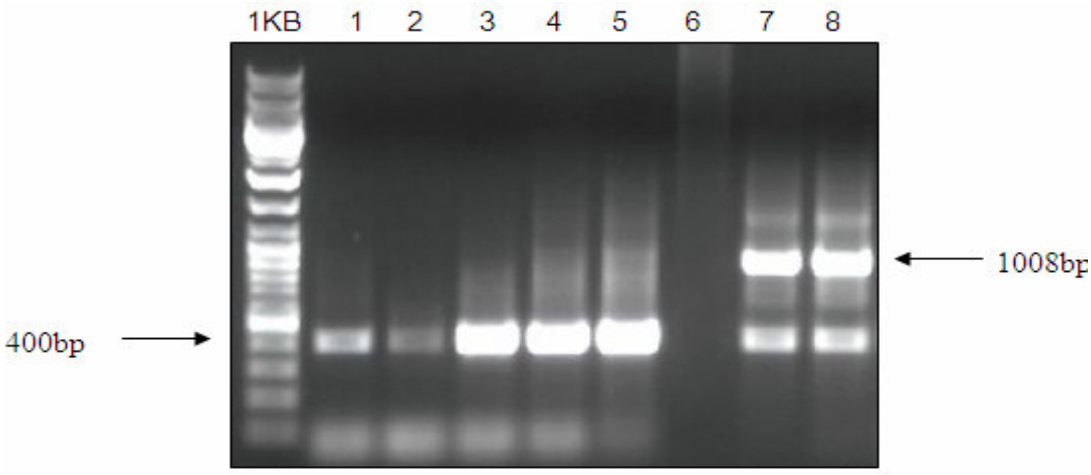

Figure 2. Nested PCR amplification product of field isolates with primers MV1 and B2. 1KB-O'GeneRuler Ladder (fermentas); Lane 1: 411/04; Lane 2: 300/05; Lane 3: F1018/97; Lane 4 and 5: 1297/98; Lane 6: D1446/95; Lane 7: 1186/04 and Lane 8: F842/97.

37℃. The remaining virus suspensions were incubated at 4℃ for 8 h. Thereafter, a second batch of SPF eggs were inoculated and incubated under the same conditions. The eggs were examined twice daily for a maximum period of 7 days by candling the eggs and embryo death times were recorded.

RESULTS

Positive amplification was obtained for all sets of primers (Figures 1 and 2). Blast searches conducted with obtained sequences, resulted in four isolates (1297/98; F842; 411/05; 1186/04) showing high homology with known NDV isolates/strains (results not shown) and from these results the deduced amino acid sequences at the F2/F1 cleavage site of strains were obtained for positively identified NDV isolates (Table 1). With the positively identified isolates MDT studies were performed. A definite difference in the time required to kill the embryos is observed when exogenous trypsin is incubated with lentogenic isolates. No difference was observed for

Table 1. Nucleotide and correlating amino acid sequence for the deduced amino acid sequences at the F2/F1 cleavage site of strains positively identified as NDV.

Isolate	Nucleotide sequence	Amino acid sequence	Virulence*
1297/98	GGG AGA CAG GGG CGC CTT	G R Q G R L	Lentogenic
F842/97	GGG AGA CAG AAA CGA CTT	G R Q K R L	Lentogenic
411/05	AGG AGA CAG AAA CGC TTC	R R Q F R F	Velogenic
1186/04	AGG AGA CAG AAA CGA TTT	R R Q K R F	Velogenic

*L-Lentogenic represented by a monobasic amino acid sequence. V-Velogenic represented by a dibasic amino acid sequence.

Table 2. For MDT's five (5) eggs per isolate was inoculated with 0.25% trypsin in a 1:1 v/v ratio. The same test was repeated 8 h later, results were observed and the mean value for the specific time frame was recorded.

Time (h)	0	24	48	72	96	120	144	168
LaSota® (control-lentogenic)	0	0	0	0	1	1	1	2
LaSota®+ 0.25% trypsin	0	0	1	4	-	-	-	-
Texas GB (control-velogenic)	0	0	2	3	-	-	-	-
Texas GB + 0.25% trypsin	0	0	2	3	-	-	-	-
F842/97	0	0	0	0	1	2	2	-
F842/97 + 0.25% trypsin	0	0	2	2	1	-	-	-
1297/98	0	0	0	1	1	2	1	-
F1297/98 + 0.25% trypsin	0	0	2	2	1	-	-	-
1186/04	0	0	2	3	-	-	-	-
1186/04 + 0.25% trypsin	0	0	3	2	-	-	-	-
411/04	0	0	2	3	-	-	-	-
411/04 + 0.25% trypsin	0	0	2	3	-	-	-	-

velogenic isolates. The mean values for the MDT's obtained were recorded (Table 2).

DISCUSSION

Amplification of the fusion protein cleavage site was achieved by means of a nested PCR. First round PCR amplification was performed using primer pair K1 and K2, in this step total RNA was reverse transcribed into cDNA. A good yield of the amplification product, a band of approximately 1008bp, was obtained, as indicated by the intensity of fluorescence Figure 1. The bands were purified from the gel and were subjected to a second round PCR step (nested PCR) using primer pair MV1 and B2. A smaller fragment of 400bp was obtained (Figure 2). Positive amplification was obtained for six (1297/98; 411/05; 300/05; 1186/04; F1018/97; F842/97) isolates as well as for the control strains LaSota® and Texas GB. Isolate 1186/04 and F842/96 (Figure 2), gave banding patterns of both the 1st and 2nd amplification product. This could be that the 1st product was highly concentrated and only a certain concentration was amplified, resulting that the first product is re-obtained.

Thus, sequencing the F protein region gives a clear indication of the level of virulence of the different isolates. Isolates 411/05; 1186/04 are velogenic, they display the characteristic dibasic amino acid residues at the fusion cleavage site compared to isolates 1297/98 and F842/97, which displays a monobasic sequence a characteristic of lentogenic isolates (Table 1) (Collins et al., 1993). The presence of the phenylalanine residue after the point of cleavage is characteristic of velogenic and mesogenic strains.

In this study 0.25% trypsin-free of EDTA in a 1:1 (v/v) ratio was added to lentogenic isolates as well as mesogenic and velogenic isolates. A MDT study was done, where the control reaction was represented by a MDT analysis as described by Hanson, (1975). The trypsin concentration was based on the recommended concentration to trypsinize cell cultures and it is free of EDTA, as this compound is toxic to cell cultures (personal communications; NCL, UFS, Bloemfontein, SA).

As indicated in the results (Table 2) there is a definite difference in MDT's obtained. For the control isolate LaSota®, the mean times decreased from 168 to 72 h. There was no significant difference for the Texas GB isolates.

Looking at the effect of trypsin on field isolates, 1297/98 and F842/97 also indicated a significant time decrease

compared to untreated isolates, again no significant difference was observed for velogenic isolates 411/05 and 1186/04. This clearly indicates that lentogenic isolates are susceptible to cleavage by trypsin-like enzymes. Upon opening of the infected embryos, haemorrhaging was also observed for lentogenic isolates treated with trypsin (results not shown). This was a clear indication that replication occurred in various organs and cells.

Conclusion

MDT analysis are laborious and time consuming to identify lentogenic isolates as this can take up to 7 days (168 hours), the proposed method is still laborious, but results are obtainable within 4 days. This reduction in time is required to make a reliable diagnosis on the virulence of the virus. This can assist in the control of the disease by allowing control strategies to be implemented sooner. It is important to obtain results in such a short period of time so that effective measures can be taken.

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