PROBING THE TRANS-SIALIDASE ACTIVITY OF THE NEURAMINIDASE DERIVED FROM HUMAN INFLUENZA A (H3N2) VIRUS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to family especially my parents Dr Meshack Aluvaala and Mrs. Rose Aluvaala for their unending support, love, and encouragement and for seeing me through my education.

To my baby Ray, mummy loves you. My fiancé Ibrahim Nambati Kishindo, for always being there for me.

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LIST OF ABBREVIATIONS

Ac	Acetyl
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
CE	Capillary Electrophoresis
DMF	N, N- dimethyl formadine
EDTA	Ethylenediamine tetra-acetic acid
Et	Ethyl
Gal	D-galactose
GalA	Galacturonic acid
GalNac	N- acetyl galactosamine
GlcA	Glucoronic acid
GlcN	A – N- Acetyl glucosamine
GSL	Glycosphingolipids
HA	Hemagglutinin
HAI	Hemagglutination inhibition
IdoA	Iduronic acid
ITROMID	Institute of tropical medicine and infectious diseases
IPTG	Isopropyl β-D-1-thiogalactopyranoside

Kdn	2-keto-3-deoxynononic acid
Man	Mannose
MDCK	Madin Darby canine kidney
Me	Methyl
NCBI	National Center for BiotechnologyInformation
Na	Neuraminidase
Neu	5Ac N-acetyl neuraminic acid
Neu- 5Gc	N glycolylneuraminic acid
PCR	Polymerase chain reaction
Sia	Sialic acid
TLC	Thin Layer Chromatography
UV	Ultraviolet light
WHO	World Health Organization

ABSTRACT

Sialo-oligosaccharides are found on the distal ends of glycans present on mammalian cell surfaces. They play major roles in cell growth, function, and are determinants of many disease states. Due to the important physiological role of glycoconjugates, there has been increased focus towards the development of drugs and vaccines that are carbohydrate based. Sialo-oligosaccharides can be synthesized through chemical means or by use of enzymes. Enzymes are preferred in the synthesis process as they are cheaper, faster and capable of catalyzing the synthesis of molecules with the correct specificity. These being the case enzymes with activities capable of synthesizing glycoconjugates are being sought. Like other glycosidases, neuraminidases, also referred to as sialidases, hydrolyse glycosidic linkages. Glycosidases are also known to catalyze the synthesis of oligosaccharides through trans-glycosylation, but the trans-sialidation potential of Influenza neuraminidases has not been investigated. This aim of this study sought was to determine whether the influenza A H3N2 neuraminidase has trans-sialidase activity. The influenza A H3N2 virus was amplified by inoculating in Madin Darby canine kidney (MDCK) cell line. Total viral RNA was extracted and reverse transcribed. The cDNA was used in the amplification of neuraminidase Type 2 (NA2) followed by cloning, sequencing and expression of the neuraminidase (NA2). The recombinant enzyme was used in desialylation of guinea pig red blood cells to establish the presence of sialidase activity. The trans-sialidase activity was assayed using sialic acid from RBC as donor and PNP β-D galactose as the acceptor molecule. Products of trans-glycosylation reaction were analysed by Thin Layer Chromatography (TLC) plate and by capillary electrophoresis.

Hemagglutination Inhibition of guinea pig RBC occurred after treatment with the recombinant influenza A type 2 neuraminidase. This showed that desialylation had taken place and hence the sialidase activity of the recombinant neuraminidase was present. The resolution of transglycosylation reaction on TLC and by capillary electrophoresis did not reveal any products. This observation suggests that there was no transfer of sialic acid residues from donor RBC to acceptor molecule PNP-Gal. These findings suggest that the trans-sialidase activity of neuraminidase derived from influenza A/Nairobi/2041/2006(H3N2) is not significant. The search for enzymes that can be used for synthesis sialo-oligosaccharides continues. In future studies on the neuraminidase (NA2) trans-sialidase activity, the purification method should be improved by incorporating a Histag on the expression vector. The analysis of the trans-glycosylation products should be carried out using approaches such as mass spectroscopy as it is highly sensitive and able to detect compounds in trace quantities that are not detectable by Thin Layer Chromatography and Capillary Electrophoresis. Since the use of chemicals in the synthesis of carbohydrates remains expensive and full of challenges in reference to regiospecificity, it would be worthwhile to carryout molecular evolution to transform A/Nairobi/2041/2006(H3N2) neuraminidase to a trans-sialidase.

CHAPTER ONE

1: INTRODUCTION

1.1 The chemistry and diversity of Sialic acids

Carbohydrates in the form of glycoconjugates are a subset of carbohydrates whose functions include cell development, growth and function (Dwek, 1995) . Inspite there being hundreds of glycoconjugates in nature, they only carry a small number of monosaccharides. These include; Pentoses (D-xylose), Hexoses (D-glucose D-galactose, D-manose), Deoxyhexoses (L-Fucose), Hexosamines (N-acetylgalactosamine), Uronic acids (Glucuronic acid, Iduronic acid) and Sialic acids (N-acetylneuraminic acid, N-glycolylneuraminic acid) (Varki and Sharon, 2009).

Amongst these monosaccharide's, Sialic acid also refered to as neuraminic acid is unique with respect to chemistry and location on glycoconjugates structure. They have a nine-carbon skeleton, an amino group and a carboxyl group (Fig1.1). The amino group mostly bears an acetyl or glycolyl group. The hydroxyl group can be substituted with acetyl, lactyl, methyl, sulfate or phosphate groups. Sialic acids are typically found on the distal end of N-glycans, O-glycans and glycosphingolipids, making them suitable molecules for interaction with other cells as well as environmental agents (Angata and Varki, 2002).

Due to the various substitutions, sialic acids are the most diverse sugars found on glycan chains of mammalian cell surfaces, contributing to distinct make up of cell types. This in turn determines their recognition by antibodies and sialic acid binding lectins. The most common sialic acids are N-acetlyneuraminic (NeuAc) and N-glycolylneuraminic acid (NeuGC) (Fig 1.2 and 1.3) respectively. The difference in

these two molecules is the addition of an oxygen atom in the N-glycolyl group of (NeuGC)(Varki, 1992).

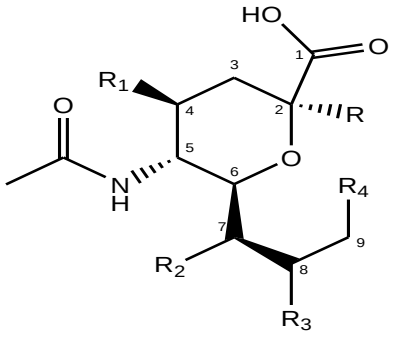

Figure 1.1: Basic structure of sialic acids

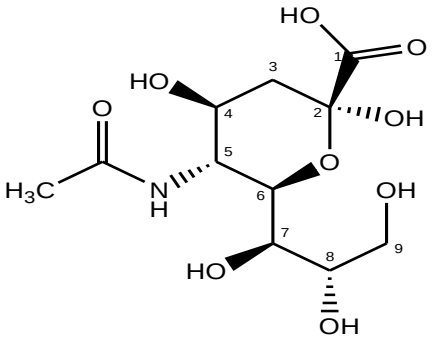

Figure 1.2: N-acetlyneuraminic acid

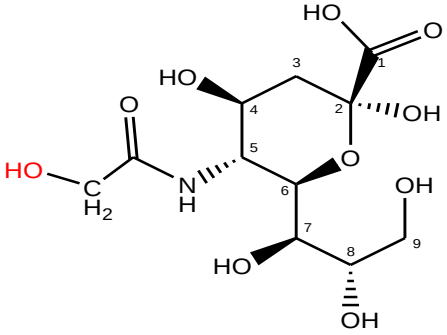

Figure 1.3: N-glycolylneuraminic acid

1.2 The roles and functions of sialo-oligosaccharides in biological systems

The location and high expression levels of sialic acids on the outer cell membranes of the human erythrocytes, interior of lysosomal membranes, secreted glyco-proteins such as blood proteins and mucins suggests that(Varki and Schauer, 2009) they have roles in the stabilization of molecules, membranes, and in control of interactions with the environment(Varki and Schauer, 2009).

They protect molecules and cells from attack by proteases or glycosidases thus extending their lifetime and function (Varki and Schauer, 2009). They regulate the affinity of receptors and modulate processes involved in transmembrane signaling, fertilization, growth, and differentiation for example apoptosis can be inhibited by Sia O-acetylation. (Varki and Schauer, 2009).Sialic acids also have a property of free-radical scavenging. This gives an antioxidative effect which is significant on endothelia of blood vessels. (Varki and Schauer, 2009). Sialic acids can act either as masks or recognition sites. They mask antigenic sites, receptors and penultimate galactose residues. When Sia residues are lost, molecules and cells can be bound to, taken up and degraded by macrophages and hepatocytes through Gal-recognizing receptors. Sialic acids serve as ligands for a variety of microbial and animal lectins (Drickamer and Taylor, 1993). This property can be influenced by chemical modifications. For example, 9-O-acetylation or N-acetylhydroxylation of Neu5Ac can create new receptor functions or decrease the affinity of binding (Varki and Schauer, 2009).

1.2.1 Role of sialic acids in pathology

1.2.1.1 Microbial pathogens

A large number of micro-organisms depend on the host cell- surface glycans as receptors for attachment and tissue colonization. Many micro-organisms do this by recognizing specific sialylated ligands.

Viruses, bacteria, and protozoa express an enormous number of glycan-binding proteins or lectins, these include; adhesins, hemagglutinins and toxins. Bacterial lectins are referred to as fimbriae or pili. In *E.coli,* K99 binds to glycolipids that contain N glycolylneuraminic acid (Neu5Gc) but doesn't bind glycolipids that contain N-acetyl neuraminic acid. Neu5Gc is present in intestinal cells of new born piglets but not in humans. This specificity explains why *E.coli* K99 causes diarhoea in piglets but not in adult pigs and humans(Esko and Sharon, 2009).

Parasites also use glycans for adhesion for example the merozoite stage of *Plasmodium falciparum* infects red blood cells. The invasion is dependent on recognition of sialic acids rich receptors (erythrocyte membrane protein/ glycophorin). The parasite adhesin, EBA-175 (erythrocyte-binding antigen-175) preferentially binds (Neu5Ac) rather than f9-*O*-acetyl-Neu5Ac or Neu5Gc. Different *plasmodium* species such as *Plasmodium falciparum* and *Plasmodium Reichnowii* have different sialic acid binding proteins that enable them to preferentially bind to human and chimpanzee host cells respectively (Martin *et al.,* 2005, Varki and Sharon, 2009, Esko and Sharon,2009)

The influenza virus binds to airway epithelial cells by recognizing sialylated receptors. The viral hemagglutinin binds to sialic acid containing receptors hence facilitating fusion of the viral envelope with the plasma membranes and uptake of virus into cells. The avian and equine viruses preferentially bind sialic acids with an α2-3 gal linkage while the human virus binds to sialic acid with an α2-6 gal linkage which is present on the upper respiratory tract (Suzuki *et al.,* 2000, Connor *et al.*, 1994).Humans also display sialic acids with an α2- 3 gal linkage but this is found in the lower respiratory tract. This implies that for humans to be infected by avian or equine viruses, certain mutations must occur on the virus' hemagglutinin to enable it to bind to the α2- 6 gal linkages. Since pigs have both linkages in their airways it is thought that they are the intermediate hosts that facilitate these mutations (Brown, 2000). In isolated cases, human beings have been infected by avian viruses that have not undergone any mutations. This has been attributed to high levels of exposure such that the virus is able to reach the lower respiratory tract where there is α 2-3 gal linkage (Shinya *et al.*, 2006, Varki, 2007).

The side chains of sialic acids can be modified by the addition of 9-*O*-acetyl esters. This modification enables rhinoviruses, influenza C viruses and Coronaviruses to bind to receptors on host cells. However, this modification effectively prevents the binding of influenza A virus to host cells (Varki and Varki, 2007)

1.2.1.2 Role of sialic acids in human physiology

There are few sialic acid specific lectins that are specific to an organism that synthesizes its own sialic acids. Examples of such lectins include; Siglecs, Factor H, Selectins and L1-CAM. These lectins can either be attached to cell surface or

secreted into the extracellular space. Factor H negatively regulates the alternative complement pathway. It recognizes and binds cell surface sialic acids. This attachment facilitates recognition of self. The recruitment on self surfaces enables factor H to perform its role of dissociating the alternative pathway convertase enzyme (Varki, 2007).Some microbial pathogens cover themselves with sialic acid hence they are able to recruit factor H and in effect evade the host cell immune response(Varki, 2007).Selectins are involved in immune responses, homeostasis and inflammation. Leukocytes, platelets and endothelial cells are involved in these processes. They express L, P and E selectins respectively. They recognize and bind to sialic acids. The homing of lymphocytes into lymphnodes through endothelial venules is mediated by sialic acids, the leukocytes recognize of sialylated ligands through (L) selectin. The adhesion of leukocytes to activated endothelial cells is mediated by E selectins which also involves recognition of sialic acids. During an inflammation process leukocytes roll along the endothelium cells and adhere tightly to the endothelium wall. This is facilitated by the binding of selectins to sialic acid receptors present on the endothelium wall. This allows the leukocytes to migrate into the inflamed tissue (Ley, 2003, Varki, 2007).

The binding of sialic acid recognizing Immunoglobulin (Ig) super family lectins (siglecs) is dependent on recognition of sialic acids (Crocker *et al.,* 2007). CD33r recognizes sialic acids as self and thus prevents immune response against self. They also recognize sialic acids on pathogens (Razi and Varki, 1999).

MAG (Myelin associated glycoprotein) recognizes gangliosides that have sialic acids. This recognition is crucial for the long term stability of myelin (Vinson *et al.,* 2001). L1CAM (L1 cell-adhesion molecule) is expressed in the nervous system and

is involved in neural development and function. It recognizes α 2-3 linked sialic acid present on CD24 a heavily glycosylated cell-surface molecule (Kleene *et al.*, 2001).The binding of sperm to the zona pellucida of the ovum has been shown to be mediated by α2-3 linked sialic acids. The sperm is hence able to penetrate the egg and this enables fertilization to take place(Varki, 2007).

1.2.1.3 The role of sialic acids in malignancy

Sialic acids can be used in monitoring of tumor progression. This is because many changes occur during malignant transformation. These changes can thus be used to detect abnormalities. Sialic acids are abundant on cell surfaces and changes in these cell surface antigens have been associated with cancers.

The over expression of Sialyl Lewis^{x} and sialyl Lewis^{a} structures on epithelial carcinomas such as ovary epithelial tumors correlates with tumor progression, metastatic spread and poor prognosis. (Dall'Olio and Chiricolo, 2001, Laubli *et al.*, 2006, Varki and Varki, 2002).

1.3 Sialidases

Sialidases also referred to as neuraminidases belong to a class of enzymes refered to as glycosyl hydrolases. Sialidases hydrolyse sialo-conjugates releasing sialic acids. Many micro-organisms also express sialidases. Bacterial fungal and invertebrate enzymes are evolutionary related to mammalian families. Viral sialidases on the other hand show two distinct families. Most sialidases share common "Asp boxes" (Ser-x-Asp-x-Gly-x-Thr-Tyr).These are involved in the maintenance of the protein enzyme protein conformation.

Most sialidases exhibit substrate specificity with regard to sias linkages. The 2-3 linkages are hydrolyzed more easily than α 2-6 linkages, the hydrolysis of α 2-8 is

intermediate. The O-methylation and O-acetylation of sias can hinder hydrolysis of glycosidic bonds by sialidases.

1.3.1 Bacterial sialidases

Pseudomonas aeruginosa is an opportunistic pathogen. It causes nosocomial pneumonia and is the chief cause of lung infections in cystic fibrosis. Secreted *P. aeruginosa* neuraminidase modifies the airway epithelial mucosal surfaces by cleaving sialic acids this produces increased numbers of asialoglycolipid receptors that have GalNAcβ1, 4Gal to which the bacteria can bind(Soong *et al.,* 2006). *S. pneumonia* also removes sialic acid from respiratory epithelial cells to expose underlying *N*-acetylglucosamine and galactose residues to which the bacterium binds with high affinity (Nizet and Esko, 2009). *Vibrio cholerae* neuraminidase aids in the pathogenesis of cholera by removing sialic acid from larger gangliosides exposing GM1, the receptor for cholera toxin (Moustafa *et al.*, 2004).

1.3.2 Viral sialidases

The influenza virus expresses neuraminidase as a surface glycoprotein. Neuraminidase is an integral type II membrane glycoprotein. The gene is 141o nucleotides long, and codes for a protein of 469 amino acids and has five potential glycosylation sites. Neuraminidase is a mushroom-shaped tetrameric protein,anchored to the viral membrane by a single hydrophobic sequence of some 29 amino acids near the N-terminus(Varghese *et al.,* 1983). From above the head, each monomer consists of six blades of a propeller. The box-like head of the neuraminidase is connected to a stalk that comprises membrane-spanning domain and the amino terminal part. The structure of the stalk remains unknown.

