



Comparison of three techniques used to characterize Newcastle disease virus isolated from chickens treated with aqueous extract of *Momordica balsamina*

SE Mantip*, EA Ogunsan, IS Tekki, J Okpara, W Bertu, M Gyang, PE Emennaa, K Adamu, R Okosi & DO Ehizibolo

National Veterinary Research Institute, P. M.B. 01, Vom. Plateau State, Nigeria.

*Correspondence: Tel.: +2348066656916, E-mail: samantipx@yahoo.com

Abstract

Despite the efforts made by stake holders towards eradication of pathogens from poultry flocks, Newcastle disease virus infection is still of continuing economic concern in Nigeria. Consequently, adapting prompt, sensitive and inexpensive diagnostic techniques will without doubt clamp down on the menace of the disease. Characterization of Newcastle disease virus isolated from laying chickens (layers) receiving aqueous extract of balsam apple (*Momordica balsamina*) was done using the intra-cerebral pathogenicity infectivity (ICPI) test, mean death time of the minimum lethal dose (MDT/MLD) and the polymerase chain reaction (PCR) techniques. The presence of Newcastle disease (ND) virus was established using the haemagglutination (HA) and haemagglutination inhibition (HI) test from samples of trachea, lungs, liver, heart, proventriculus and intestines of chicken carcasses necropsied. Polymerase Chain Reaction (PCR) has a higher advantage over other techniques with respect to sensitivity while in terms of cost and availability of materials; the mean death time and intra-cerebral pathogenicity infectivity have higher advantage over PCR.

Keywords: Characterization, mesogenic, *Momordica balsamina*, Newcastle disease.

Introduction

Newcastle disease (ND) is an acute rapid spreading contagious, disease of birds of all ages, the disease is categorized as velogenic, lentogenic, mesogenic or apathogenic, depending on the degree of virulence (Hanson & Brandy, 1995). It is caused by Newcastle disease virus (NDV), belonging to the genus *paramyxovirus*, in the family *paramyxoviridae* (David West, 1972). The disease was first described by Doyle in Newcastle upon Tyne, in England, in 1927 (Baudette & Black, 1946), and it is usually associated with gasping as a result of respiratory discomfort (avian pneumoencephalitis) (Beach, 1942) and twisting of the neck (torticollis) as a result of brain infection (encephalitis) (Hitchner & Johnson, 1948).

Haemagglutination (HA) and haemagglutination inhibition (HI) tests are commonly employed in the diagnosis of the disease (Hill, 1953; Crowther, 1964), while relatively newer techniques include intracerebral pathogenicity infectivity (ICPI) test, mean death time of the minimum lethal dose (MDT/MLD) and characterization by molecular biology using the polymerase chain reaction (PCR).

Momordica balsamina (Grieve, 1995), also called balsam apple (Ejirin-dudu in Yoruba and Daddagu in Hausa languages), is a plant commonly found in the dry

savannah and clearings in secondary push of Northern Nigeria (Mgbojikwe *et al.*, 2002; Bot *et al.*, 2006). It is a climber or trailer with an annual stem length of 4-5 metres and belongs to the family *Curcubitacea* (Grieve, 1995). The fruit is orange-yellow beaked, 2½ inches in length containing red-brown seeds; and is easily recognizable by its colorful spike-like projections (Hutchinson, 1954). The fruit pulp is commonly used by poultry farmers in Plateau State, Nigeria for general well being of birds (Mgbojikwe *et al.*, 2002).

In this study, we intended to access the respective merits and demerits of ICPI, MDT/MLD and PCR in the characterization of Newcastle disease.

Materials and Methods

Case History

A poultry farmer in Bukuru, Jos South Local Government Area of Plateau State raising 5,000 birds of which 3,000 were pullets, observed morbidity and mortality rates of 15 and 3%, respectively, in one of the pens and subsequently reported same to the ECWA Veterinary Clinic, Bukuru, as well as the Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI), Vom, for diagnosis and treatment. The birds were 17 weeks old and the breed was Shika Brown breed. They were on deep litter system and had all the

necessary vaccines except the second dose of Newcastle disease vaccine, (Komarov). Consequently, the birds were placed on Neomycin, Chloroamphenicol, Oxytetracycline Mix (NCO-Mix® by ANUPCO U.K for Polons Investment Company, Nigeria) at a dose rate of 20mg/kg weight in drinking water for 5 days. Worried by the morbidity rate, the client further introduced aqueous extract of *Momordica balsamina* fruit pulp in drinking water *ad libitum* during the day, for 3 days. A visit to the farm by a team from the National Veterinary Research Institute for on the spot assessment and further sample collection revealed the following signs: gasping, torticollis, coughing, tracheal rales, dyspnea, greenish diarrhoea, ruffled feathers, cloudy eyes and prostration in some of the birds. However, the owner reported that mortality reduced by above 60% in each pen following introduction of *M. balsamina* fruit.

