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In vitro Production and Some Properties of Neuraminidase of a Nigerian Newcastle Disease Virus Strain

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Abstract: Experiments were performed to determine some properties of neuraminidase of Newcastle Disease Virus (NDV) Kudu 113 strain, in order to deduce the possible effects of this enzyme on hosts cells during NDV infections in poultry; and to see how its properties are similar or different from neuraminidases of some viruses that have been previously characterized. The activity and properties of neuraminidase of NDV Kudu 113 strain were determined by periodate thiobarbituric acid assay method. Neuraminidase activity was detected in NDV Kudu 113 strain *in vitro*. The neuraminidase activity increased gradually from 24 h with the mean value of 101.4±2.8 μmol min^{-1} and attained its mean maximum activity of 167.2±5.4 μmol min^{-1} by 72 h post-inoculation into embryonated chicken's eggs, after which the activity of the enzyme declined. The enzyme was precipitated at 55% by ammonium sulphate [$(NH_4)_2SO_4$] saturation. The optimum pH and temperature of the enzyme were 5.5 and 40°C, respectively. The Michaelis constant (K_m) and maximum velocity of reaction (Vmax) of this enzyme were 1.0×10^{2} μmol L^{-1} and 3.03×10^{-4} μmol min^{-1}, respectively. It was concluded that NDV Kudu 113 strain produced neuraminidase *in vitro*. The neuraminidase activity had a linear relationship with the dilution of the virus at higher serial dilutions. Neuraminidase of NDV Kudu 113 strain also cleaved sialic acid from fetuin (substrate) *in vitro*. It is likely that neuraminidase of this virus strain may cleave sialic acid from the surfaces of erythrocytes and other cells during *in vivo* NDV infections in poultry, thereby exposing the erythrocytes to destruction by reticulo-endothelial system.

Key words: Newcastle disease virus, neuraminidase, property, production, disease

INTRODUCTION

Newcastle disease is a recognised disease problem of poultry in many parts of the world. It is an infectious, highly contagious and fatal disease that affects both domestic and wild birds (Roy *et al*., 2000). In Nigeria, both the virulent and avirulent NDV strains have been isolated from both wild and domestic birds and these birds have been considered as sources of infection to susceptible commercial poultry (Ezeifek *et al*., 1992).

Newcastle disease virus Kudu 113 is a Nigerian NDV strain isolated from the duck. The virus has been classified as velogenic NDV strain (Echeonwa *et al*., 1993). Isolation of velogenic NDV strain from apparently healthy, free-roaming ducks is of epizootiological importance as these birds may serve as reservoir of infection to the intensively reared chickens.

It is believed that NDV Kudu 113 strain is one of the NDV strains causing infection in poultry in Nigeria (Echeonwu *et al*., 1993). It is, therefore, important to study the properties of neuraminidase of this new NDV strain. This is because neuraminidases play an important role in many disease processes of animals (Corfield, 1992; Lichtensteiger and Vimr, 2003). It has been observed that neuraminidase of NDV induced cytopathic changes in infected cells. Furthermore, NDV strains isolated from different hosts or those with different virulence were found to have different neuraminidase contents and biochemical properties (Mc Nulty *et al*., 1975; Reuter and Schauer, 1994). It is therefore, imperative to study the properties of neuraminidase of NDV Kudu 113 strain, in order to see how its properties differ or similar to neuraminidases of other viruses that have been previously characterized.

The pathogenicity indices of NDV Kudu 113 strain have been determined (Echeonwu *et al*., 1993) but no information on the properties of neuraminidase of this NDV strain has been reported. This is the first report on the *in vitro* production and properties of neuraminidase of NDV Kudu 113 strain.

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The objective of this study was, therefore, to determine some properties of neuraminidase of NDV Kudu 113 strain *in vitro*. Such studies could give further insights into the pathogenesis and role(s) of neuraminidase during NDV infections in poultry.