Neuraminidase also referred to as sialidase is a tetrameric protein and the structure is such that all the four subunits are identical. It is made up of a globular head, a thin stalk region and a small hydrophobic region. The hydrophobic region anchors the protein in the virus membrane. The active site of this enzyme is such that each subunit consists of a pocket that is formed by 15 charged amino acids (Colman *et al.,* 1983, Reid *et al.,* 2000).These amino acids are conserved in all influenza A viruses. There are nine known neuraminidase subtypes, N1-N9 (Murphy and Webster, 1996). Being a surface glycoprotein neuraminidase is targeted by the host immune system. The host cell produces antibodies against this virus glycoprotein. Neuraminidase undergoes mutation and has the highest level of genetic variability when compared to any other influenza gene which ensures their evasion from the host cell antibodies.

1.4 Chemical versus enzymatic synthesis of glycoconjugates

1.4.1 Chemical synthesis of glycoconjugates

The synthesis of glycoconjugates using chemical methods is challenging due to the need to control stereochemistry and regiochemistry. The synthesis involves the use of numerous protecting groups. The protecting groups mask the hydroxyl groups and prevent them from reacting with other chemical reagents. These hydroxyl-protecting groups are selectively added and removed from glycan structures during the synthesis process hence allowing regiospecific addition of monosaccharides only on the exposed hydroxyl groups. The choice of the protecting group, sequence of protecting group installation is essential in ensuring the success of the synthetic process. The advantage of chemical synthesis is that it allows for the preparation of diverse natural and unnatural structures that enzymatic methods cannot achieve (Seeberger *et al.,* 2009).

1.4.2 Enzymatic synthesis

In nature there are enzymes that can be used in the synthesis of glycans. Glycosyltransferases and glycosidases have been used in the synthwesis of glycans. In the case of glycosidases whose natural function is the cleavage of glycosidic bonds they can be manipulated to catalyse the reverse reaction and hence function as glycosylating enzymes.

The synthesis of glycans using glycosyltransferases gives high yields with the correct regiochemistry and stereospecificity without the use of numerous protecting groups. The availability of glycosyltransferases is however limited and they have to be discovered, cloned and overexpressed before they can be used in the synthesis. Glycosidases are more stable and tolerant to variations in substrate structure as compared to glycosyltransferases. However if they are to be used in synthetic purposes, their normal function must be reversed. All enzymatic processes are essentially in equilibrium it is thus possible to make the glycosidases to run in reverse direction and synthesise glycans. This can be done by exposing the glycosidase to a large excess of reaction products. This will in effect shift the equilibrium. However the reverse reaction is endothermic and the equilibrium will always favor the cleavage products. However when the anomeric hydroxyl group of the glycosyl donor is replaced with a good leaving group, such as *para*-nitrophenol (PNP), it shifts the equilibrium further toward the glycosylation product (Seeberger *et al.*, 2009).

2: PROBLEM STATEMENT

Carbohydrates and particularly sialo-oligosacharides have become increasingly important in the medical field. Carbohydrates have been used in the reduction of inflammatory responses by blocking selectins (Varki, 1997). They have been used in immunization against cancer and in the prevention of adhesion of micro-organisms to host cells (Kudryashov *et al.,* 2001, Gilewski *et al.,* 2001). Sialo-Oligosaccharides can also be used in cell targeting for gene therapy (Gao *et al.,* 2003).

Oligosaccharides can be synthesized using two methods: chemical methods and enzymatic methods. Chemical methods are expensive and require the use of complex substrates. They also require multiple protection and deprotection sequences and there is the problem of control of configuration at the anomeric centre**.** Enzymatic methods are cheaper and require the use of simple substrates. Sialyl- transferases can catalyse sialylation regio-specifically and in high yield. Transglycosidases are examples of enzymes that can be used in the synthesis of oligosaccharides.

3: JUSTIFICATION

The glycosidase (sialidase) activity of influenza virus neuraminidase is known but its trans-glycosidase (trans-sialidase) activity has not been established. Establishing the presence of trans-sialidase activity will provide a novel enzyme that can be used in the synthesis of sialo-oligosaccharides.

4: HYPOTHESIS

4.1. Null hypothesis

The influenza A H3N2 neuraminidase (NA2) has a trans-sialidase activity that can be used in the synthesis of sialo-oligosaccharides.

5: OBJECTIVES

5.1 General objective

To probe trans-sialidase activity in influenza NA2 neuraminidase

5.2. Specific objectives

1. To clone, sequence and perform phylogenetic analysis of the neuraminidase gene (NA2) from influenzaA/Nairobi/2041/2006) virus strain.
2. To express the Neuraminidase protein in *E.coli.*
3. To assess trans-sialidase activity of the expressed neuraminidase (NA2).

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Trans-Sialidases

Trans-sialidase activity has been shown to be present in some pathogenic trypanosome species and some bacteria. Trans-sialidases are unique sialidases which directly transfer sialic acids (Sia) from one glycosidic linkage to another (on galactose), without using CMP-activated Sia. Trans-sialidases specifically generate α2-3 glycosidic linkages but the nature of donor or acceptor molecule is not specific. Trans-sialidases just like sialidases are important virulence factors and play roles in invasion, unmasking binding sites and provide nutrients for the pathogen(Varki and Schauer, 2009)

2.1.1 Bacterial trans-sialidase

Corynebacterium diptheriae colonises mucosal surfaces and hence just like other microbes colonizing this surface it has sialidase activity that facilities the invasion process.*C. diptheriae* has been shown to express both neuraminidase and trans-sialidase activities. *C.diptheriae trans*-sialidase produces asialoglycoconjugates from a Sialyl substrate and at the same time generates Sialyl derivatives of β-gal acceptors (Mattos-Guaraldi *et al.,* 1998).

2.1.2 Trypanosome trans-sialidase

The trans-Sialidase of the human parasite *Trypanosoma cruzi* presents a unique type of sialyl-transferase that is highly selective in its substrate specificity for sialic acid linked α 2,3 to β-D-galactose (Vandekerckhove *et al.,* 1992, Scudder *et al.,* 1993). The *T. cruzi* trans-sialidase displays two catalytic activities, a neuraminidase

activity(Pereira, 1983)that results in sialic acid release from complex carbohydrates, and a sialyl-transferase activity that catalyses the transfer of sialic acid to terminal β-D-galactose acceptors. The transferase activity is predominant at 97% while the neuraminidase activity is 3% (Scudder *et al.,* 1993). The trans-sialidase activity is responsible for sialic acid supply in *T.cruzi*. *T.cruzi* is unable to synthesize sialic acid itself (Schauer *et al.,* 1983)and acquires it from mammalian host glycans with the help of the trans-sialidase (Schenkman *et al.,* 1991, Previato *et al.,* 1985)

The *T.cruzi trans*-sialidase has several distinct domains (Pereira *et al.,* 1991), these includes an N-terminal domain of 380 amino acids which shares up to 30% sequence identity with bacterial sialidases, a second domain of 150 residues which shows no similarity to any known sequence, a third domain containing a fibronectin type III (FnIII) motif, and a long C-terminal tandem repeat of 12 amino acids which is not required for the enzyme activity (Campetella *et al.,* 1994). The first N-terminal domain contains two copies of the Asp-box motif, a signature typical for bacterial sialidases (Roggentin *et al.,* 1989), which have been found to be in topologically identical positions in the sialidase β-propeller fold (Crennell *et al.,* 1993). This domain also appears to contain all of the key residues identified to be important for catalysis in the bacterial sialidases (Cremona *et al.,* 1995) and is therefore likely to adopt a very similar fold to the catalytic domain of the enzymes. Studies have shown that certain residues are essential for trans-sialidase activity. There is a key tyrosine, predicted to be in the active site that differentiates between active and inactive members of the *T. cruzi* TS family (Cremona *et al.,* 1995). In addition, site-directed mutagenesis studies have revealed the importance of proline in the N-terminal domain for TS activity (Smith and Eichinger, 1997).

2.2 The Biology of Influenza Viruses

Influenza is an infectious disease that can be caused by one of three types of influenza viruses, A, B or C. These three types of the virus are distinguished based on antigenic differences between their nucleocapsid (NP) and matrix (M) proteins. Type A is the most virulent form and has been known to cause epidemics and occasionally pandemics. Type B causes epidemics while C doesn't seem to cause any significant disease (von Itzstein, 2007, Wagner *et al.*, 2002).

The genomes of these viruses are composed of segmented negative strand RNA. The influenza viruses belong to the *orthomyxoviridae* family(Bhatia and Kast, 2007).

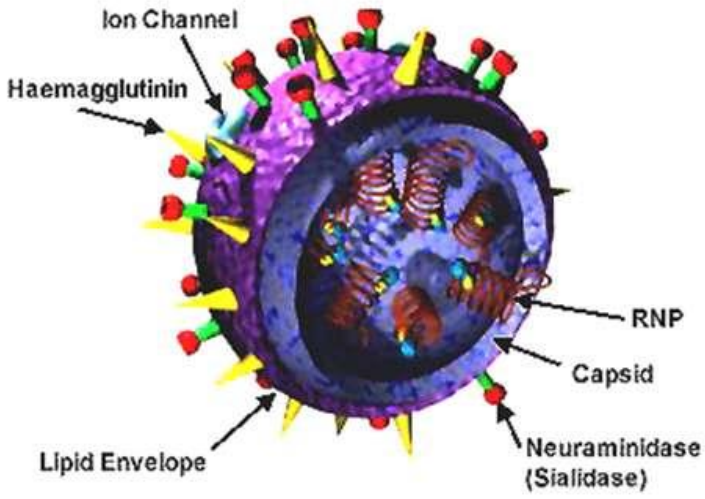

Figure 2.1: Structure of the influenza virus (Image courtesy of medicine world 2006 Virulence of 1918 Influenza Virus)

The influenza A and B genomes' have 8 segments while the C has 7 segments. Each of these segments encodes a particular gene.

The negative-strand RNA acts as a template for the synthesis of mRNA as well as a template for the synthesis of the antigenome (+) strand. Influenza A viruses can further be classified into types and subtypes. There are 16 subtypes of Hemaggluttinin and 9 of Neuraminidase. This is based on the presence of two key

viral surface proteins hemaggluttinin and neuraminidase (Fig 2.1). These glycoproteins' are the most important immune determinants of the virus. The subtypes most commonly associated with human disease are H1N1 and H3N2. The gene encoding hemagglutinin is located on segment 4 and neuraminidase is located on segment 6.

Influenza viruses infect a number of species these include humans, pigs, horses, sea mammals, poultry, wild ducks and other migratory waterfowl (Murphy and Webster 1996). Wild waterfowl act as reservoirs for all influenza viruses. Although the influenza virus infects all these species the host range is limited due to varying distribution and type of sialic acid present across species.

2.3 The life cycle of the influenza virus

The infection of host cells by the influenza virus begins with adhesion to the target cell via surface glycoprotein haemagglutinin which recognizes terminal ∝-linked N-acetylneuraminic acid (∝-Neu5Ac) residues. The virus is then endocytosed, fusion occurs and the host-cell machinery begins to produce the necessary viral components. (Fig 2.2) Subsequent viral protein synthesis and particle assembly in the host cell prepares the virion progeny for the budding process to exit the host cell. The enzyme sialidase/neuraminidase cleaves the terminal ∝-Neu5Ac residues from both the newly synthesized virion progeny glycoproteins as well as from the host-cell surface. This enables the host-cell-surface aggregated virion progeny to elute away from the infected cell and seek new host cells to infect.

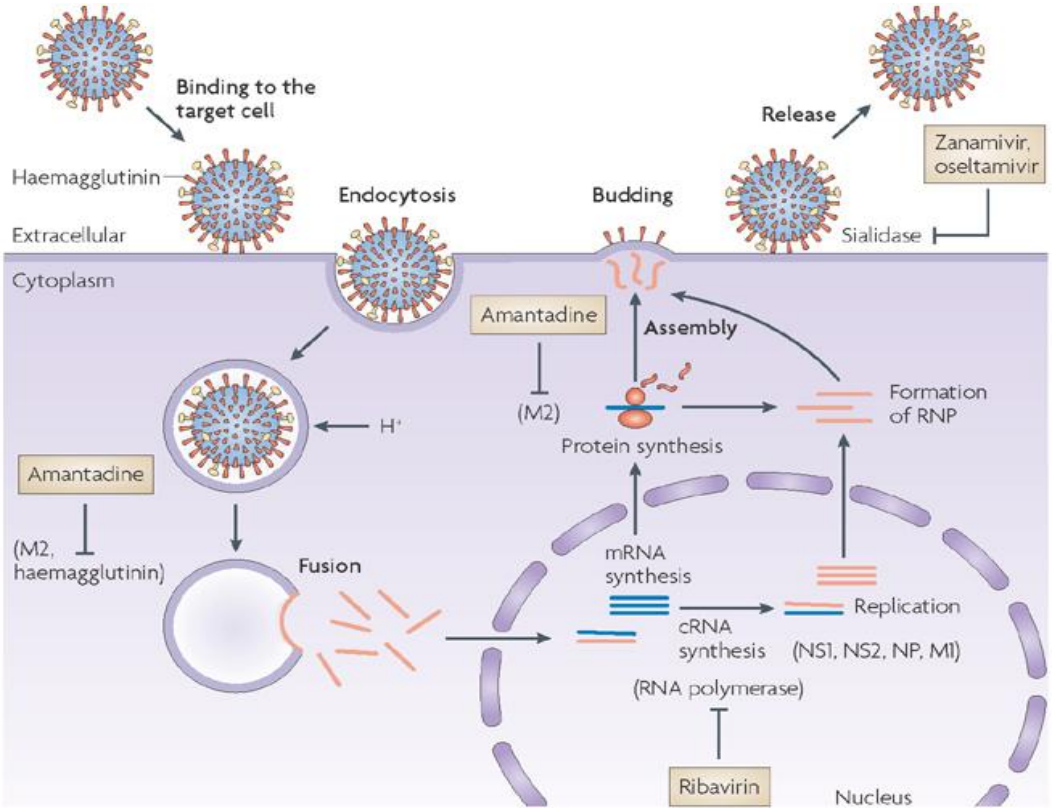

Figure 2.2: Life cycle of the influenza virus. Image courtesy of Nature Reviews Drug Discovery 2007 The war against influenza: discovery and development of sialidase inhibitors

2.4 The role of neuraminidase in influenza virus

Newly formed viruses are found attached to the sialic acid residues present on the host cell. Neuraminidase cleaves this and hence facilitates the release of the new virions which allows the viruses to invade new cells (Fig 2.3). Without neuraminidase, infection would be limited to one round of replication, rarely enough to cause disease**.** (Moscona, 2005).

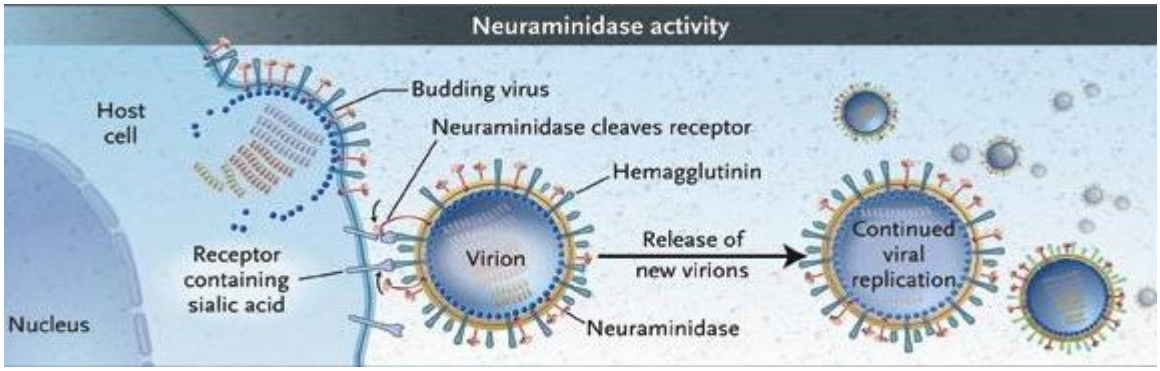

Figure 2.3: Role of neuraminidase in influenza virus. Image courtesy of the Moscona, 2005.Neuraminidase Inhibitors for Influenza New England Journal of Medicine.

Neuraminidase prevents the viruses from clumping and aggregating together this way they are free to move individually. This effectively increases the chance of infecting more cells. Neuraminidase also makes infection of the epithelial cells easier. Epithelial cells are bathed in mucin, which is essentially rich in sialic acid. This forms a barrier but since neuraminidase is able to cleave this sialic acid residues infectivity is increased(Bhatia and Kast, 2007).