Postmortem examination of 10 carcasses presented to the Central Diagnostic Laboratory of the NVRI Vom, revealed haemorrhages on the proventricular mucosa, congested kidneys, atrophic Bursa of Fabricius and spleen. Ulceration of the ilio-cecal mucosa was also prominent. Samples of trachea, lungs, liver, heart, proventriculus and intestinal segments were aseptically collected and preserved in 50% glycerin for viral identification, isolation and characterization. Three methods viz: ICPI, MDT/MLD and PCR were used in the characterization of NDV associated with the disease process.

ICPI

Ten percent (10%) suspension (w/v) of the virus isolate collected was prepared in sterile phosphate buffered saline (PBS) at pH 7.5, and 100 µl of the suspension was aseptically inoculated intracerebrally (i/c) to each of 10 (day-old) cockerel chicks. The birds were then observed for clinical signs and death patterns. In each day of incubation, numbers of sick chicks were recorded and multiplied by one (1), which is the variant for sick chick. Daily death and previous deaths were summed-up and multiplied by two (2) which is the variant for dead chick. Total scores of sick chicks and dead chicks were summed up as indicated on Table 1. The ICPI was computed after day 8 from the commencement of the study, where the sum total scores of the number of sick and dead chicks when computed, totaled 90 (Table 1).

Table 1: (ICPI) Daily observation

Day	No sick	score	Daily death	Previous death	score	Total score
1	0	0	0	-	0	0
2	3X1	3	1	-	X2=2	3+2=5
3	4X1	4	2	+1	X2=6	4+6=10
4	3X1	3	1	+3	X2=8	3+8=11
5	1X1	1	3	+4	X2=14	1+14=15
6	1X1	1	0	+7	X2=14	1+14=15
7	1X1	1	1	+7	X2=16	1+16=17
8	1X1	1	0	+8	X2=16	1+16=17
					Total	90

Note: 0=normal chick, 1=chick chick, 2= dead chick

MDT/MLD

A ten-fold serial dilution of the isolate suspension ranging from 10^{-6} to 10^{-10} was inoculated into 5 embryonated eggs each, sealed and incubated at 37°C. Inoculation took place at specified time of 8 a.m (representing morning incubation) and at 4 p.m (representing afternoon incubation). Another set of 5 uninoculated eggs served as control. All eggs were candled after every 12 hours of incubation for 7 days and dead eggs were separated from viable eggs and counted.

PCR

The tissue samples collected from postmortem lesions were subjected to conventional PCR to confirm the presence of NCD virus. An ALL/Alle primer set which gives a 362 base pair (bp) PCR product for ND virus was used to determine the presence of NDV mesogenic strain and to verify the 362 bp Amplicon size of the ND virus.

Results

HA and HI Tests

The samples of brain, trachea, lungs, proventriculus and intestines subjected to the HA test were positive for Newcastle disease virus with titres of 1:64, 1:512, 1:128 and 1:128 respectively. The result of the haemagglutination inhibition test from a twofold serial dilution of collected sera revealed titres of 1:512, 1:2048, 1:2048 and 1:2048 in pens A, B, C and layer's pen respectively.

Intracerebral Pathogenicity Infectivity (ICPI) Test

The ICPI daily observations are presented in Table 1, and the survival index was found to be 1.125, which classifies the isolate as a Mesogenic ND virus.

Mean death time of the minimum lethal dose (MDT/MLD)

Number of death eggs and number positive for haemagglutinating activity by spot test from the dead eggs were recorded on 12 hourly basis for 7days as indicated on Table 2.

Table 2 indicates numbers of eggs inoculated by diluted sample. The MDT of morning and afternoon observations were 60.0 and 67.2 hours respectively, the average of which was 63.6 hours. The isolates were therefore considered as mesogenic ND virus because the average MDT of mornings and afternoons is within the range of 60-90.

Table 2: Mean time of the minimum lethan dose daily observation (hour)

Sample dilution	No of eggs inoculated	12 hrs Dd. +ve	24 hrs Dd. +ve	36 hrs Dd. +ve	48 hrs Dd. +ve	60 hrs Dd. +ve	72 hrs Dd. +ve	96 hrs Dd. +ve	
Morning									
10 ⁻⁶	5	- -	1/5 0/1	- -	¾ 2/3	0/1 0/1	1/1 1/1	- -	
10 ⁻⁷	5	- -	1/5 0/1	- -	¾ 2/3	1/1 1/1	- -	- -	
10 ⁻⁸	5	- -	- -	- -	2/5 0/2	1/3 1/1	1/1 1/1	- -	
10 ⁻⁹	5	- -	- -	- -	2/5 0/2	0/3 -	- -	2/3 0/2	
10 ⁻¹⁰	5	- -	2/5 0/2	- -	0/3 -	0/3 -	- -	- -	
Afternoon									
10 ⁻⁶	5	- -	- -	- -	- -	3/5 3/3	- -	- -	
10 ⁻⁷	5	- -	- -	- -	- -	1/5 1/1	- -	- -	
10 ⁻⁸	5	- -	1/5 0/1	¼ 0/1	0/3 -	- -	- -	- -	
10 ⁻⁹	5	- -	- -	- -	- -	- -	- -	- -	
10 ⁻¹⁰	5	- -	- -	- -	- -	- -	- -	- -	

Note: Dd=Death

+ve=positive for haemagglutination activity by spot test.

