

***In vitro* Effects of Calcium, Magnesium, Mercury and Copper Ions on Neuraminidase of a Nigerian Newcastle Disease Virus Strains**

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Abstract: This study was carried out to determine the effects of cations on neuraminidase during NDV infections in poultry. A total of two hundred samples from dialyzed neuraminidase of NDV Kudu 113 strain were used for the experiment. Fifty samples each were used on four divalent cations, namely calcium (Ca^{2+}), magnesium (Mg^{2+}), mercury (Hg^{2+}) and copper (Cu^{2+}) ions. Neuraminidase activity of NDV Kudu 113 strain was determined *in vitro* in the presence of these cations, tested as their chloride form by periodate thiobarbituric acid assay method. Neuraminidase activity was also determined in the control samples that were without cations. Out of the four divalent cations examined, Hg^{2+} induced the highest neuraminidase activity of 75% at the concentration of 0.24M. Magnesium and Ca^{2+} ions also increased the activity of neuraminidase of NDV Kudu 113 strain *in vitro*. On the other hand, Cu^{2+} significantly inhibited the activity of this enzyme by 69.23% at the concentration of 0.47M. It was concluded that Ca^{2+} , Mg^{2+} and Hg^{2+} induced increase in the activity of neuraminidase of NDV Kudu 113 strain, while Cu^{2+} inhibited its activity *in vitro*. Further studies should be carried out on the roles of these cations in Newcastle disease (ND) and the possible use of Cu^{2+} in the future as inhibitor of the activity of neuraminidase of NDV *in vivo*, so as to reduce the menace of ND in poultry.

Key words: Cation, effect, neuraminidase and newcastle disease virus

Introduction

Newcastle disease is a recognized disease problem of poultry worldwide. In Nigeria, ND is rated as one of the greatest constraints to the development of poultry industry (Adene, 1990; Saidu *et al.*, 2006).

Newcastle disease virus is known to have a wide range of susceptible avian hosts. Newcastle disease virus Kudu 113 strain is a Nigerian NDV strain isolated from free-roaming ducks in Nigeria. This strain has been classified as velogenic NDV based on the following pathogenicity indices: intracerebral pathogenicity index, 1.56; mean lethal dose, \log_{10} 8.00; mean death time, 49.60h; 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, 120min; and virus elution rate, >26h (Echeonwu *et al.*, 1993). The isolation of this virulent NDV from free-roaming ducks is of epizootiological importance. This is because ducks could serve as reservoir of infections to intensively reared chickens (Majiyagbe and Nawathe, 1981).

Viral neuraminidases are believed to assist in viral spread by cleaving local sialyl residues during budding of infectious particles that would have been trapped by haemagglutinin-host-sialyl interactions, hence playing an important role in spreading of disease agents (Corfield, 1992; Roggentin *et al.*, 1993).

It is believed that cations could act as activators or inhibitors of neuraminidases (Muller and Hinz, 1978; Heuermann *et al.*, 1991; Martinez-Zorzano *et al.*, 1994), therefore, it is important to study the effects of some divalent cations on neuraminidase of NDV. This is because ND is endemic in most developing countries of the world, as a result, supportive therapy is often administered to surviving birds after ND outbreaks. Some of these drug preparations and feed supplements used as supportive therapy by poultry farmers are also known to contain compounds of some cations (Cromwell *et al.*, 1980; Odunsi and Onifade 1998), while some compounds and drugs form complexes with these cations. For example, quinolones, a group of very important antibacterial drugs in human and veterinary medicine, have been found to form complexes with divalent cations, such as calcium, manganese and zinc ions (Lacomte *et al.*, 1994).

Since enzymes and biocatalysts are now becoming increasingly important tools for synthesizing veterinary and pharmaceutical products, it therefore, becomes imperative to study the effects of cations on neuraminidase of NDV, in order to see if cations could modify its activity during NDV infections in poultry.

In our previous study, the level of serum neuraminidase was determined in unvaccinated and chickens vaccinated against ND. It was suggested that further

studies should be carried out to isolate and characterize neuraminidase of a local NDV strain, in order to shed more light on the role of this enzyme during NDV vaccination or infection in poultry (Oladele *et al.*, 2007). The present study is the first report on the *in vitro* effects of divalent cations on neuraminidase of NDV Kudu 113 strain. Therefore, the objective of this study was to determine the effects of Ca^{2+} , Mg^{2+} , Cu^{2+} and Hg^{2+} ions on neuraminidase of NDV Kudu 113 strain *in vitro*.