2.5 Mechanism of action of influenza neuraminidase

Neuraminidase cleaves terminal sialic acid residues. When a Neu5Ac-containing glycoconjugate binds to the neuraminidase active site, it is orientated through a cluster of three arginine residues and the Neu5Ac moiety makes contact with eight amino acid residues. The orientation of sialic acid residue in the active site is followed by the formation of a putative sialosyl cation intermediate which adapts a distorted half chair arrangement. The active site of neuraminidase contains tyrosine residues this residues carry out a neucleophillic attack on the intermediate. The intermediate is consequently hydrolysed and released as an α-Neu 5Ac (von Itzstein, 2007).

2.6 Neuraminidase inhibitors

Neuraminidase is a good target for antiviral drug development because it is essential for virus infectivity and its active site is highly conserved.

The highly conserved active sites ensure that they are less prone to selecting for drug resistance. Selective competitive neuraminidase inhibitors are used as they are able to bind tightly to the active site. This inhibition prevents the release of new virion progeny from an infected cell this is by preventing the hydrolysis of neuraminic acid in mucus this in turn reduces the ability of the virus to spread through the respiratory epithelium. (Fig 2.4) NA inhibitors are effective against human and non-human subtypes of influenza A and B (Democratis *et al.*, 2006, McKimm-Breschkin, 2000). Zanamivir has poor bio-availability and is administered directly into the respiratory tract oseltamivir is administered orally.

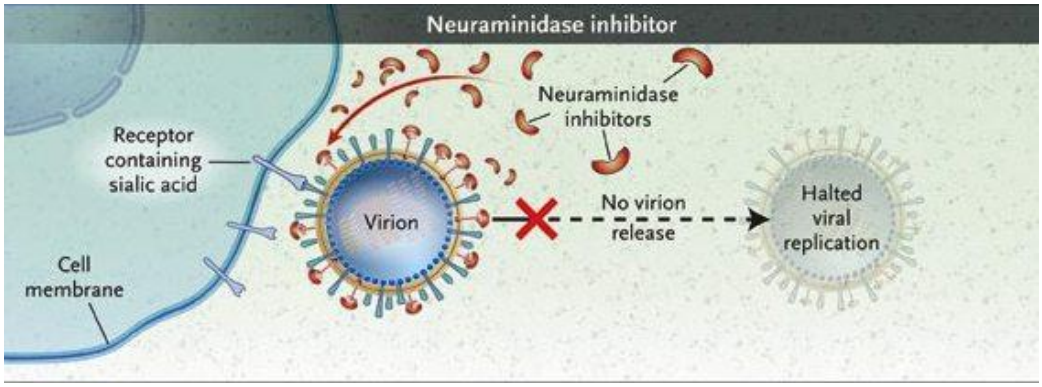

Figure 2.4: Neuraminidase inhibitor. Image courtesy of the Mascona, 2005. Neuraminidase Inhibitors for Influenza New England Journal of Medicine.

2.7 Chemical structure and mechanism of action of neuraminidase inhibitors

2.7.1 Mechanism of action of the inhibitors of neuraminidase

Zanamivir and oseltamivir (Fig 2.5) are examples if NA inhibitors. These inhibitors prevent the hydrolysis of the bond between sialic acid, preferably *N*-acetylated and the adjacent carbohydrate molecule, of cellular glycoprotein(Bucher and Kilbourne, 1972).

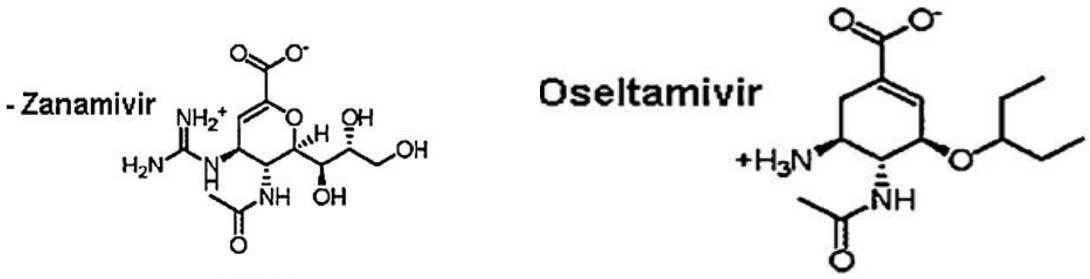

Figure 2.5: Structure of zanamivir and Oseltamivir

The carboxylate group attached at C2 on Neu5Ac, zanamivir and oseltamivir interact with a positively charged and hydrogen binding environment formed by amino acid residues R118, R292, and R371 on neuraminidase. The guanidino group present at C4 only on zanamivir interacts with a negatively charged region formed by the amino acid residue E227 and E119. The same interaction is observed with the amino group present on the oseltamivir. The amino nitrogen side chain of DANA, zanamivir and oseltamivir is common to Neu5Ac, and seems to interact with amino acid R152K (based on N2 structure interaction). The glycerol side chain present at C6 on zanamivir interacts with amino acid residues R224 and E276 (Yen et al., 2006, McKimm-Breschkin, 2000, Smith *et al.*, 2002, Stoll *et al.*, 2003).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 The Sample

The neuraminidase gene (NA2) in this study was derived from influenza A/Nairobi/2041/2006(H3N2) virus (Bulimo et al., 2008). This isolate was provided courtesy of Dr Wallace Bulimo of the GEIS Human Influenza Program at the National Influenza Centre, KEMRI Kenya.

3.2 Primer Design for amplification of Neuraminidase (NA2)

Gene specific primers were designed based on neuraminidase (NA2) gene sequences from the flu database at http://www.ncbi.nlm.nih.gov/genomes/FLU/Database. The accession numbers of the reference sequences used were CY037489, CY037505, CY037585, CY037601, CY037625, CY037809, EF566190, EU103981, EU885537 and GU968161. To identify conserved region at the 5' and 3' ends of the open reading frames (ORFs) in these gene sequences, the nucleotide sequences were fetched from the database and aligned using MUSCLE3.6 (Edgar 2004). Using the default parameters of the online Web Primer software (http://www.yeastgenome.org/cgi-bin/web-primer), the CY037489 sequence was initially used to design two gene-specific forward and reverse primers that would amplify the entire NA2 ORF. Subsequently, degeneracy observed in the multiple sequence alignments was incorporated in the primers to increase the chance of amplifying the NA2 from not sequenced A/Nairobi/2041/2006(H3N2) virus. This generated the primers NA2-Foward 5'-ATG AAT CCA AAT CAA AAG ATA ATA ACR-3' and NA-Reverse 5'- TTA TAT AGG CAT GAG ATT GAT GTC C-3'. To

aid in directional cloning of the amplified PCR products in pQE30 expression vector, KpnI and HindIII restriction sites were incorporated at the 5'ends of NA-Fwd and NA-Rev to give NA-Fwd-KpnI 5'-TTT TGG TAC CAT GAA TCC AAA TCA AAA GAT AAT AAC R-3' and NA-Rev-HindIII 5'-TTT TAA GCT TTT ATA TAG GCA TGA GAT TGA TGT CC-3. Since KpnI and HindIII are endonucleases, three T's were added at the 5' ends of the primers to flank these restriction recognition sites. The primers were ordered from MWG Biotech (http://www.mwg-biotech.com) at the 0.1 nM scale.

3.3 Extraction of RNA from influenza isolate and nasopharyngeal samples.

The RNA was extracted from A/Nairobi/2041/2006(H3N2) and three other control samples using QIAamp Viral RNA (Qiagen USA) extraction kit according to the manufacturer's instructions. Briefly, 100µl of each virus sample, (10^7 pfu) was added to 500µl of lysis buffer and allowed to incubate at room temperature for 10 minutes to allow lysis. 500µl of absolute ethanol was added and pulse vortexed for 15 seconds to give a homogeneous solution. 630µl of the lysed solution was applied to the spin columns and centrifuged at 6000 x g for 1 minute at 4 ^{0}C. The remaining 370 µl was applied to the spin column and centrifuged at 6000 x g for 1 minute at 4 ^{0}C and column placed in a clean collection tube. 500µl of Buffer AWL (wash buffer 1) was added to the spin column and centrifuged at 6000xg for 1 minute at 4^0C. The column was placed in a clean collection tube and washed with 500µl of Buffer AW2 (wash buffer 2) and centrifuged at 6000 x g for 3minutes at 4^0C. The spin column was placed in a clean 1.5 ml micro centrifuge tube and 60 µl of Buffer AVE (elution buffer) added to the column and allowed to incubate for 1 minute at room

temperature. The column was centrifuged at 6000 x g for 1 minute and the filtrate (RNA) stored at -80^{0}C.

3.4 Detection of Human Influenza A virus using Real Time PCR.

The extracted viral RNA was used to detect influenza A virus genetic material using influenza A matrix gene specific primers in a real time PCR assay. The CDC provided the primers and probes used in this assay. The primers and probes were manufactured by Applied Biosystems (California, USA).The PCR master mix was prepared by mixing, 0.5 µl Flu A Forward primer (5 pmoles), Flu A reverse primer (5 Pico moles) 0.5 µl, Flu A Probe, (2.5 Pico moles /µl.) 0.3 µl, Buffer 12.5 µl, enzyme 0.625 µl, water 5.575 µl, template 5 µl giving a total volume/reaction volume of 25 µl.

The thermo cycling conditions for the 1 step RT PCR comprised of reverse transcription at 48°C for 30 min followed by polymerase activation at 95°C for 10 min and thirty five cycles of denaturation at 95°C for 15mins, annealing at 50 °C for 1min and final extension at 50 °C for 1min.

3.5 Amplification of (A/Nairobi/2041/2006(H3N2)) virus using MDCK cell line.

Isolate (A/Nairobi/2041/2006(H3N2)) and the control patient samples tested positive for influenza A by real time PCR and were inoculated in MDCK cells monolayer at 80% confluence. Flat sided culture tubes were washed thrice using sterile phosphate buffered saline pH 7.4 containing 0.25% trypsin and innoculated with 100µl of patient sample.

These were incubated for 1hour at 33^{0}C to absorb the virus. Maintenance media was then added. The tubes were incubated at 34^{0}C in a 5% CO2 incubator to allow

growth of the viruses until a cytopathic effect was observed. The supernatant from cultures showing CPE and therefore containing virus were stored as isolates at -80^{0}C.

3.6 Hemagglutination Test (HA)

The amplified virus stocks were then used in hemagglutination test. This was done so as to confirm that the isolate provided was influenza. The isolate (A/Nairobi/2041/2006(H3N2)) and the 3 controls were partially thawed and kept on ice. 25µl of sterile PBS was added from well (2A-2D) – (12A-12D) (Fig 3.1) on a flat-bottomed 96-well plate. Using a pipette, 50µl of the controls and isolate were added into wells 1A-1D respectively and two-fold serial dilutions carried out by transferring 25µl from well to well starting with well 1-12. 25µl of PBS was added to all the wells and 50µl of 1% guinea pig red blood cell added to all wells. The plate was incubated at room temperature for 30 minutes. A record was kept where agglutination occurred. The titre of the virus was expressed as the reciprocal of the dilution that caused complete agglutination. For example, if the last dilution showing complete agglutination was 1: 64, then the HA titre was 64.

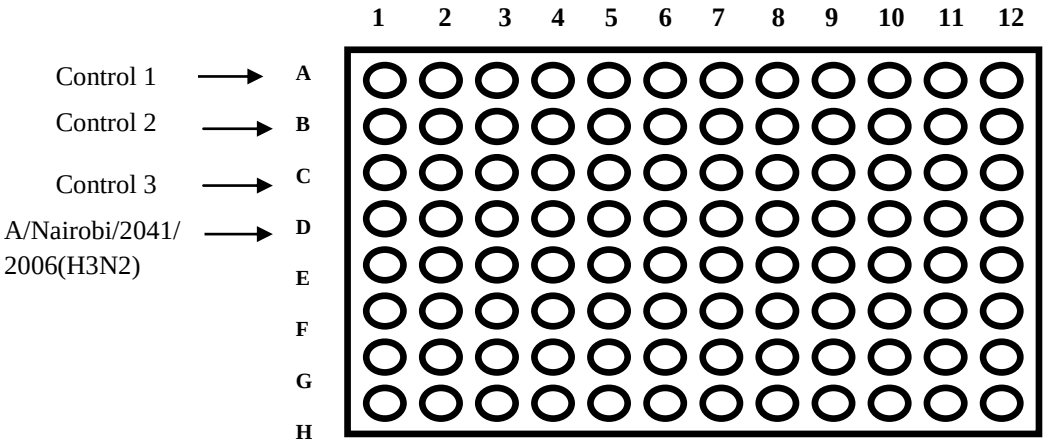

Figure 3.1. Layout of hemagglutination (HA) assay

3.7 Hemagglutination Inhibition Test (HAI)

Antigenic characterization was performed on the isolate (A/Nairobi/2041/2006(H3N2)) and one of the 3 controls using reference reagents prepared by the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia to confirm that the influenza sample was subtype H3N2.

First, the isolate, control and the reference strains were adjusted to have 4 HA units before testing. A 96 U-well plate was labelled with the reference antisera as follows:

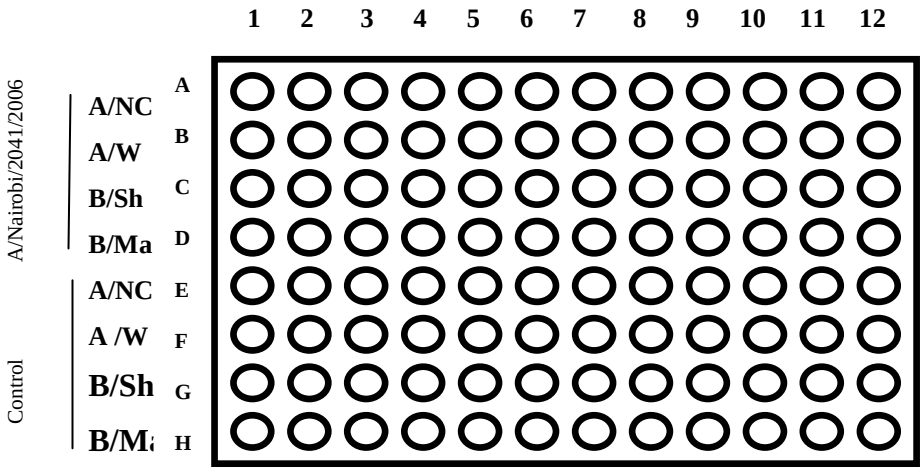

Figure 3.2: Layout of hemagglutination inhibition test (HAI)

A/NC = A/New Caledonia/20/99 (H1N1)-like

A/W = A/Wisconsin/67/2005 (H3N2)-like

B/Sh = B/Shanghai/361/2002-like

B/Ma= B/Malaysia/2506/2004-like

25µl PBS was added from well (2A-2H)-(12A-1H) (Fig.3.2). 50µl of the reference antiserum was added to well 1 of the appropriate row (1A-IH). Two-fold serial dilution of the antisera was carried out by transferring and mixing 25µl from well 1-11. 25µl of the 4HA units of the isolate and control was then added to all wells of the diluted set of antisera, wells 1 to 11. After mixing, the plate was incubated at room

temperature for 30 minutes. 50µl of 1% guinea pig red blood cells was added to all the wells and incubated for 1 hour at room temperature to allow hemagglutination inhibition to take place. Virus sample causing hemagglutination inhibition with reference strain antisera was characterized as being like that strain.

3.8 Amplification of influenza NA2 gene using RT PCR.

Once the isolate (A/Nairobi/2041/2006(H3N2)) strain was confirmed to be H3N2, the extracted RNA (3.3) was used in RT-PCR to amplify the NA2 gene. The gene was amplified using conventional 2 steps RT PCR.

3.9 cDNA Synthesis

2µl of forward primer (40 pmol/µl), was added to 10 µl of 0.2µg/µl RNA template and vortexed at 1200rpm for 1 minute in a 5415R eppendorf micro-centrifuge. The sample was incubated at 70^0C for 10 minutes to allow denaturation. The sample was cooled at 4^0C for 5 minutes.

4 µl of 5X 1st strand Buffer (BD worldwide), 2 µl of 0.1 M DTT, 1 µl MM-LV RT (Clontech powerscript), and 1 µl 10mM dNTPS were added to the denatured sample and used in cDNA synthesis. The reaction was incubated at 50^0c for 50 minutes to allow synthesis to take place followed by incubation at 70^0c for 15 minutes to inactivate the MM-LV RT then incubated at 4^0C until used in a PCR.