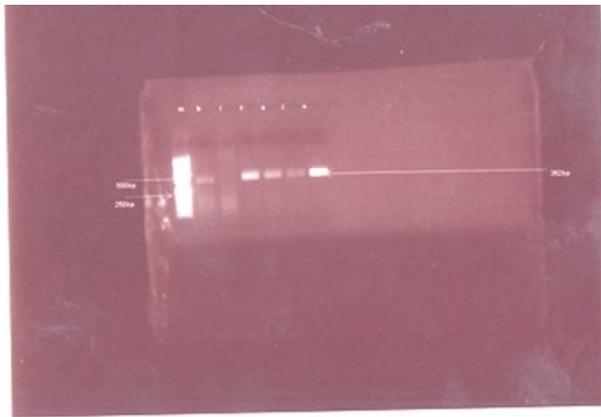


Figure 1: Polymerase Chain Reaction SDS gel photograph result

NOTE: B –brain, I – intestine, T – trachea, S – spleen, L- lung, PC - positive control NC - negative control

Polymerase Chain Reaction (PCR)

An ALL/Alle primer set which gives a 362 bp PCR product for ND virus was used to determine the amplitude of the various tissue tested in which a 50 bp DNA marker (m) verified the 362 bp Amplicon size of the ND virus as mesogenic.

As noticed in figure 1, brain, trachea, spleen and lungs samples were positive in the pattern of the positive control lane, while intestine was negative in the pattern of the negative control lane.

Discussion

Mesogenic Newcastle disease can be severe, especially in young chickens, but mild in adult birds as described by Baudette & Black (1946). Attenuated strains of

mesogenic ND virus have been used as vaccines in older birds because they give a longer duration of immunity than lentogenic ND virus (Crowther, 1964; Hitchner & Johnson, 1984).

In analyzing the MDT, when the figure is less or equal to 60, the virus is velogenic, 60-90 indicates a mesogenic strain and when the figure is from 90 and above, the virus is a lentogenic strain (David West, 1972). The isolate was thus classified as a mesogenic ND virus because the MDT at 8:00 a.m. and at 4:00 p.m. were 60 and 67.2 respectively, yielding the average MDT of 63.6. For the ICPI, a mesogenic ND virus usually has high mortality with a survival index range of 1.1-7.5 (Kaschuca, 1961). After computing the observation, the survival index was found to be 1.125 which classifies the

virus isolates as a mesogenic ND virus. For the molecular biology analysis, the 50 bp DNA marker (m) used to verify the 362 bp amplicon size also indicated a mesogenic strain of ND-virus. In comparing the three methods used in characterization of the ND isolate, criteria such as sensitivity/specificity, cost, effectiveness, convenience and duration of the technique, as well as availability of materials were considered.

Polymerase Chain Reaction (PCR) is most sensitive and specific due to its ability to specifically identify, and amplify the ND-RNA virus, while Intra-cerebral pathogenecity Infectivity (ICPI) was considered more sensitive than mean death time of the minimum lethal dose (MDT/MLD) due to its pathogenic effects on the day old chicks, which was physically noticed as depicted through the mortality pattern of the chicks (Table 1 and Table 2).

PCR was however most expensive due to the high cost of PCR machine, reagents and primers involved in running the test. MDT/MLD was cheaper than ICPI because the cost of day old chick was higher than cost of embryonated egg.

The time limit for running PCR was shortest compared to the other 2 tests, because time taken from extraction to amplification of the virus takes a maximum of 6 hours, while in MDT/MLD inoculated eggs were candled in the

mornings and afternoons, giving a 24 hours period. In ICPI, it takes 7days duration to check for living, sick and dead chicks.

As in the case of duration of test procedure, PCR is the most convenient of all the techniques when all required materials are available. However, even when the PCR equipment are available, the reagents especially primers are in most instances not readily available, while materials notably, day old chicks and embryonated eggs for running MDT/MLD and ICPI are readily available commercially.

The use of extracts of *momordica balsamina* in the management of Newcastle disease in Nigeria had been described (Mgbojikwe *et al.*, 2002). It cannot be ascertained that reduction in mortality is due to effect of *momordica balsamina* since NCO-Mix was earlier administered, and might have only potentiated the effect of NCO-Mix or vice versa. A separate study is necessary to answer this question.

Consequently, based on the different criteria used to compare the three methods of characterization, it implies therefore that PCR has higher advantages than the others in terms of duration, convenience and sensitivity/specificity. While in terms of cost, availability and accessibility of materials, MDT and ICPI have higher advantages over PCR.

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