MATERIALS AND METHODS

Virus strain: Newcastle disease virus Kudu 113 strain stock (embryo infective dose 50% end point of $10^{8.46}$ per mililitre, obtained from National Veterinary Research Institute, Vom, Plateau State, Nigeria) was used for this experiment. The virus was isolated from free-roaming ducks in Kuru, Plateau State of Nigeria and the pathogenicity indices have been determined by Echeonwu *et al.* (1993) as follows: Intracerebral pathogenicity index, 1.56; mean lethal dose, $\log_{10}$ 8.00; mean death time, 49.60 h; intravenous pathogenicity index, 2.18; embryo infective dose 50% end point per mL, 8.46; percentage adsorption of chicken brain cell, 97.66%; thermostability of haemagglutinin at 56°C, 120 min; and virus elution rate, >26 h.

Virus growth: One vial of NDV Kudu 113 strain stock was diluted with 2 mL of sterile cold phosphate buffered saline, pH 7.2. Procaine penicillin at 200 i.u mL^{-1} and streptomycin at 200 μg mL^{-1} were added to the final concentrations. About 0.2 mL of the solution was inoculated into the allantoic cavity of 20 ten-day-old embryonated chicken's eggs. After inoculation, the eggs were incubated in an electric egg turner incubator (Ovo-Lux, BP 500 4000 Liege, Belgium) at 37.5°C for 6 days. The eggs were candled twice daily to check for dead embryos.

Virus isolation: Dead embryos were chilled to 4°C before the allantoic fluids were harvested. The allantoic fluid was harvested at 24, 48, 72, 96, 120 and 144 h after inoculation into embryonated chicken's eggs. The presence of NDV in the allantoic fluid was detected by haemagglutination test (Beard and Wilkes, 1989). Thereafter, the allantoic fluid was clarified by high-speed centrifugation at 9,000 g for 5 min. The sediment was then stored at -20°C until used.

Ammonium sulphate fractionation and dialysis of neuraminidase of newcastle disease virus kudu 113 strain: Ammonium sulphate fractionation of neuraminidase of NDV Kudu 113 strain was carried out to determine the $(NH_4)_2SO_4$ fraction with the highest neuraminidase activity. This was done by modifying the procedures of Nees *et al.* (1975) as follows: Ten per cent, 20, 30, 40, 50, 55, 60, 70 and 80% $(NH_4)_2SO_4$ precipitation of the enzyme was determined by adding 1 mL each of the allontoic fluid, containing NDV Kudu 113 strain to 56, 70, 176, 242, 313, 351, 390, 472 and 820 mg of $(NH_4)_2SO_4$, respectively in a 10 mL capacity polythene tubes. All the $(NH_4)_2SO_4$ fractions and control were set in duplicates. The experiment was repeated three times.

The mixture for each fraction was homogenized by violent agitation to apparent homogeneity and centrifuged using refrigerated centrifuge (MSE, mistral 4L, Beckman, USA) at 9,000 g for 1 h at 4°C. The supernatant for each fraction was aspirated using Pasteur pipette, after which 0.5 mL of sodium phosphate citrate buffer, pH 5.0 was added to each sediment.

The enzyme fraction with the highest activity was dialysed by modifying the methods of Heuermann *et al.* (1991) thus: Thirty mililitre of $(NH_4)_2SO_4$ fraction with the highest neuraminidase activity was transferred into a 20 cm long and 6cm wide dialysis bag and immersed in a 2 L capacity dialysis beaker, containing 2 L of sodium phosphate citrate buffer, pH 5.0. The dialysis beaker and its contents were placed on a speed plate magnetic stirrer (Beckman, USA) at 4°C for 28 h.

Assay for neuraminidase activity at different viral dilutions: Serial two fold dilutions of 50 μL of the virus was made in a series of 5 test tubes which had already contained 50 μL each of sodium phosphate citrate buffer, pH 5.0. About 50 μL of fetuin (subtrate) was then added to each mixture in each tube and incubated at 37°C for 1 h. Both the samples and the control were set in duplicates and the experiment was repeated three times. The activity of neuraminidase in each tube was assayed by modifying the procedures of Aminoff (1961) as follows: About 25 μL of sodium periodate was added to each tube, the mixture was shaken and incubated for 20 min in a water bath at 37°C, after which 10 μL of sodium arsenite was added. A brown colour immediately appeared. Thereafter, 100 μL of 2-thiobarbituric acid (4, 6-Dihydroxypyrimidine-2-thiol) was added. The mixture was shaken and put into boiling water for 10 min. A pink colour appeared after about 10 min. After this, the test tubes, containing all the mixture was cooled by placing them under a running tap. Following this, 250 μL of acid butanol was added and vigorously shaken. The mixture was centrifuged at 9,000 g for 5 min. The supernatant for each mixture was carefully aspirated into cuvettes using Pasteur pipettes. Absorbance was read against blank on Sp6-400 spectrophotometer (Beckman, USA) at 549 nm.