3.10 PCR amplification of NA2 gene

A master mix was prepared to have a final concentrations of (1X) PCR buffer, 0.2 mM dNTPS, 3mM $MgCl_2$ and 0.8 pmoles/µl forward & reverse primers as well as 1.5U of *Taq* polymerase. Each reaction tube had a final volume of 25 µl after adding 2 µl (0.5ug) cDNA as the template.

The PCR conditions used comprised an initial denaturation at 94 ^{0}C for 4 min., followed by thirty five cycles of 94 ^{0}C 30 sec., annealing at 55 ^{0}C for 30 sec. and extension at 68 ^{0}C for 1 min. To complete the amplification of the of the PCR products, a final extension step at 72 ^{0}C for 7 min was included. The GeneAmp® PCR system 9700 thermo cycler from Applied Biosystems was used.

3.11 Agarose gel electrophoresis

The PCR product was run on a 1% Agarose gel. 1g of Agarose and 100ml of 0.5X TBE buffer was used. The solution was heated to boiling point and allowed to cool to 55^{0}C before adding 5µlof ethidium bromide (10mg/ml). The gel was poured on a gel caster with a comb and allowed to set for 30 minutes at room temperature then transferred to the electrophoresis tank. 0.5X TBE buffer was used as the gel running buffer. 1 µL of Promega 6x loading dye was used per 5µL sample. 5µl, of Promega 1kb DNA molecular marker was loaded alongside the samples.

3.12. Gel purification of the amplified NA2 PCR product

The DNA fragment corresponding to the neuraminidase gene (1500 bp) was purified using GFX PCR band gel purification kit (GE health care Amersham place UK) following the manufacturer's instructions.

The bands were excised from 1% agarose gel using sterile scalpel blades and placed in a pre-weighed 15ml centrifuge tube.

10µl of capture buffer per 10mg of gel was added and the mixture vortexed. The sample was incubated in a water bath at 60^{0}C for 15 minutes until the gel slices dissolved. The sample was transferred to a GFX column and centrifuged at 13000 rpm for 30 sec in a model 5415R Eppendorf micro-centrifuge. The column was

transferred to a micro-centrifuge tube and 15µl of elution buffer added. The column was centrifuged at maximum speed for 1 min to recover the DNA

3.13 Cloning NA2 gene fragment

3.13.1 Ligation

pGEM-T Easy Vector Systems (Promega) (Fig 3.3) was used in cloning of the neuraminidase gene. The pGEM-T Easy vector was spun down briefly to bring the contents down followed by preparation of 3 ligation reactions. The positive control was prepared by mixing 5µl 2X Rapid ligation Buffer, 1µl pGEM-T Easy Vector (50ng), 1µl control insert DNA, 1µl T4 DNA ligase and 1µl Nuclease-Free Water to give a final volume of 10µl.The negative control was prepared by mixing5µl 2X Rapid ligation Buffer, 1µl pGEM-T Easy Vector (50ng),

Rapid ligation Buffer, 1µl pGEM-T Easy Vector (50ng), 1µl (NA2), 1µl T4 DNA ligase and 1µl Nuclease-Free Water to give a final volume of 10µl.The ligation reactions were gently mixed and incubated at 4^0C overnight.

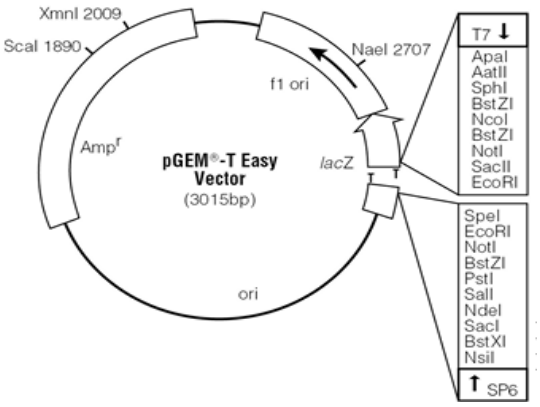

Fig 3.3 pGEM®-T Easy
Image courtesy of pGEM®-T and pGEM®-T Easy vector Systems technical manual no TM042 2009.

3.13.2 Preparation of competent cells

LB tetracycline plate (10µg/ml) was prepared and used for revival of XL1- Blue cells. (Cloning host. *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K-, m_K+), *sup*E44, *rel*A1, *lac*, [F', *proAB*, *lac*$I^qZ\Delta M15$::Tn10(tet^r)])

The XL1-Blue cells were removed from -80^{0}C and allowed to thaw on ice for 5 minutes. 20µl of the XL1- Blue cells were spread on LB tetracycline plate and incubated overnight at 37^{0}C. A single colony was then inoculated into 5ml of LB media with a final tetracycline concentration of 10µg/ml. The cells were incubated at 37^{0}C, with rocking at 130rpm for 6 hours and the cells stored at 4^{0}C. 7.5ml of the pre-culture was inoculated into 250ml LB media with 20mM $MgSO_4$ and incubated with shacking at 37^{0}C, 130rpm for 5 hours till an optical density (O.D) of 0.6 was obtained. The cultured XL1 blue cells were used in the preparation of competent cells using $CaCl_2$ method. Briefly, the cells were spun at 6,000 rpm at 4^{0}C for 5mins and re-suspended using 25ml of a solution containing 80mM $MgCl_2$ and 20mM $CaCl_2$. The cells were incubated on ice for 30 minutes till the pellet was completely dispersed. The cells were spun at3, 000 x g for 5minutes at 4^{0}C and the pellet re-suspended using 10ml of 0.1M $CaCl_2$ and glycerol to make a final concentration of 10% v/v. These were mixed by gently pipeting and placed on ice for 30 minutes till the pellet had dispersed. The cells were aliquoted and stored at -80^{0}C.

3.13.3 Transformation of competent XL1Blue cells

The ligation reactions, (positive control, negative control and the pGEMT-NA2) were spun briefly to bring the contents down and 5µl of each of the ligation reactions added to 200µl tubes. 20µl of competent cells were added to each tube.1 µl of control vector DNA (Promega pGEMT) was added to 20 µl of competent cells. The cells

were mixed by gently pipetting and incubated at 4°C for 30 minutes. The cells were heat shocked at 42°C for 1 minute and cooled at 4°C for 2minutes. 180 µl of freshly prepared SOC media was added and the reactions incubated at 37°C with shaking at 130 rpm for 1 hr 30minutes. 20 µl of the transformation cultures were plated on LB ampicillin100µg/ml IPTG/XGAL plates. The plates were incubated overnight at 37°C without shaking.

3.13.4 Plasmid midi prep extraction using alkaline lysis method.

The method of (Birnboim and Doly, 1979)was used. Briefly, a starter culture was prepared by inoculating a single colony of the transformation culture into 5ml LB broth containing 100µg/ml ampicillin and incubated at 37°C with shaking at 130rpm overnight. 50 µl of the starter culture was inoculated in 100ml LB broth with final concentration of 100µg/ml ampicillin. This was grown overnight at 37°C with shaking at 130rpm.

The cells were spun at 5,000 x g using a bench top centrifuge for 10 minutes at 4°C. The pellet was completely resuspended using 5ml of ice cold re-suspension buffer. 5ml of lysis solution was added and allowed to incubate on ice for 5minutes. 5ml of precipitation solution (Potassium acetate pH 4.8) was added and the solution vigorously mixed 5 times. The solution was then stored on ice for 15 minutes to enhance precipitation. The solution was spun at 5, 000 x g for 5minutes at 4^0C and the supernatant transferred to a fresh tube. 1.5µl 10mg/ml of RNase A was added to each tube and incubated at 37°C for 3 hours. The plasmid DNA was extracted using phenol chloroform isoamyl. An equal volume of 25:24:1(phenol: chloroform: isoamyl) was added to the RNA and protein free lysate and tubes spun at 5,000 x g

for 5minutes at 4°C. The upper aqueous layer containing the plasmid DNA was transferred to a clean tube using a pipette.

Equal volume of 24:1(chloroform: isoamyl) was added and spun at 5,000 x g for 5minutes at 4°C. This was repeated twice each time the upper aqueous layer was transferred to a clean tube using a pipette.

The plasmid DNA was then precipitated overnight using 0.1 volume of 3M sodium acetate pH 4.8 and 2X volume of absolute ethanol and the sample stored overnight at -20°C.

The sample was spun at 5, 000 x g for 5minutes at 4°C and washed twice with 70% ethanol at 6,000rpm for 5minutes and allowed to dry. The pellet was reconstituted by adding 100µl of 10mM Tris pH 8 and allowed to stand for 30min. 5µl of the Plasmid DNA was run on a 1% Agarose gel in TBE buffer. The remaining DNA was stored at -20°C.

3.13.5 Restriction enzyme digestion of the recombinant plasmid

The recombinant plasmid was restricted using Not 1(Promega) to confirm presence of the neuraminidase insert. 2 µl of 10X buffer D, 1 µl of Not 1(10u/ µl), 1 µl Acetylated BSA (10µg/ml), 1µg of recombinant plasmid DNA and nuclease free water were mixed in a sterile 1.5 eppendorf tube giving a final volume of 20 µl. The sample was mixed and spun down briefly followed by incubation in a water bath overnight at 37^0C. The restricted products were run on a1% Agarose gel in TBE buffer.

3.13.6 Confirmation of presence of insert in the recombinant plasmid

A PCR was performed using the extracted plasmid to ascertain the presence of the neuraminidase insert in the pGEMT –Easy vector.

25 µl of the PCR reaction was prepared with a final concentrations of (1X) PCR buffer, 0.2 mM dNTPS, 3mM $MgCl_2$,0.8 pmoles/µl gene specific forward and reverse primers, 0.3 µl, Taq polymerase (ampliTaq)(5U/µl). 0.5ng/ml of the plasmid DNA was used as template.

The PCR programme used consisted of an initial denaturation step at 94^0C, for 4 min followed by 35 cycles of denaturation at 94^0C for 30 sec, annealing at 55^0C for 30 sec. and extension at 68^0C for 1 min. A final extension step at 72^0C for 7 min was included to amplify the ends of the PCR products.

To visualize the amplification products, amplified PCR products were run on an ethidium bromide stained 1% agarose gel in TBE alongside DNA size markers.

3.13.7 Sequencing of the neuraminidase (NA2) insert

The insert was sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977) using ABI prism 3100 DNA analyzer at ILRI Segoli laboratory. Prior to sequencing, the plasmid DNA samples were cleaned using GFX kit (GE Healthcare Amersham place UK) and eluted using nuclease free water. The inserts was sequenced using T7 and Sp6 universal primers.

3.14 Sub cloning and expression of neuraminidase

3.14.1 Linearization and purification of the expression vectors

The NA2 gene was previously cloned into a pGEMT-easy which is a cloning vector. In order to express the neuraminidase protein from this cloned gene, it was necessary to sub-clone the gene into the expression vectors pQE30 and pBK.

pQE30 was double restricted using 14µl nuclease-free water, 2 µl 10X restriction buffer , 2µl acetylated BSA (1mg/ml), 20µl pQE30,1µl Kpn1(10 U/ µl) and 1 µl Hind111 (10 U/ µl) giving final volume of 40 µl. The reaction was incubated overnight in a water bath at 37^0C.

pBK was Single restricted using 14µl Nuclease-Free Water, 2 µl 10X Restriction Buffer D, 2µl Acetylated BSA (1mg/ml), 20µl pBK, 2 µl Not 1 (10u/ul) giving final volume of 40 µl. The reaction was incubated overnight in a water bath at 37^0C.

The restricted plasmids were run on a 1% Agarose gel which was prepared using 0.5X TBE. The plasmids were purified from gel using GFX kit as described in (§ 2.2.11).

3.14.2 Digestion of neuraminidase (NA2) insert from pGEMT-easy

The neuraminidase gene was digested from pGEMT-easy for subcloning in the pBK expression vector using Not 1(Promega) in order to liberate insert. The restriction reaction was prepared by mixing 2 µl of 10X buffer D, 2 µl of Not 1(10U/ µl), 2 µl acetylated BSA (10mg/ml), 20 µl of recombinant plasmid DNA and 14 µl of nuclease free water were mixed in a sterile 1.5 eppendorf tube giving a final volume of 40 µl. The sample was mixed and spun down briefly followed by incubation in a water bath overnight at 37^0C. The restricted NA2 was run on a 1% Agarose gel

which was prepared as described in (3.11) and purified from gel using GFX kit as described in (3.12).

3.14.3 PCR amplification and cleaning of the NA2 gene insert

For ligation into pQE30 the NA2 was first amplified using primers with restriction sites for Kpn1 and Hind 111. Forward primer 5'-TTT TGG TAC CAT GAA TCC AAA TCA AAA GAT AAT AAC R-3' and reverse primer 5'-TTT TAA GCT TTT ATA TAG GCA TGA GAT TGA TGT CC-3' The product (NA2) was double restricted by mixing 14µl Nuclease-Free Water, 2 µl 10X Restriction Buffer 2µlAcetylated BSA (1mg/ml), 20µl DNA, 1µl kpn1(10U/ µl) and 1 µl Hind111 (10U/ µl) giving final volume of 40 µl. The reaction was incubated overnight in water bath at 37^0C.

3.14.4 Ligation of restricted neuraminidase (NA2) into linealized pQE30 and pBK

The reaction for ligation of NA2 into pQE30 was prepared by mixing vector DNA pQE30 100ng, (2µl) Insert DNA neuraminidase 150ng, (3µl), 1µl 10X Ligation Buffer Roche, 2µl T4 DNA Ligase (3U/µl) and 2µl Nuclease-free water to 10µl.These were incubated at 4°C overnight. The ligation reaction for ligation of NA2 into pBK was prepared by mixing vector DNA pBK 100ng, (2µl) Insert DNA neuraminidase 150ng, (3µl), 2µl 10X Ligation Buffer Roche, 3µl T4 DNA Ligase (3u/µl) and 10µl Nuclease-free water to 20µl.These were incubated at 4°C overnight

3.14.5 Confirmation of sub cloning of NA2 gene into pBK using PCR

The transformation of competent cells using the ligation reaction was carried. pQE30 did not give any colonies but pBK gave colonies and the plasmids were screened for recombinants using PCR. Five colonies were picked randomly and master mixes

prepared by mixing 1X PCR buffer, 0.2 mM dNTPS, 3mM MgCl2, 0.8 pmoles/µl forward and reverse NA2 gene specific primers. 0.3 µl, Taq polymerase (5U/µl), each reaction tube had a final volume of 25 µl after adding 2ul (0.5 ug) DNA as template.

PCR conditions used comprised an initial denaturation at 94 ^{0}C for 4 min., followed by thirty five cycles of denaturation at 94 ^{0}C 30 sec., annealing at 55 ^{0}C for 30 sec. and extension at 68 ^{0}C for 1 min. To complete the amplification of the of the PCR products, a final extension step at 72 ^{0}C for 7 min was included. The GeneAmp® PCR system 9700 thermo cycler from Applied Biosystems was used. The PCR products were resolved in a 1% Agarose gel prepared as described in (3.11).

3.14.6 Induction of protein expression and sonication of recombinant cultures

Two colonies gave a product with PCR and were separately cultured in 100ml of LB Kanamycin media (20µg/ml) and incubated at 37^{0}C with shaking at 130 rpm overnight. 200µl of 100mM IPTG was added to the media and allowed to induce expression for 2 hours. The cells were centrifuged at 5, 000 x g for 5 minutes at 4^{0}C and the supernatant discarded. The pellet was resuspended and washed twice using 1X PBS pH 7.4 each time the samples were spun at 5, 000 x g for 5 minutes at 4^{0}C and the supernatant discarded.

After the second wash the cells were resuspended using 750 µl of 1X PBS pH7.4 and the cell samples kept on ice for 30 minutes. The cells were sonicated using 2min bursts at 22µm amplitude till the cells were disrupted and the solution changed colour from milky to translucent. The ultrasonic Processor Sonicator PR-250 was used. Care was taken to ensure that the tip was inserted into a depth of at least 1cm.