Materials and Methods

Virus strain: Newcastle disease virus Kudu 113 strain stock, containing embryo infective dose 50% end point of $10^{8.46}$ per ml was obtained from National Veterinary Research Institute, Vom, Plateau State, Nigeria, for this experiment. The virus was isolated from ducks in Nigeria and the pathogenicity indices have been determined (Echeonwu *et al.*, 1993).

Virus growth: One vial of NDV Kudu 113 strain stock was diluted with 2ml of sterile cold phosphate buffer saline, pH 7.2. Procaine penicillin at 200 international units per ml and streptomycin at 200mg per ml were added to the final concentrations, according to the methods of Allan *et al.* (1978). About 0.2ml of the solution was inoculated each 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 fluid was harvested. The allantoic fluid was harvested at 72 hours post-inoculation into embryonated chicken's eggs. The presence of NDV in the allantoic fluid was detected by haemagglutination test (Allan *et al.*, 1978). Thereafter, the allantoic fluid was clarified by high - speed centrifugation at 9,000g for 5 minutes. After which the sediment was stored at -20°C until used.

Ammonium sulphate fractionation and dialysis of neuraminidase of Newcastle disease virus Kudu 113 strain:

Neuraminidase was precipitated at 55% ammonium sulphate fractionation as described by Nees *et al.* (1975). The enzyme fraction was then dialyzed as previously described by Heuermann *et al.* (1991).

Effect of divalent cations on the activity of neuraminidase of Newcastle disease virus Kudu 113 strain:

A total of 200 samples from dialyzed neuraminidase of NDV Kudu 113 strain were used for the experiment. Fifty samples each were used on four compounds, which include calcium chloride, magnesium chloride, mercury chloride and copper

chloride at the concentrations of 0.12M, 0.24M, 0.35M and 0.47M, for each compound. The effect of divalent cations on this enzyme was determined by mixing 10 μ l each of the dialyzed NDV Kudu 113 strain neuraminidase in a set of 200 test tubes with the various concentrations of these compounds. The enzymatically liberated N-acetylneuraminic acid (neuraminidase activity) was then determined by using periodate thiobarbituric acid assay method of Reuter and Schauer (1994). Neuraminidase activity was also determined for samples without cations, which served as control.

The experiment was repeated three times for each sample in both the control and samples containing the cations.

Statistical analysis: All the data were subjected to statistical analysis using Student's t-test analysis. Values were expressed as mean \pm SD. The analyzed data were used to plot graphs.

Results and Discussion

When calcium ion was incubated with neuraminidase of NDV Kudu 113 strain *in vitro*, there was an increase in the activity of the enzyme as the concentration of Ca^{2+} increased. The activity of the enzyme increased from the value of $113.27 \pm 6.21 \mu\text{mol}/\text{min}$ at the concentration of 0.12M to the value of $145.63 \pm 3.12 \mu\text{mol}/\text{min}$ at the concentration of 0.47M. These values were higher than the corresponding values of $97.08 \pm 4.31 \mu\text{mol}/\text{min}$ and $140.24 \pm 2.23 \mu\text{mol}/\text{min}$ obtained from the enzyme without Ca^{2+} (control) at the same concentrations of 0.12M and 0.47M, respectively (Fig. 1).

In Fig. 2, magnesium ion also induced an increase in the activity of neuraminidase of NDV Kudu 113 strain from $140.24 \pm 5.30 \mu\text{mol}/\text{min}$ at the concentration of 0.12M to the value of $199.57 \pm 3.47 \mu\text{mol}/\text{min}$ at the concentration of 0.47M. The result shows that Mg^{2+} induced an increase in the activity of the enzyme by $43.16 \pm 1.29 \mu\text{mol}/\text{min}$; that is, 30.78% higher than the value obtained from the enzyme without Mg^{2+} (control) at the same concentration of 0.12M. Similarly, Mg^{2+} increased the activity of the enzyme by $59.33 \pm 2.15 \mu\text{mol}/\text{min}$ (30%) higher than the value obtained from enzyme activity without Mg^{2+} (control) at the concentration of 0.47M.