Assay for neuraminidase activity of newcastle disease virus kudu 113 strain at different hours post-inoculation into embryonated chicken's eggs: The activity of neuraminidase of NDV Kudu 113 strain was assayed at 24, 48, 72, 96, 120 and 144 h post-inoculation into embryonated chicken's eggs by using the modified procedures of Aminoff (1961) as described above, in order to determine the sequential changes in neuraminidase activity as the virus grew in the embryonated chicken's eggs. The experiment was repeated three times for each hour mentioned above and the control.

pH dependent studies of neuraminidase of Newcastle disease virus Kudu 113 strain: The pH dependent studies of neuraminidase of NDV Kudu 113 strain were determined at pH of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. About 10 μL each of dialysed neuraminidase of NDV Kudu 113 strain was equilibrated in sodium phosphate citrate buffer at the different pH mentioned above, at room temperature (25°C) for 30 min. The samples and the control were set in duplicates. Thereafter, each mixture was incubated with 10 μL of fetuin for 1 h. The activity of the enzyme at different pH values mentioned above was then assayed by the modified methods of Aminoff (1961) described above. The experiment was repeated three times for each pH value.

Temperature studies of neuraminidase of newcastle disease virus kudu 113 strain: The temperature studies of neuraminidase of NDV Kudu 113 strain were carried out at temperatures of-20, 0, 4, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C. About 10 μL each of dialysed neuraminidase of NDV Kudu 113 strain was first equilibrated at the various temperatures mentioned above for 10 min. Thereafter, 10 μL of fetuin was incubated with the equilibrated enzyme at each temperature for 1 h. The samples and the control were set in duplicates. The activity of the enzyme for each temperature was then assayed by the modified methods of Aminoff (1961) described above. The experiment was repeated three times for each temperature value.

Kinetic properties of neuraminidase of newcastle disease virus kudu 113 strain: Fetuin concentrations of 10-50 μL were used to study the kinetic properties of neuraminidase of NDV Kudu 113 strain. The K_m and Vmax of the enzyme were determined by graphic extrapolation using Lineweaver Burk plots.

Statistical analysis: Statistical analysis was performed with Student's t-test. All data were expressed as mean±standard deviation. Results were displayed graphically. For all statistical analysis, values of $p<0.05$ were considered significant.

RESULTS

The activity of neuraminidase of NDV Kudu 113 strain rose gradually from 10% $(NH_4)_2SO_4$ fractionation

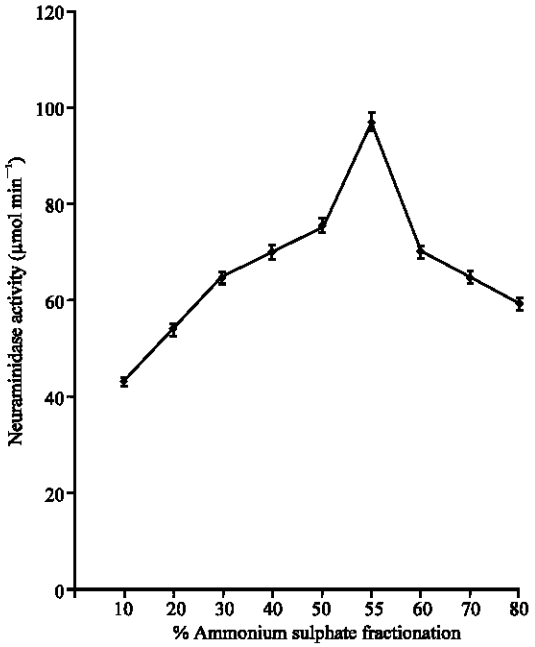

Fig. 1: Ammonium sulphate fractionation (±SD) of neuraminidase of Newcastle disease virus Kudu 113 strain