The solution was then centrifuged at 5, 000 x g at 4°C and the supernatant (enzyme lysate) aspirated and stored at -20°C**.**

3.15 Assay for neuraminidase activity

3.15.1 Hemagglutination inhibition assay as a proxy for neuraminidase assay.

The two enzyme lysates were used in hemagglutination inhibition assay to check for presence of functional neuraminidase activity (Table 3.1)

The basis of this assay was that neuraminidases cleave sialic acid bearing receptors on RBC thus causing hemagglutination inhibition upon incubation with virus/ control H3N2 control antigen. (Fig 3.4)

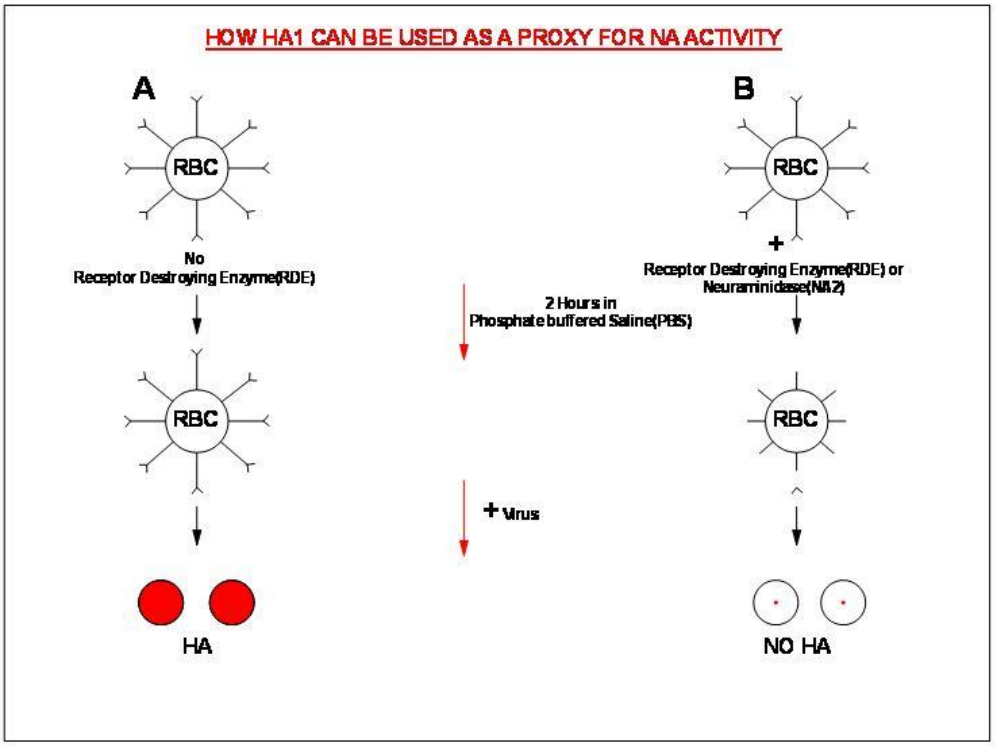

Figure 3.4: Use of hemagglutination inhibition assay to demonstrate neuraminidase activity

Table 3.1 Treatment of the guinea pig RBC using receptor destroying enzyme (Vibrio cholerae) and recombinant (NA2) enzyme lysate.

Sample	**Volume of Sample**	**Volume of RB**C
Positive control Receptor destroying enzyme (Vibrio cholerae)	600µl	200 µl of guinea pig red blood cells
Enzyme lysate from colony 2	600µl	200 µl of guinea pig red blood cells
Enzyme lysate from colony 3	600µl	200 µl of guinea pig red blood cells
Negative control lysate from non recombinant *E.Coli*	600µl	200 µl of guinea pig red blood cells

The samples were allowed to incubate at 37^0 C for 6 hours.

The reactions were washed twice with PBS buffer pH 7.4 and centrifuged at 1500 rpm for 15 mins at 4^0C using eppendorf centrifuge model 5810R and diluted to make 1% guinea pig blood.

The samples were used in HAI assay as a proxy for neuraminidase assay (Fig 3.5).

The assay was set up by adding 50µl of sterile PBS pH 7.4 to well (2A-2D), (12A-12D) using a multi channel micro-pipette.

50µl of each of the treated 1% guinea pig red blood cells was added to respective wells. 50µl of control H3N2 antigen was added to wells 1A-1H and two- fold serial dilutions carried out by transferring 25µl from well to well starting with (1A-1H) and the plate incubated at room temperature for 30minutes.

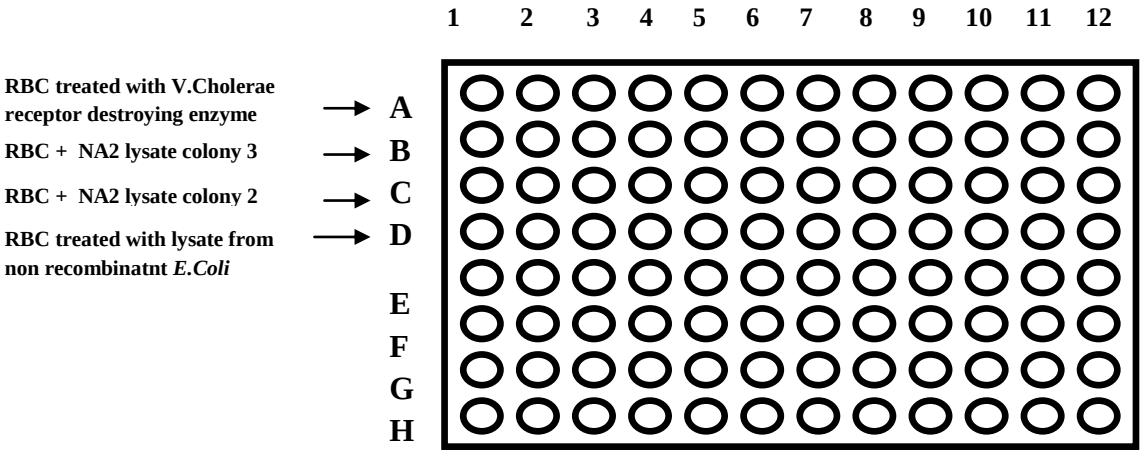

Figure 3.5: Layout for hemagglutination inhibition assay (proxy for neuraminidase assay)

3.16. Trans-Sialidase assay

The neuraminidase enzyme lysate from colony 2 was used in trans-sialidase assay as it caused hemagglutination inhibition an indication that neuraminidase activity was present. The assay was set up as shown in table 3.2

Table 3.2 Trans-sialidase assay set up

Sample	**Volume of Sample**	**Volume of Guinea pig RBC**	**Acceptor Molecule**
Enzyme lysate from colony 2	600µl	200 µl	40 µl of 100mM PNP- Gal
Enzyme lysate from colony 2	600µl	200 µl	

The samples were allowed to incubate at 37^0 C for 6 hours. The cells were spun at 1500 rpm for 15 minutes at 4^0C using eppendorf centrifuge model 5810R to recover the supernatant. The supernatant was aspirated and placed in a clean 1.5 eppendorf tube. The cells were washed twice with PBS buffer pH 7.4 and spun at 1500 rpm for

5 minutes at 4^0C using eppendorf centrifuge model 5810R. The pellet was diluted to make 1 % guinea pig RBC using PBS buffer pH 7.4.

3.16.1 Acetone precipitation of proteins in serum

Before running the samples on the Thin Layer Chromatography (TLC) plate, it was necessary to precipitate proteins present in the serum.10ml of acetone was added into a 15ml centrifuge tube and allowed to chill at -20^0C for 30 minutes. 50 µl of each of the serum samples (table 3.2) was pippeted into a 1.5 eppendorf tube and 200 µl of pre-chilled acetone added to each tube. The tubes were vortexed and incubated at -20^0C for I hour. The samples were spun at 13,000 rpm for 5 minutes at 4 ^{0}C using 5415R eppendorf micro-centrifuge and the clear supernatants aspirated into clean 1.5 eppendorf tubes. The precipitated proteins were discarded.

3.16.2 TLC analysis of trans-glycosylation products

Thin layer chromatography is a technique used to separate compounds in a mixture. TLC is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel referred to as stationary phase. Samples are applied on the plate, and a solvent mixture (mobile phase) is drawn up the plate by capillary action. Since different compounds ascend the TLC plate at different rates, separation is achieved.

The mobile phase used was ethyl acetate/methanol/ acetic acid/water at a ratio of 120:30:30:20 respectively. The stationary phase was silica gel analytical grade. 10µl of each of the precipitated samples were spotted on TLC plates. When the migration of the spots reached the end point, the plate was dried and then observed under UV. Fluorescent spots were circled using a fine pencil. The plate was dipped in revelation

solution (Appendix VI) and dried before heating on a hot plate at ≈ 60°C to reveal blue spots.

3.16.3 Capillary electrophoresis of trans-glycosylation products

Capillary electrophoresis (CE) can be used to separate ionic species by their charge, frictional forces and hydrodynamic radius. The separation of compounds by capillary electrophoresis is dependent on the differential migration of analytes in an applied electric field. 30 µl of the trans-glycosylation product and controls were diluted with sodium borate buffer pH 10.5 to give a final volume of 500 µl.The samples were loaded and run at 30.00 kV under injection pressure of 50.psi for 4 seconds. The spectrum was from190 nm-600nm.

CHAPTER FOUR

4. RESULTS

4.1 Cloning, sequencing and phylogenetic analysis of the neuraminidase gene from the influenza A/Nairobi/2041/2006(H3N2)

4.1.1 Detection of influenza Virus from nasopharyngeal swabs

Isolate (A/Nairobi/2041/2006(H3N2 previously obtained from a nasopharyngeal swab specimen from a patient presenting with influenza-like-illness (Bulimo et al., 2008) was confirmed to be influenza H3N2. Fig. 4.1 shows that with a Ct value of 21, indeed the aliquot contained influenza A virus.

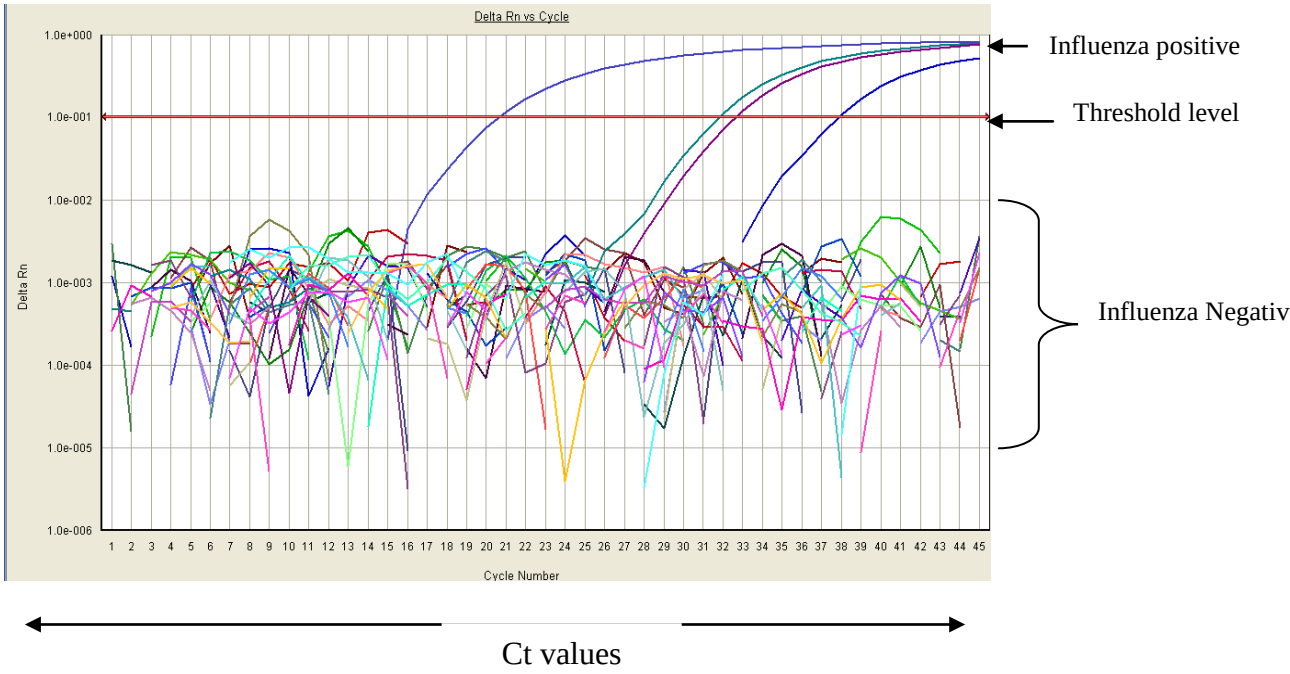

Fig. 4.1: Detection of influenza virus A using isolate aliquot sample using real time RT-PCR.

Ct value (the point at which amplification curves crosses the threshold line) for RNA derived from the Influenza A virus (A/Nairobi/2041/2006(H3N2) isolate aliquot was 21.

4.1.2 Typing and sub typing of virus samples.

In order to confirm the subtype of the influenza A virus in the aliquot, it was necessary to carry out sub typing of the virus in the aliquot. The hemagglutination and hemagglutination inhibition assays were applied. Two of the four isolates caused hemagglutination after incubation with the guinea pig blood hence confirming presence in the aliquot of a virus with hemagglutinating activity (Fig 4.2), control 2 and A/Nairobi/2041/2006(H3N2)).Since hemagglutinating activity can be caused by other viruses other than influenza virus, presence of influenza virus was confirmed using hemagglutination inhibition assay (HAI). This influenza-specific assay is able to distinguish between the H3N2 and H1N1 subtypes of influenza A viruses. Hemagglutination inhibition was observed in wells containing H3N2 reference antisera with A/Nairobi/2041/2006(H3N2) isolate and control 2. These results showed that the samples did not cause hemagglutination inhibition with H1N1 antisera (Fig4.3). However, the A/Nairobi/2041/2006(H3N2) isolate and the virus in control 2 were indeed influenza A viruses of the H3N2 subtype containing the NA2 gene segment (Fig 4.3).

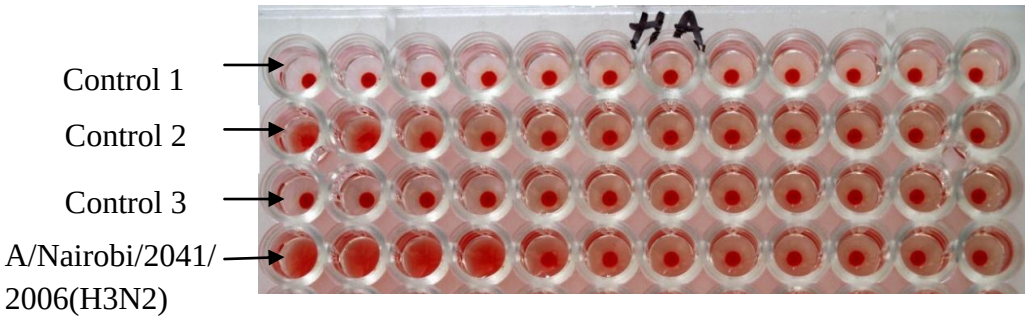

Fig 4.2: Hemaglutination assay

Control1 and 3: Formation of rings (no hemagglutination)

Control 2 and isolate A/Nairobi/2041/2006(H3N2): Formation of mesh (hemagglutination)

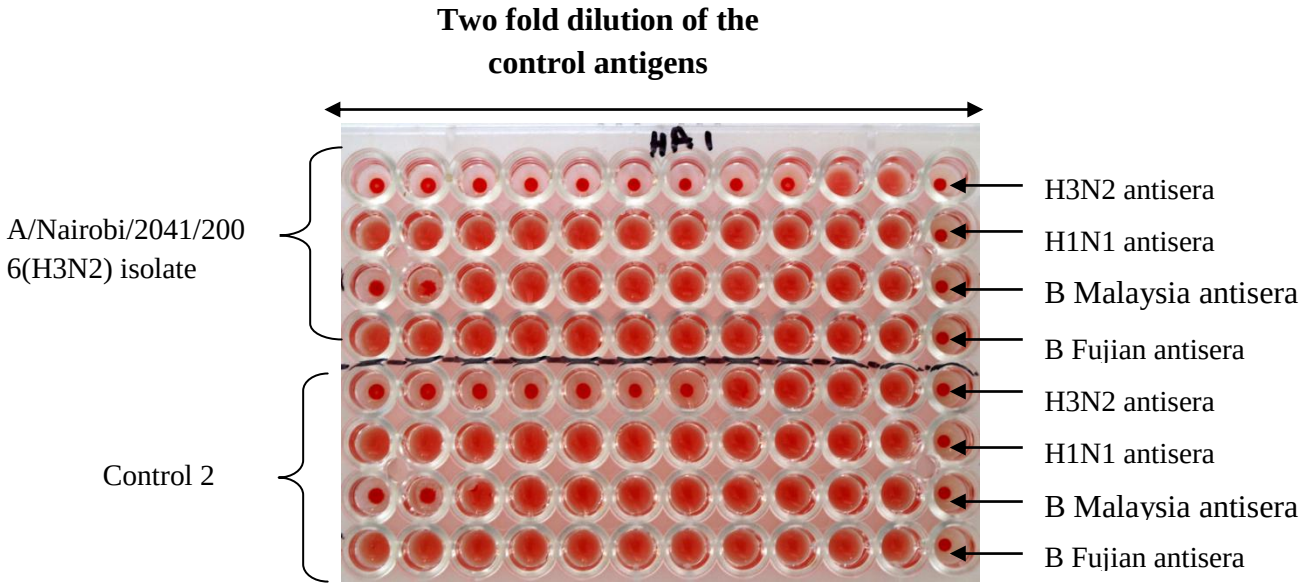

Fig 4.3: Hemagglutination inhibition assay

A/Nairobi/2041/2006(H3N2) isolate: Hemagglutination inhibition with H3N2

Control 2: Hemagglutination inhibition with H3N2

4.1.3 Amplification of type NA2 neuraminidase from the influenza A/Nairobi/2041/2006(H3N2) virus

Conventional RT PCR was applied in amplification of the NA2 gene and the amplification products resolved on a 1% Agarose gel. The expected NA2 size of approximately 1500bp was obtained (Fig 4.4, lanes 1 & 2). Amplification of the hemagglutinin gene (HA3) from the same virus isolate was used as the internal positive control for this experiment (Fig. 4.4, lane 3). Since the amplified product had a non specific band, multiple aliquots of the amplified product were loaded on a 1% agarose gel to facilitate gel purification of the main 1.5kbp fragment from the minor non specific amplification products (Fig. 4.5) to allow for efficient cloning. An aliquot of this gel-purified NA2 fragment was electrophoresed in a 1% agarose gel and the purity of the gene fragment confirmed prior to ligation (Fig. 4.6).