This study shows that Ca^{2+} and Mg^{2+} induced increase in the activity of neuraminidase of NDV Kudu 113 strain *in vitro*. This finding is in agreement with the results of Heuermann *et al.* (1991) who found increase in the activity of bacterial neuraminidase by Ca^{2+} and Mg^{2+} . Shehu *et al.* (1991) also found increase in the activity of neuraminidase of intestinal mucosa cells of goats by Ca^{2+} and Mg^{2+} . Similarly, Useh (2002) found elevated activity of neuraminidase of *Clostridium chauvoei* (Jakari strain) by Ca^{2+} and Mg^{2+} .

Oladele et al.: The Effects of Cations on Neuraminidase During NDV Infections in Poultry

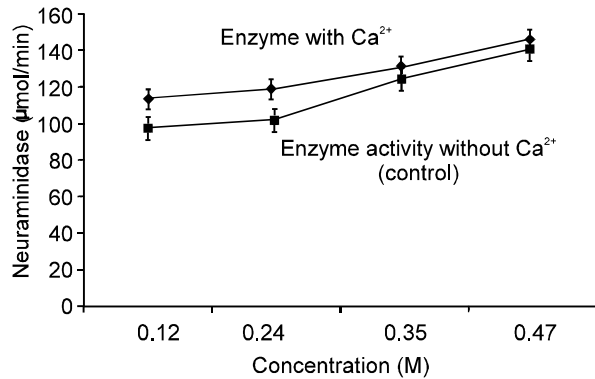


Fig. 1: Effect (mean±SD) of calcium ion (Ca²⁺) on neuraminidase of Newcastle disease virus Kudu 113.

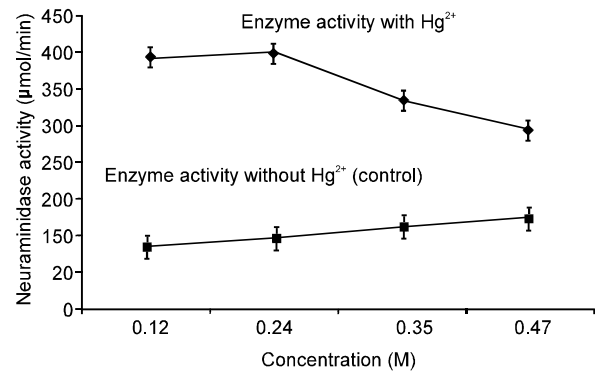


Fig. 3: Effect (mean±SD) of mercury ion (Hg²⁺) on neuraminidase of Newcastle disease virus Kudu 113.

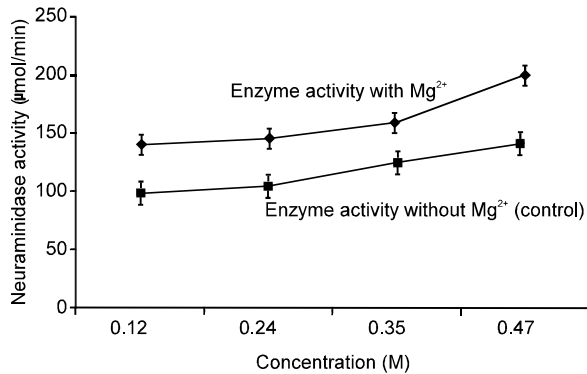


Fig. 2: Effect (mean±SD) of magnesium ion (Mg²⁺) on neuraminidase of Newcastle disease virus Kudu 113

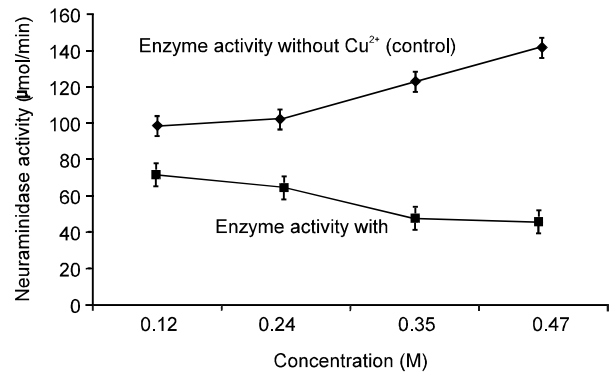


Fig. 4: Effect (mean±SD) of copper ion (Cu²⁺) on neuraminidase of Newcastle disease virus Kudu 113.