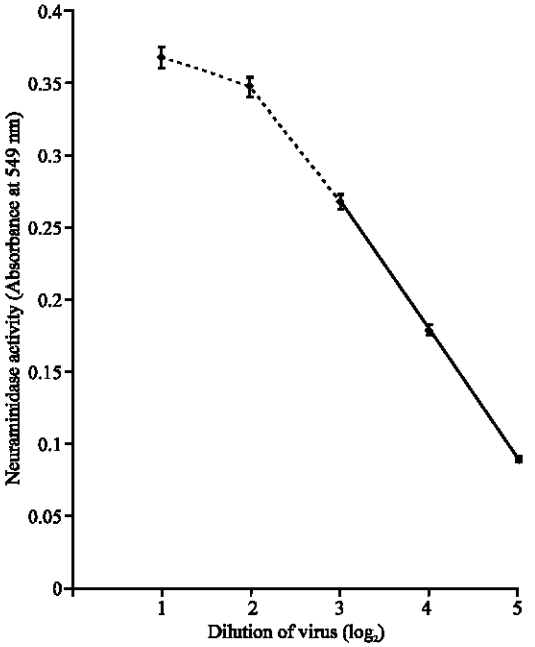

Fig. 2: Relationship (±SD) between activity of neuraminidase and virus dilutions of Newcastle disease virus Kudu 113 strain

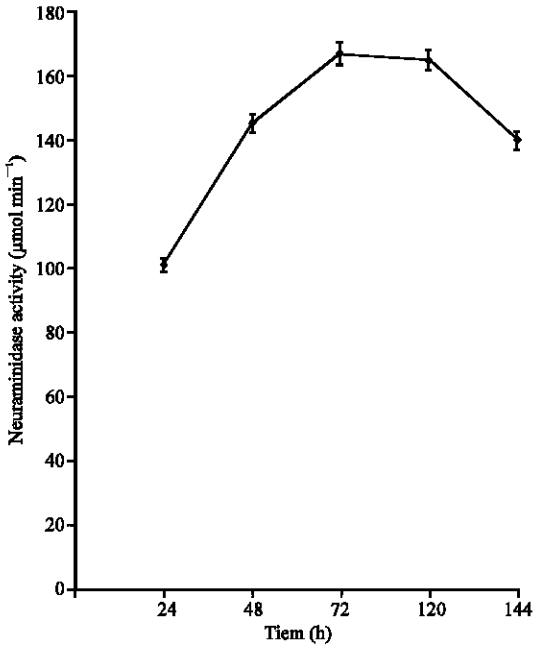

Fig. 3: Hourly activity (±SD) of neuraminidase of Newcastle disease virus Kudu 113 strain

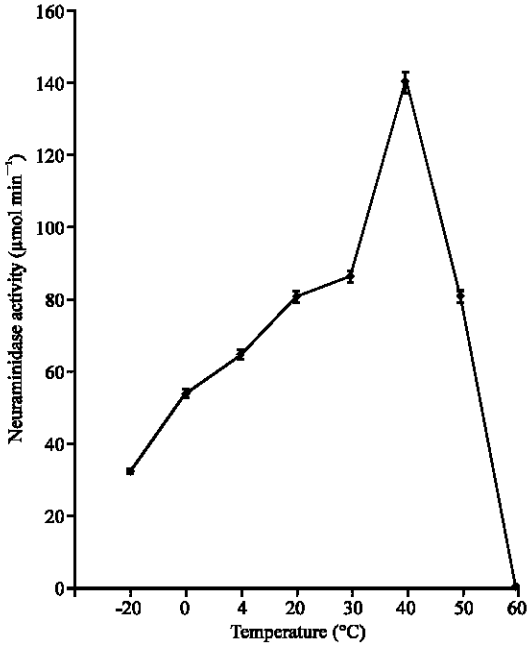

Fig. 5: Temperature studies (±SD) of neuraminidase of Newcastle disease virus Kudu 113 strain