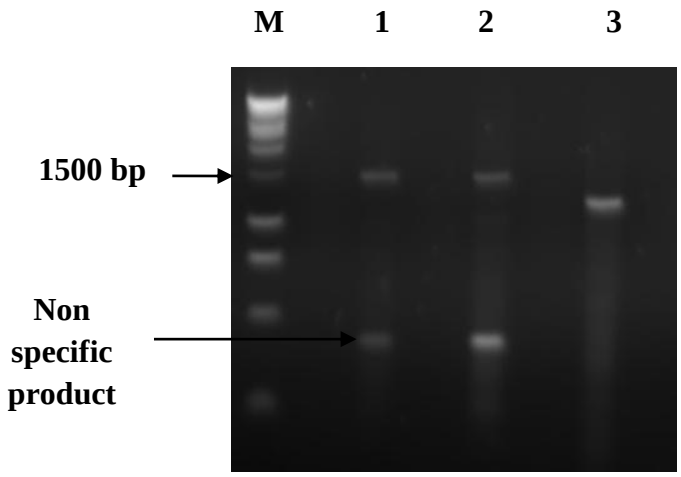

Fig 4.4: RT-PCR of the neuraminidase and hemagglutinin derived from human H3N2 influenza A virus.

Viral RNA was extracted using Qiagen QIAamp virus RNA extraction kit and reverse transcribed using invitrogen's superscript. The cDNA was used in the amplification of neuraminidase.

M= Promega 1Kb molecular Weight Marker.

1 = Amplified neuraminidase gene (first aliquot)

2 = Amplified neuraminidase gene (second aliquot)

3= HA positive control amplified with 40 pmoles forward and reverse primers

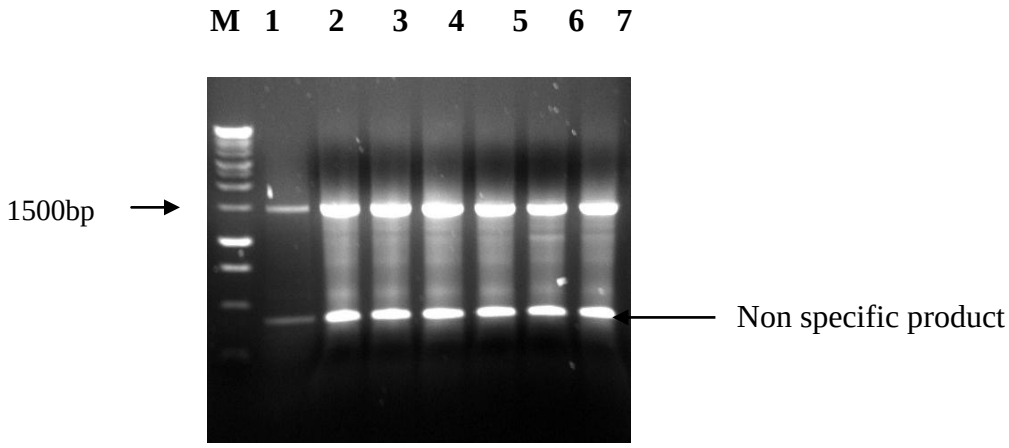

Fig 4.5: Multiple aliquots of the amplified NA2 product

M= Promega 1Kb molecular Weight Marker.

1-7 = Aliquots of amplified neuraminidase.

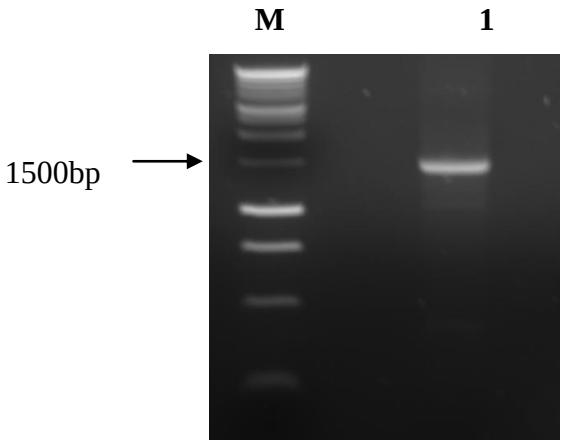

Fig 4.6: Gel purification of the amplified neuraminidase

The neuraminidase gene was gel purified using GFX kit. 2 µl was loaded on a 1% Agarose gel and electrophoresed to check for purity.

M= Promega 1Kb molecular Weight Marker

1= Gel purified Neuraminidase

4.1.4 Ligation and transformation of the neuraminidase gene fragment.

Upon ligation of the purified neuraminidase gene fragment into the pGEMT-Easy vector followed by transformation of competent *E.coli,* the transformed cells were cultured and plasmid DNA extracted and resolved on a 1% agarose gel (Fig 4.7).

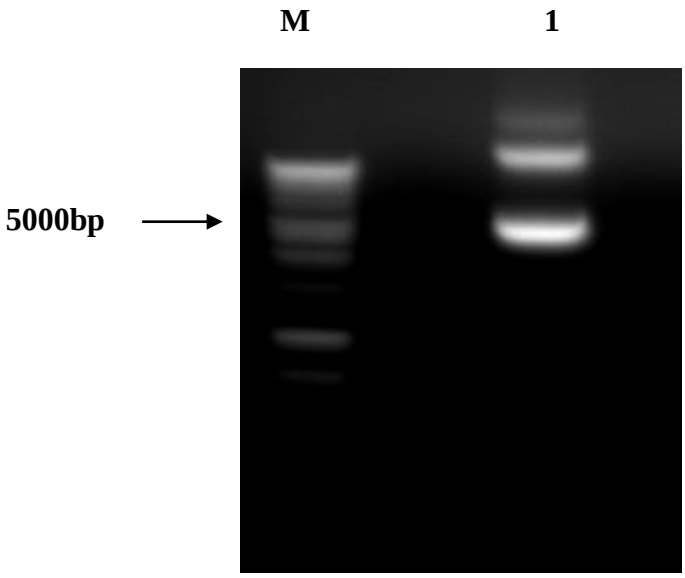

Fig 4.7 Analysis of plasmid from transformed *E. coli*

M = Promega 1Kb molecular Weight Marker

1 = Extracted recombinant plasmid (lower band) circular and (upper band) super coiled) form

4.1.5 Screening of the recombinant plasmid DNA for the presence of human NA2.

To identify transformants with the NA2 insert, plasmids obtained from several independently transformed *E. coli* colonies were screened with NA2-specific primers using conventional PCR. The amplifications gave a fragment size that corresponded to 1500bp which is the expected size of neuraminidase (Fig. 4.8). Additionally, Not1

restriction endonuclease was used to digest the putative recombinant plasmids derived from the transformed *E. coli* because Not1 restriction sites had been incorporated in the forward and reverse primers of PCR insert but are absent in the neuraminidase gene.

The restriction reaction yielded two fragments, one of 3.5kb corresponding to the linealized backbone vector and a 1.5kb fragment corresponding to the NA2 gene fragment (Fig 4.9).

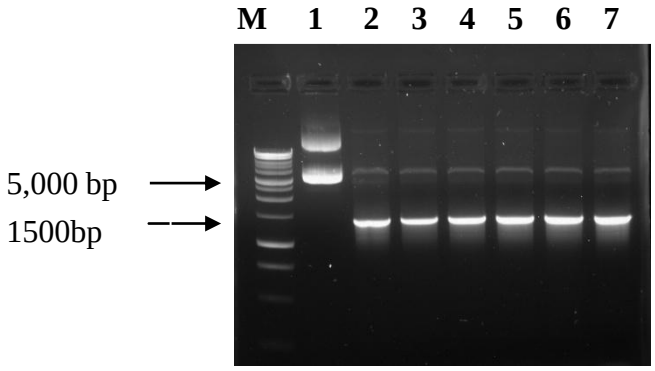

Fig 4.8: Amplification of the recombinant plasmid (pGEMT-NA2).

The extracted plasmid was used in amplification of neuraminidase gene to confirm that the insert in the plasmid is neuraminidase

M = Molecular weight marker Promega 1Kb

Lane 1 = Recombinant pGEMT-NA2

Lane 2-7= Aliquots of amplified neuraminidase using the recombinant plasmid as template.

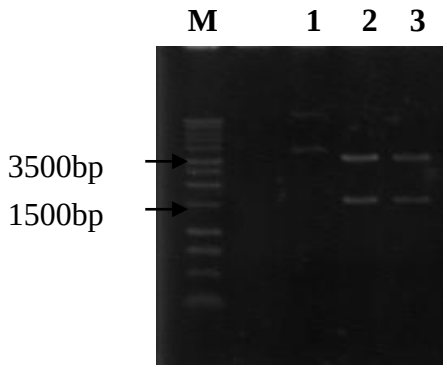

Fig 4.9: Restriction of the recombinant pGEMT–Easy using promega's Not1.

The recombinant plasmid was digested using Not 1 and resolved on a 1% agarose gel.

M = Promega's 1 kb molecular weight marker

Lane 1 = Undigested recombinant plasmid

Lane 2-3 = **(**Upper band**)** Linealized pGEMT and (lower band) liberated neuraminidase.

4.1.6 Sequencing of the recombinant pGEMT-Easy plasmid

To definitively confirm that the putative recombinant plasmid had the influenza A/Nairobi/2041/2006(H3N2) NA2 insert, the nucleotide sequence of the insert was determined. This was also important to determine the orientation of the insert in the plasmid relative to the T7 promoter. Only a clone with the insert in the forward orientation relative to the T7 promoter would be amenable for further use in recombinant protein expressions. One such clone was obtained. Two partial sequences were generated using the T7 and SP6 Primers. The forward primer (T7) gave a sequence that was 956 bp while the reverse primer (SP6) gave a fragment that was 1002 bp long (appendix vii).

4.1.7 Contig Assembly and Cleaning of the NA2 nucleotide sequence

Since the two partial sequences obtained using the T7 and SP6 sequencing primers overlapped, it was necessary to assemble them into a single contiguous sequence in order to get the full complement of NA2 gene fragment. Upon contig assembly using

the contig assembly program (CAP) found in the Bioedit suite (Hall, 1999), a contig of 1616 nucleotides was generated (appendix). In order to clean this nucleotide sequence of contaminating vector sequences, the sequence was scanned against a database containing known DNA vector sequences using the VecScreen algorithm found at the National Center for Biotechnology Information (NCBI). The result showed that a segment of 55 nucleotides towards the 3' end of the insert (position 1545-1599) was perfectly matched to the Promega pGEM-T easy vector within the region containing the T7 promoter-multiple cloning site-SP6 promoter junction (Fig. 4.10). Downstream to this at position 1600-1616, the nucleotide sequence was of suspect origin (Fig. 4.10). Thus, the correct nucleotide size of the NA2 insert in the contig was 1522nt (Fig. 4.11).

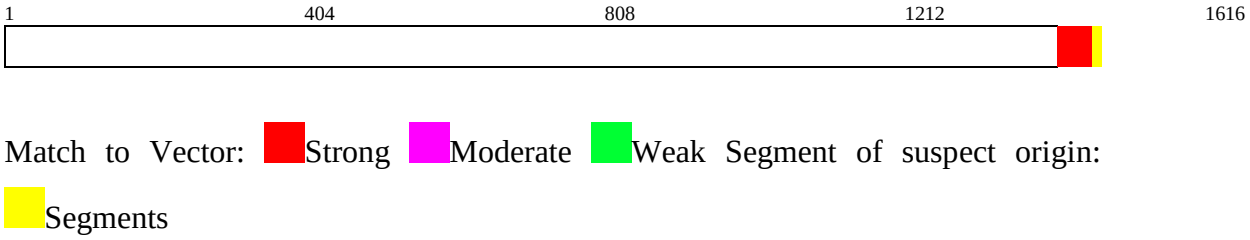

Fig 4.10. Distribution of Vector Matches on the Query Sequence
Matching vector: Strong match: 1545-1599, Suspect origin: 1600-1616

```
   1 TTTTATAATG TAAATTCCAT CCAGCTCCGG CGCCAGGCGG CCGCGGGATT CGATTAGCAA
  61 AAGCAGGAGT GAAGATGAAT CCAAATCAAA AGATAATAAC GATTGGCTCT GTTTCTCTCA
 121 CCATTTCCAC AATATGCTTC TTCATGCAAA TTGCCATCTT GATAACTACT GTAACATTGC
 181 ATTTCAAGCA ATATGAATTC AACTCCCCCC CAAACAACCA AGTGATGCTG TGTGAACCAA
 241 CAATAATAGA AAGAAACATA ACAGAGATAG TGTATCTGAC CAACACCACC ATAGAGAAGG
 301 AAATATGCCC CAAACTAGCA GAATACAGAA ATTGGTCAAA GCCGCAATGT GACATTACAG
 361 GATTTGCACC TTTTTCTAAG GACAATTCGA TTAGGCTTTC CGCTGGTGGG GACATCTGGG
 421 TGACAAGAGA ACCTTATGTG TCATGCGATC CTGACAAGTG TTATCAATTT GCCCTTGGAC
 481 AGGGAACAAC ACTAAACAAC GTGCATTCAA ATGACACAGT ACGTGATAGG ACCCCTTATC
 541 GGACCCTATT GATGAATGAG TTAGGGGTTC CTTTCCATCT GGGGACCAAG CAAGTGTGCA
 601 TAGCATGGTC CAGCTCAAGT TGTCACGATG GAAAAGCATG GCTGCATGTT TGTATAACCG
 661 GGGATGATAA AAATGCAACT GCTAGCTTCA TTTACAATGG GAGGCTGGTA GATAGTATTG
 721 TTTCATGGTC AAAAGAAATC CTCAGGACCC AGGAGTCAGA ATGCGTTTGT ATCAATGGAA
 781 CTTGTACAGT AGTAATGACT GATGGGAGTG CTTCAGGAAA AGCTGATACT AAAATACTAT
 841 TCATGGAGGA GGGGAAAATC GTTCATACTA GCACATTGTC AGGAAGTGCT CAGCATGTCG
 901 AGGAGTGCTC CTGCTATCCT CGATACCCTG ATGTCAGATG TGTCTGCAGA GACAACTGGA
 961 AAGGCTCCAA TAGGCCCATC GTAGATATAA ACATAAAGGA TCATAGCATT GTTTCCAGTT
1021 ATGTGTGTTC AGGACTTGTT GGAGACACAC CCAGAAAAAA CGACAGCTCC AGCAGTAGCC
1081 ATTGTTTGGA TCCTAACAAT GAAGAAGGTG GTCATGGAGT GAAAGGCTGG GCCTTTGATG
1141 ATGGAAATGA CGTGTGGATG GGAAGAACGA TCAGCGAAAA GTCACGCTTA GGGTATGAAA
1201 CCTTCAAAGT CATTGAAGGC TGGTCCAACC CTAAGTCCAA ATTGCAGATA AATAGGCAAG
1261 TCATAGTTGA CAGAGGTAAT AGGTCCGGTT ATTCTGGTAT TTTCTCTGTT GAAGGCAAAA
1321 GCTGCATCAA TCGGTGCTTT TATGTGGAGT TGATAAGGGG AAGAAAAGAG GAAACTGAAG
1381 TCTTGTGGAC CTCAAACAGT ATTGTTGTGT TTTGTGGCAC CTCAGGTACA TATGGAACAG
1441 GCTCATGGCC TGATGGGGCG GACATCAATC TCATGCCTAT ATAAGCTTTC GCAATTTTAG
1501 AAAAAAACTC CTTGTTTCTA CT
```

Fig 4.11. NA2 cleaned of the contaminating vector sequence

The contig sequence (Fig 4.11) was cleaned using VecScreen algorithm of NCBI. The contaminating sequence was found to be 55 nucleotides long and of pGEM-T vector origin. The correct nucleotide size of the NA2 insert in the contig was 1522 nt.