It is established that metals which are similar to vitaminous co-enzymes are supplied to organisms in food. Therefore, the normal functioning of a large number of metalloenzymes is dependent on regular supply of metal ions, such as Ca²⁺ and Mg²⁺ which activate Ca-Mg pump in cells (Consolazio, 1983). This explains the high biological activity of neuraminidase of NDV Kudu 113 strain in the presence of Ca²⁺ and Mg²⁺ in this study. The deficiency of these metal ions may lead to metabolic disturbances of NDVs which possess neuraminidase.

Since Ca²⁺ and Mg²⁺ were found to induce increase in the activity of neuraminidase of NDV Kudu 113 strain *in vitro* in this study, one could surmise that, if excessive quantity of Ca²⁺ or Mg²⁺ is present in drug preparations or feed supplements administer to surviving birds after ND outbreaks, it may probably cause an *in vivo* increase in the activity of neuraminidase, known to play important role in spreading of infectious particles during disease processes (Corfield, 1992) and consequently, resulting in further outbreaks of ND. Therefore, any effort adapted to reduce the levels of Ca²⁺ and Mg²⁺ in feeds and drugs

to the barest minimum during NDV infections could be of help in reducing ND in poultry.

Mercury ion induced a significant increase in neuraminidase activity of NDV Kudu 113 strain at the concentrations of 0.12M and 0.24M with the enzyme activity of 382.96 ± 10.21µmol/min and 393.74 ± 7.01µmol/min, respectively. Thereafter, the activity of the enzyme decreased. Nevertheless, Hg²⁺ induced the highest neuraminidase activity between the concentrations of 0.12M and 0.47M when compared with the other cations used in this study. The Hg²⁺ increased the enzyme activity by 74% (285.88 ± 4.09µmol/min), 61% (194.17 ± 2.01µmol/min) and 49% (134.84 ± 1.23µmol/min) higher than the corresponding values obtained from the enzyme without Hg²⁺ (control) at the concentrations of 0.12M, 0.35M and 0.47M, respectively (Fig. 3).

The significant increase in the activity of neuraminidase of NDV Kudu 113 strain more than the other cations used in this experiment, especially at the concentrations of 0.12M and 0.24M by Hg²⁺ is an important finding because Hg²⁺ has been described as a powerful

Oladele *et al.*: The Effects of Cations on Neuraminidase During NDV Infections in Poultry

inhibitor of enzymes, even at a very low concentration. Furthermore, it is known that mercury containing compounds are capable of non-competitively inhibiting enzymes in the organisms' cells or in the cells of pathogenic organisms, which actually determines their medicinal effects (Consolazio, 1983; Araujo *et al.*, 1993; Martinez-Zorzano *et al.*, 1994).

Copper ion inhibited the activity of neuraminidase of NDV Kudu 113 strain. The activity of the enzyme decreased with increase in the concentrations of Cu^{2+} . There was an inhibition of the activity of the enzyme by 28% ($26.96 \pm 3.01 \mu\text{mol}/\text{min}$) when the enzyme was incubated with Cu^{2+} at the concentration of 0.12M. Similarly, there was a significant inhibition of the enzyme activity by 69.23% ($97.09 \pm 2.61 \mu\text{mol}/\text{min}$) at the concentration of 0.47M by Cu^{2+} (Fig. 4).

The inhibition of the activity of neuraminidase of NDV Kudu 113 strain by only Cu^{2+} in this study is similar to the result of Gastald *et al.* (1993) who found that Cu^{2+} was the only cation out of the six cations studied which inhibited enzymic reactions.

The Cu^{2+} acted as an inhibitor of neuraminidase of NDV Kudu 113 strain at both low and high concentrations. Therefore, this study demonstrates that Cu^{2+} is not only a moderate, but a strong inhibitor of neuraminidase of NDV Kudu 113 strain.

In conclusion, this study has shown the effects of Ca^{2+} , Mg^{2+} , Hg^{2+} and Cu^{2+} on neuraminidase of NDV Kudu 113 strain. Further studies should be carried out on the roles of these cations in ND and the possible use of Cu^{2+} in the future as inhibitor of activity of neuraminidase of NDV. This is because in recent times, enzymes and biocatalysts have become important tools for synthesizing, improving diagnostic assays and enhancing efficiency of industrial processes. Therefore, protein engineering can be used to modify specific properties of natural enzymes of NDV to suit the needs of biotechnology, veterinary drug industries and poultry farmers.

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Oladele et al.: The Effects of Cations on Neuraminidase During NDV Infections in Poultry

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