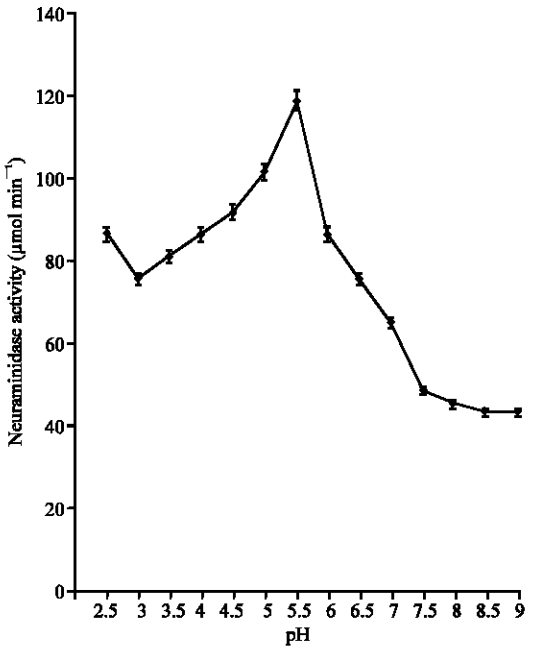

Fig. 4: pH dependent studies (±SD) of neuraminidase of Newcastle disease virus Kudu 113 strain

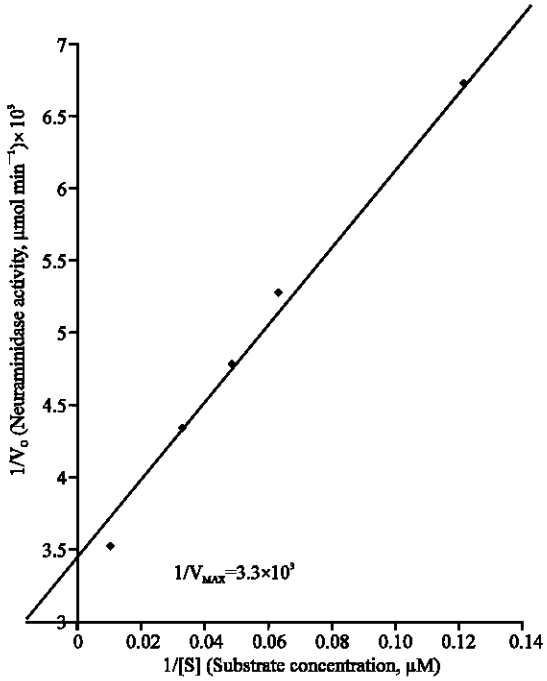

Fig. 6: Lineweaver Burk plot of initial velocity data and substrate concentration of Newcastle disease virus Kudu 113 strain neuraminidase

and attained its mean maximum activity of 97.1±3.0 µmol min^{-1} at 55% $(NH_4)_2SO_4$ fractionation. Thereafter, the activity of the enzyme decreased with increase in per cent $(NH_4)_2SO_4$. No neuraminidase activity was detected in the control samples (Fig. 1).

The results of various serial two-fold dilutions of the virus and the activity of neuraminidase of NDV Kudu 113 strain showed that there was a linear relationship between the neuraminidase activity and dilution of the

virus at higher serial dilutions (log_2 3-5). However, the relationship was no longer linear at lower serial dilutions (log_2 1 and 2). No neuraminidase activity was detected in the control samples (Fig. 2).

The activity of neuraminidase increased gradually from 24 h post-inoculation of the NDV Kudu 113 strain into embryonated chicken's eggs and attained its mean maximum value of 167.2±5.4 μmol min^{-1} by 72 h. Thereafter, the activity of the enzyme declined. No neuraminidase activity was detected in the control samples (Fig. 3).

The results on pH studies show that the activity of neuraminidase of NDV Kudu 113 strain dropped from the mean value of 86.3±3.7 μmol min^{-1} at pH 2.5 to 75.5±3.5 μmol min^{-1} at pH 3.0, after which the activity of the enzyme increased until it attained the mean optimum activity of 118.6±5.2 μmol min^{-1} at pH 5.5. Thereafter, the activity of the enzyme decreased with increase in pH. No neuraminidase activity was detected in the control samples (Fig. 4).

The activity of neuraminidase of NDV Kudu 113 strain rose gradually at -20°C and attained its optimum temperature at 40°C. After which the activity of the enzyme declined and it was completely inactivated at 60°C. No neuraminidase activity was detected in the control samples (Fig. 5). The K_m and Vmax were 1.0×10^{2} μmol L^{-1} and 3.03×10^{-4} μmol min^{-1}, respectively (Fig. 6).