4.1.8 Similarity search using NCBI BLAST and the generated contig sequence.

The contig sequence was analyzed against the non redundant nucleotide database using the default parameters of BLASTN algorithm at NCBI. The search revealed that the sequence was closely related to other human influenza A H3N2 neuraminidase sequences in the database and most closely related to that of A/New York/UR06-

0607/2007(H3N2). The search also revealed that the orientation of the neuraminidase fragment is plus- plus, implying that it was in the right orientation for translation upon sub-cloning. To further analyze the sequence, ten sequences with the highest BLAST bit scores and lowest expectation values were selected for alignment against the NA2 sequence. (Fig. 4.12) shows a multiple sequence alignment of the NA2 (designated Neuraminidase) against the ten sequences designated by their gi database number. From this figure it is evident that the NA2 is identical to 90% of these reference sequences.

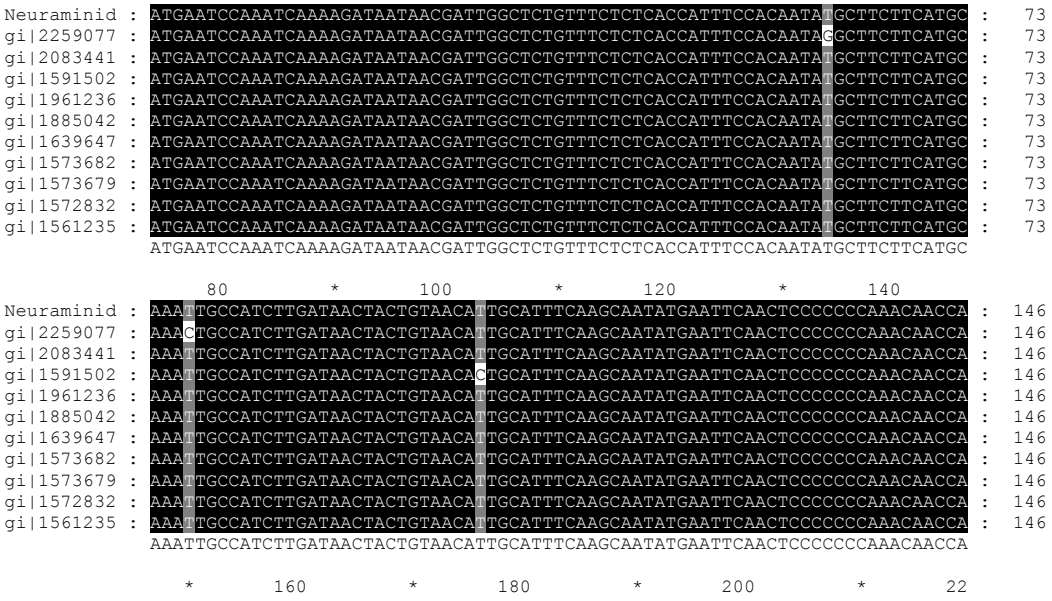

Figure 4.12: Alignment of influenza A H3N2 neuraminidase gene sequence with similar neuraminidase genes.

The MUSCLE 3.6 program (Edgar, 2004) was used to align the sequences. The first row labeled Neuraminidase is the sequence under study. The other sequences were derived from NCBI the sequence labels refer to their gene bank identification numbers. The Gene Doc program (Nicholas and Nicholas, 1997) was used to visualize the alignment.

4.1.9 Determination of the amino acid sequence of the neuraminidase.

To further analyze the NA2 gene fragment, the nucleotide sequence was conceptually translated into amino acid (protein) code. The "translate" algorithm of the Sequence Manipulation Suite (SMS) program (Stothard, 2000) was used in the conceptual translation. This gave a sequence that was 449 amino acids residues long (Fig. 4.13). The major open reading frame and any other minor ones as well as other signals present in the gene were determined and visualized using Accelrys DS Gene1.5 (Accelrys Software Inc) (Fig. 4.14). The visualization showed that the sequence had a single ORF of 1409 bp long (Fig. 4.14) spanning positions 75 to1484 implying that the full length neuraminidase gene was indeed present in the insert. The amino acid sequence was analysed using NCBI protein BLAST and found to be identical to other influenza A NA2 neuraminidase protein sequences (Fig. 4.15).

4.1.10 Determination of Phylogenetic relationship of NA2 with other human influenza A (H3N2) neuraminidases

To compare how closely related the NA2 was to other sequences in the database, those sequences at GenBank showing the highest bit scores and the lowest expectation values were used to draw a phylogenetic tree with a swine NA2 sequence as an out group. The result showed that NA2 sequence was closely related to a number of human influenza virus nucleotide sequences including A/Brisbane/10/2007(H3N2), the 2008 season's vaccine strain (Fig. 4.16).

```
  1 MNPNQKIITI GSVSLTISTI CFFMQIAILI TTVTLHFKQY EFNSPPNNQV MLCEPTIIER
 61 NITEIVYLTN TTIEKEICPK LAEYRNWSKP QCDITGFAPF SKDNSIRLSA GGDIWVTREP
121 YVSCDPDKCY QFALGQGTTL NNVHSNDTVR DRTPYRTLLM NELGVPFHLG TKQVCIAWSS
181 SSCHDGKAWL HVCITGDDKN ATASFIYNGR LVDSIVSWSK EILRTQESEC VCINGTCTVV
241 MTDGSASGKA DTKILFMEEG KIVHTSTLSG SAQHVEECSC YPRYPDVRCV CRDNWKGSNR
301 PIVDINIKDH SIVSSYVCSG LVGDTPRKND SSSSSHCLDP NNEEGGHGVK GWAFDDGNDV
361 WMGRTISEKS RLGYETFKVI EGWSNPKSKL QINRQVIVDR GNRSGYSGIF SVEGKSCINR
421 CFYVELIRGR KEETEVLWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI
```

Fig 4.13: Amino acid sequence of the NA2 neuraminidase

The NA2 nucleotide sequence gave a sequence of 469 amino acids residues long upon translation with the "translate" algorithm of the Sequence Manipulation Suite (SMS) program (Stothard, 2000).

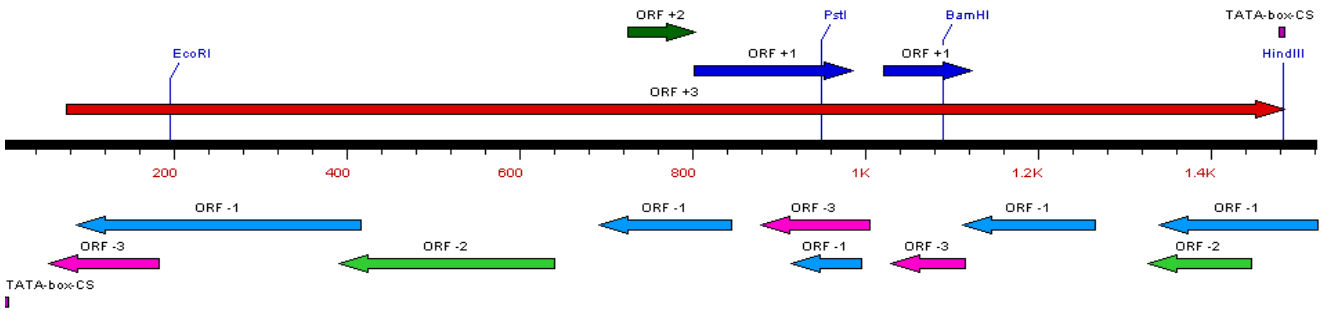

Fig 4.14: Visualization of the nucleotide sequence

The continuous red arrow ORF+3 is the neuraminidase open reading frame the visualization also shows the restriction sites present, EcoR1, Pst1 and BamH1. This information is important in sub cloning.

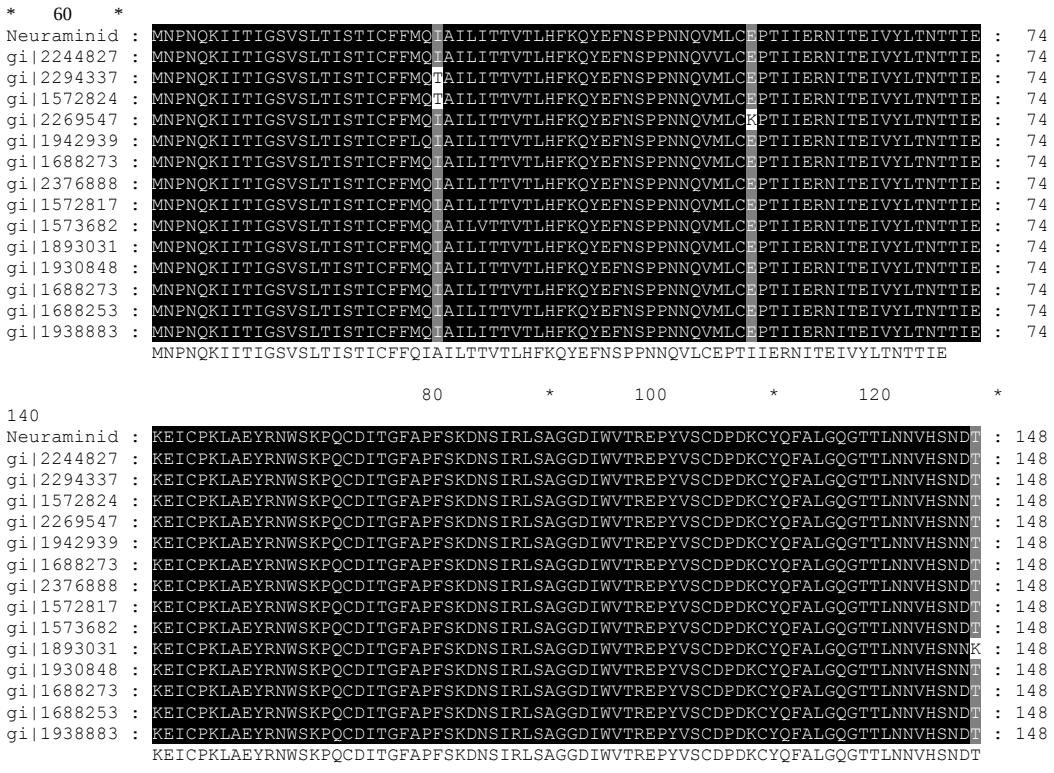

Fig 4.15: Alignment of influenza A H3N2 neuraminidase protein sequence with similar neuraminidase protein sequence.

The MUSCLE 3.6 program (Edgar, 2004) was used to align the sequences. The sequence labels refer to their gene bank identification numbers. The Gene Doc program (Nicholas and Nicholas, 1997) was used to visualize the alignment.

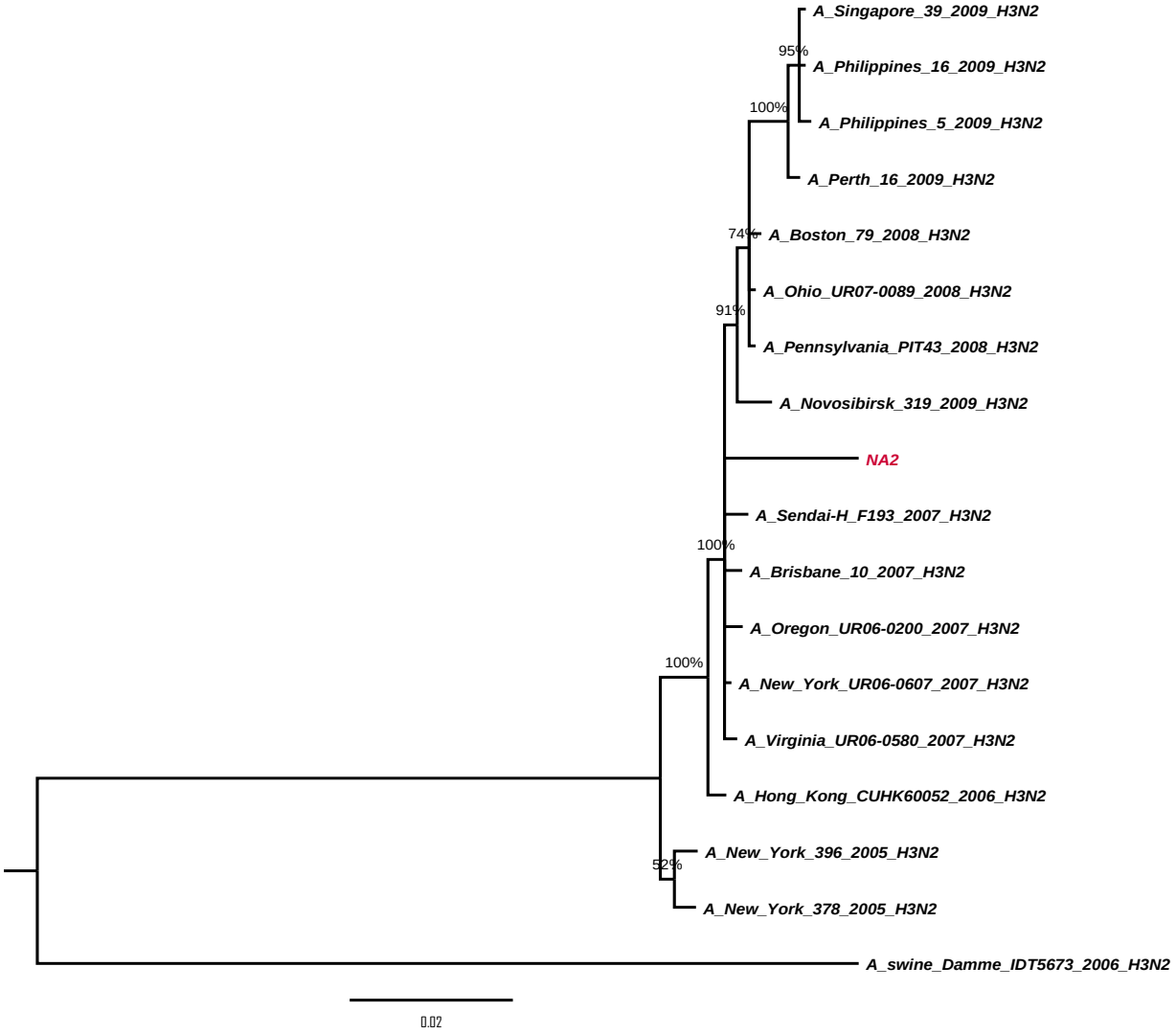

Fig 4.16. Phylogenetic relationships amongst influenza A (H3N2) NA2 genes

Maximum-likelihood phylogenetic tree of strain influenza A/Nairobi/2041/2006(H3N2) NA2 and the most closely related human influenza A (H3N2), viruses created using NA2 contig sequence & reference nucleotide sequences from GenBank and MrBayers software, version 3.1.2 (Huelsenbeck and Ronquist, 2001). The percentages are branch Bayesian posterior probability branch labels. The sequence of A/swine/Damme/IDT5673/2006(H3N2) was used as the out-group. Bar represents 2% sequence divergence.

4.2 Expression of the H3N2 influenza A neuraminidase

4.2.1 Restriction enzyme digestion of expression vectors pBK, pQE30 and the recombinant neuraminidase (NA2).

Once it was confirmed that the insert was indeed neuraminidase in the right orientation and with a single ORF of ~1400bp, the NA2 gene was sub cloned and expressed. The sub cloning was performed on two vectors pBK and pQE30

The pBK (Fig 4.17)and pQE30 were digested and neuraminidase gene digested to ensure compatibility (Fig 4.18). Transformation into pQE30 did not yield colonies but pBK gave several colonies

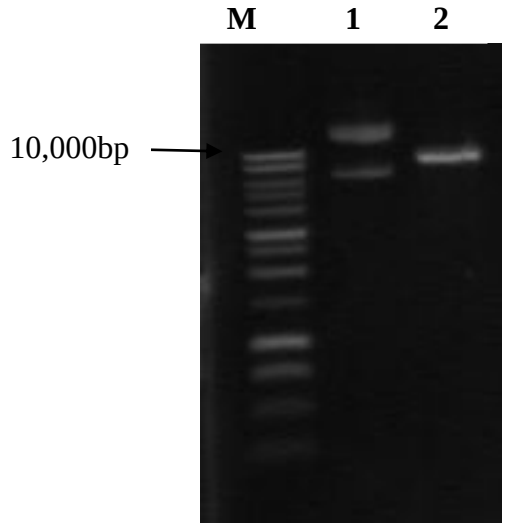

Fig 4.17 Digested PBK using Not1

The PBK plasmid was digested using promegas' Not 1 to ensure compatibility with the NA2 upon sub cloning.