DISCUSSION

In this study, it was observed that NDV Kudu 113 strain produced neuraminidase *in vitro*. There was loss of linear relationship between the activity of the neuraminidase and virus dilution at lower serial dilutions. Similar results were obtained from the neuraminidase of influenza virus (Webster and Campbell, 1972).

The activity of the neuraminidase increased from 24 h and reached its peak by 72 h. Thereafter, the neuraminidase activity decreased. The increase in neuraminidase activity from 24-72 h post-inoculation of NDV Kudu 113 strain in embryonated chicken's in this study could probably be that, as the virus grew in the allantoic fluid, more neuraminidase was produced and the virus consequently, reached its maximum growth at 72 h, when the maximum neuraminidase activity was also attained. This is a significant finding, as this result shows that the activity of neuraminidase may be related to antibody production during the pathogenesis of virulent NDV, when it was observed that the haemagglutination inhibition antibody titres to NDV increased from 24 h and attained the highest titres by 96 h post-infection (Kouwenhoven, 1993).

In this study, the pH optimum of 5.5 was obtained for neuraminidase of NDV Kudu 113 strain. This result is similar to the optimum pH range of 5.0 to 5.4 obtained from neuraminidases of other paramyxoviruses (Schauer *et al*., 1995). The pH results obtained in this study, therefore, indicate that the neuraminidase of NDV Kudu 113 strain will be more active in acidic medium like other neuraminidases that have been characterized previously.

The optimum temperature of activity for NDV Kudu 113 strain neuraminidase in this study was 40°C. This value is similar to the optimum temperature range of 37°C to 40°C obtained from neuraminidases of human parainfluenza viruses (Hu *et al*., 1992). However, the optimum temperature of activity of 40°C obtained in this study is lower than the optimum temperature of 44°C recorded for bacterial neuraminidase of *Haemophilus avium*, a pathogen of poultry (Muller and Hinz, 1978).

The enzymic activity of neuraminidase of NDV Kudu 113 strain was destroyed at 60°C. This temperature is higher than the results obtained by Peeples *et al.* (1983) who found that the neuraminidase activity of NDV was completely inhibited at 56°C. The temperature studies, therefore, show that neuraminidase of NDV Kudu 113 strain is more likely to be resistant to heat than the neuraminidase of NDV Australia-Victoria wild-type strain previously described by Peebles *et al.* (1983).

Maximum velocity of reaction of 3.03×10^{-4} μmol min^{-1} was recorded for NDV Kudu 113 strain neuraminidase in this study. The K_m value of 1.0×10^{2} μmol L^{-1} obtained in this study for neuraminidase of NDV Kudu 113 strain is lower than the values quoted by Alexander (1974) for neuraminidases of NDV (1.02×10^{3} μmol L^{-1}), Bangor virus (3.16×10^{3} μmol L^{-1}) and Yucaipa virus (1.97×10^{3} μmol L^{-1}). The K_m value of 1.0×10^{2} μmol L^{-1} obtained in this study is also lower than the value of 5×10^{3}μmol L^{-1} recorded for neuraminidase of human parainfluenza 1 virus (Kessler *et al*., 1977) when N-acetylneuramin lactose was used as substrate. It could be that NDV Kudu 113 strain neuraminidase has a higher affinity for fetuin than neuraminidases of other viruses that were mentioned above. This is because the lower the K_m, the higher the enzyme's efficiency, its affinity for the substrate and catalytic conversion of the substrate (Stroev, 1989). Therefore, it could be inferred that during NDV infections *in vivo*, NDV Kudu 113 strain neuraminidase may cleave sialic acids from the surfaces of erythrocytes and other cells at a faster rate, thereby exposing its hosts to secondary bacterial, viral and fungal infections, in addition to loss of sialic acids which are known to mask the erythrocytes and other cells from biological recognition of subterminal galactose residues (Schauer *et al*., 1995).

CONCLUSION

Newcastle disease virus Kudu 113 strain produced neuraminidase *in vitro*. The neuraminidase activity had a linear relationship with the dilution of the virus at higher serial dilutions. Since neuraminidase of NDV Kudu 113 strain cleaved sialic acid from the fetuin (substrate) *in vitro*, it is likely that neuraminidase produced by this virus strain *in vivo* may cleave sialic acid from erythrocytes during NDV infections and expose them to destruction by the reticulo-endothelial system. Further studies are required to confirm this hypothesis.

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