M = 1 kb Molecular weight marker

Lane 1 = Undigested PBK plasmid (upper band super coiled) (lower band circular)

Lane 2 = Not 1 digested PBK

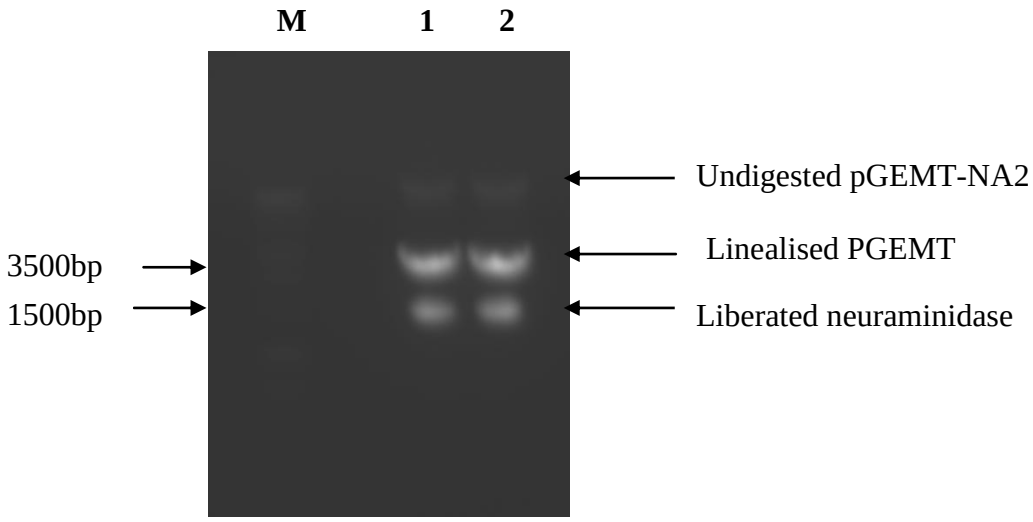

Fig 4.18: NA2 liberated from recombinant pGEMT-NA2 using promegas' Not1

The NA2 was digested from recombinant pGEMT-NA2 using promegas' Not 1 to ensure compatibility with Not 1 digested pBK (Fig 4.17) upon sub cloning.

M = Promegas' 1kb ladder Molecular weight marker

Lane 1, 2 = Linealized pGEMT (upper band) and liberated neuraminidase (lower band) after digestion with Not 1

4.2.2 Analysis of the recombinant colonies using colony PCR

pBK yielded several colonies that were used in screening of recombinant plasmids. Five colonies were picked randomly, cultured and the plasmids extracted and screened by PCR. Two of the plasmids gave a product implying that they had the NA2 insert and are thus recombinant. The remaining three plasmids did not amplify implying that they were products of self ligation of the PBK vector (Fig 4.19).

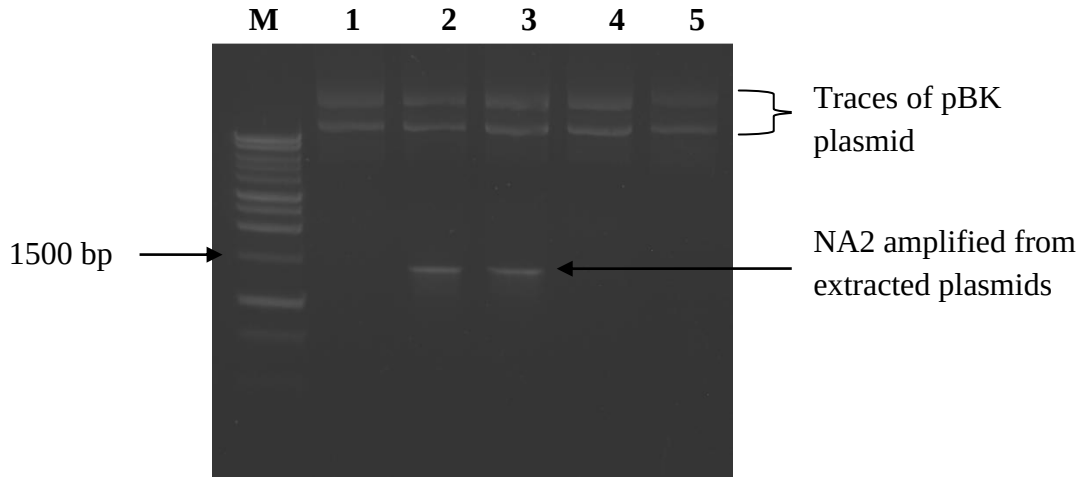

Fig 4.19: PCR using plasmids extracted from colonies

Five colonies were picked randomly and cultured for plasmid extraction. The extracted plasmids were then used in PCR reaction for screening for recombinants.

M **=** Promega 1kb Molecular weight marker

Lane = 1, 4, 5 Self ligated PBK plasmid

Lane = 2, 3 (Upper band> 10,000bp) recombinant pBKNA2 (Lower band =1500bp) NA2 insert)

4.3 Assay for presence of neuraminidase (NA2) activity in recombinant colonies.

Since the NA2 was sub cloned into pBK expression vector, using Not 1, it was not possible to have directional cloning. This implies that the insert could be incorporated in either reverse or forward orientation. Expression of the protein could only be possible from those that had the insert in the forward orientation. In order to determine if expression was possible from the two selected clones with the insert, a neuraminidase functional assay was done. The assay involved digestion of

neuraminic (sialic) acid moieties off guinea pig RBC using neuraminidase lysates from bacteria transformed with pBK-NA2 plasmids. If neuraminidase activity was present in the lysates, the consequence would be inhibition of hemagglutination of the RBC. The two pBK-NA2 plasmids that were positive in PCR screening were used in the assay. Lysate derived from bacterial cells transformed with pBK-NA2 from the second colony inhibited hemagglutination of the RBCs while those from colony 3 did not (Fig 4.20). From the result it was concluded that pBK-NA2 from colony 2 had the insert in the corrected orientation and was expressing neuraminidase activity.

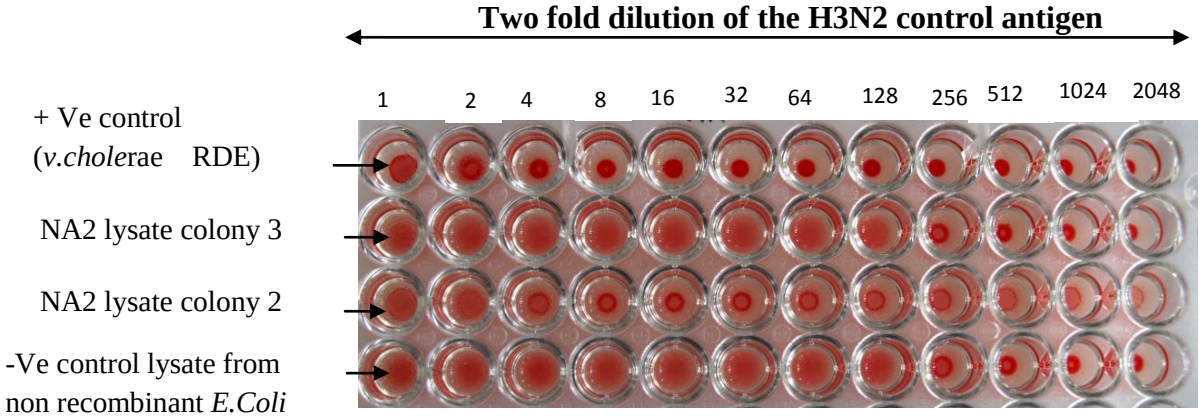

Fig 4.20: Assay for neuraminidase activity

Enzyme lysate from colonies (2 and 3) that gave positive result with PCR (Fig 4.19) were used in assay for neuraminidase activity. Four aliquots of the guinea pig red blood cells were treated separately using *vibrio cholerae* receptor destroying enzyme(RDE)-which destroys sialylated receptors, NA2 enzyme lysates (Colony 3and 2) and lysate from non recombinant *E.Coli* for 6 hours before adding H3N2 control antigen.

+ve control = (RDE) causing hemagglutination inhibition

NA2 lysate colony 3 = No inhibition of hemagglutination

NA2 lysate colony 2 = Inhibition of hemagglutination

-ve control = No inhibition of hemagglutination

4.4 Assay for presence of trans-sialidase activity in the recombinant neuraminidase

The pBK-NA2 plasmid that inhibited hemagglutination (NA2 lysate colony 2) encoded neuraminidase activity. The lysate from this colony was therefore used to assay for presence of trans-sialidase activity from the bacterially-expressed neuraminidase (Table 3.2). After adding the acceptor PNP-Gal molecule to the enzyme lysate and guinea pig RBC, and assaying for trans-glycosylation activity on a TLC plate (Fig.4.21) and capillary electrophoresis (Fig 4.22), no formation of trans-glycosylation products was observed. This implied that the human H3N2 influenza A virus neuraminidase could not transfer the sialic acid to the acceptor molecules PNP-Gal, hence lacked trans-glycosylation activity.

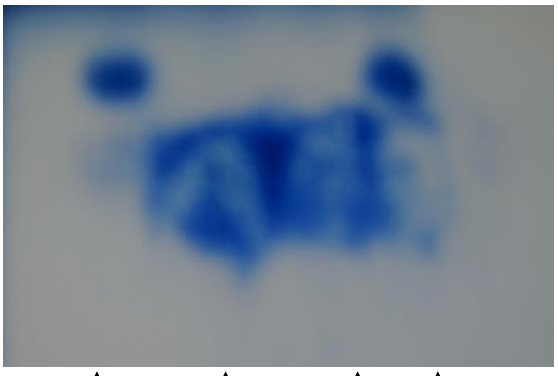

Figure 4.21: Analysis of trans-glycosylation products on TLC

The trans-glycosylation products were spotted on a TLC plate and allowed to migrate. The spots were visualized under UV and revealed by use of a revelation fluid (appendix vi) followed by heating of the plate. PNP-Gal and PNP-Gal NA2 migrated the same distance implying that there was no product formation through trans-glycosylation.

1 = PNP-Gal **2** = Non recombinant lysate + RBC **3** = NA2 + RBC **4** = PNP-Gal + NA2 + RBC

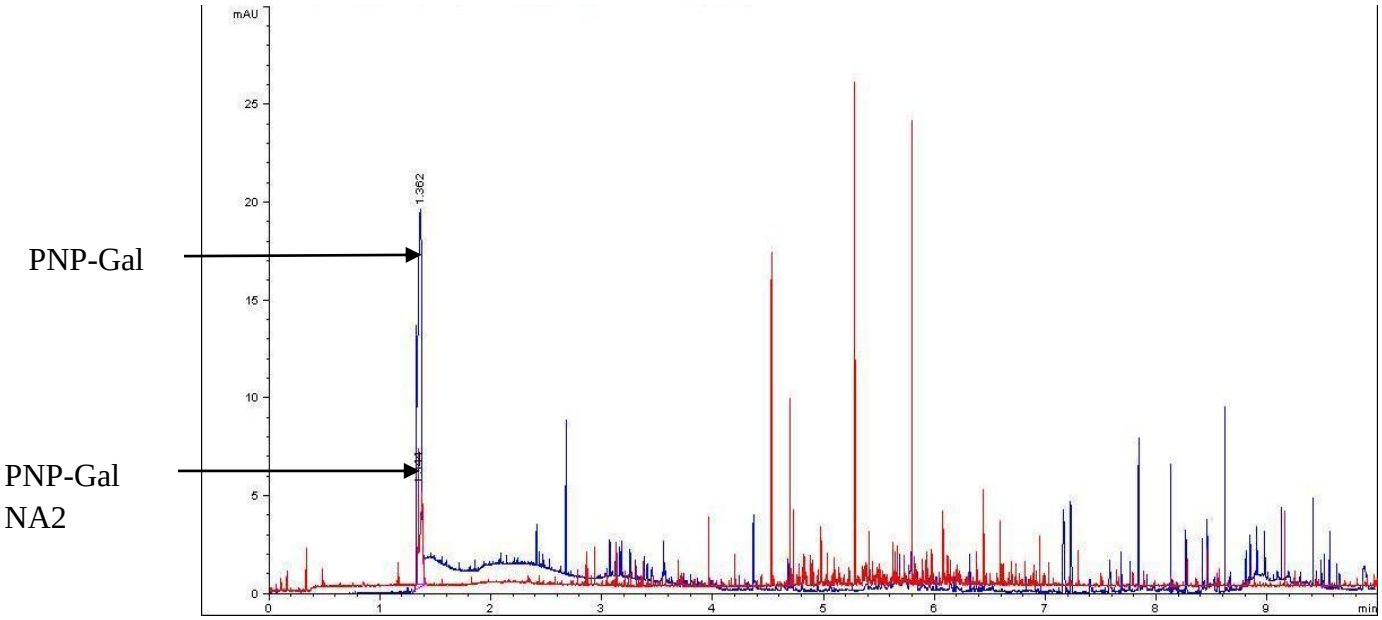

Fig 4.22: Analysis of the trans-glycosylation products using capillary electrophoresis

The trans-glycosylation products were run by capillary electrophoresis. The electropherograms were over layed in order to compare the peaks generated. There was formation of a lot of background peaks due to the nature of the assay which included the use of blood that has many components which can be detected using capillary electrophoresis. Although this was the case it was still clear from the overlaying that there was no trans-glycosylation product as both sample (red peak) and control (blue peak) gave a PNP-Gal peak with similar retention time.

Blue: PNP-Gal

Red: PNP-Gal NA2

CHAPTER FIVE

5. DISCUSSION

Neuraminidases are important due to their role as virulence factors for influenza viruses, ability to elicit immune reactions, as drug targets and their potential for use in synthesis of sialic acid bearing compounds (Ley, 2003, Varki, 2007, Democratis et al., 2006, Seeberger *et al.*, 2009, McKimm-Breschkin *et al.*, 2003). The potential use of the influenza H3N2 neuraminidase (NA2) in the synthesis of sialic acid bearing compounds is important as it would provide novel enzyme activities that can be used in the synthesis of sialo-oligosaccharides that can be used as drugs or vaccines. This was the first time that the influenza (NA2) was being investigated for trans-sialidase activity.

In this study, the influenza A virus type 2 neuraminidase was successfully amplified, cloned, expressed and investigated for trans-sialidase activity in order to determine potential for use in synthesis of sialyloligosaccharides. The amplified neuraminidase (NA2) fragment was 1522 nt long which was within the expected length of ~1500bp for full length gene (Varghese *et al.*, 1983). The exact length of neuraminidase however varies from strain to strain. In this case the fragment size was longer (1522 nt) owing to the primer design that spanned the untranslated regions that are present upstream and downstream of start and stop codon respectively these regions are conserved hence appropriate in primer design (Appendix viii). In the initial experiments, a non specific product was amplified alongside the fragment of the expected size and this was attributed to non optimal amplification conditions. Further optimization to ensure that only the neuraminidase gene was amplified were carried out using different conditions. However, under all

these conditions the non specific band was also co-amplified albeit to very low extent when the conditions became stringent. This co-amplification of this non specific band when the NA gene is amplified has been reported before (Chan *et al.,* 2006). The unwanted band was separated from the 1.5kb putative neuraminidase gene band through excision from gel and purification. The nucleotide sequence of the insert in the pGEMT-Easy vector was ascertained by Sanger dideoxy sequencing (Sanger *et al.,* 1977).

Analysis of the nucleotide sequence of the insert gene showed the presence of a continuous open reading frame of 1410 bp. The sequence encoded a polypeptide of 469 amino acids residues. This is in agreement with published literature (Varghese *et al.,* 1983). Phylogenetic analysis of the nucleotide sequence showed it was related to the nucleotide sequences of a number of other NA2 genes of human influenza A viruses including A/Brisbane/10/2007(H3N2), the 2008 season's vaccine strain. However the NA2 gene diverged slightly from these other NA2 sequences. This characterization of A/Nairobi/2041/2006(H3N2) virus was important as the influenza A viruses undergo antigenic drifts and shifts. These mutations are generally meant to ensure that the virus is able to evade the host immune system. However these mutations are random and can be deleterious to the virus. If such a mutation occurs on neuraminidase, its activity would be affected negatively. In natural systems a virus that has such unfavorable mutations would not continue to propagate. The sequence analysis showed that no deleterious mutations had occurred on the neuraminidase (NA2) gene.

Expression of neuraminidase (NA2) in pQE30 was attempted but failed to yield any clones. However pBK yielded several clones. These two vectors were used

as they offered disparate multiple cloning sites and therefore different strategies by which the neuraminidase gene could be ligated at the multiple cloning site. Using pQE30 it would be possible to perform directional cloning whereas the multiple cloning site in pBK did not allow for directional cloning. Two of the pBK clones had insert of the NA2 fragment as assessed by colony PCR.

To screen for expression of the neuraminidase activity among the clones, a functional assay was used. The basis of this assay was the ability of the expressed influenza A neuraminidase to cleave sialic acid moieties on red blood cells (RBC) (Stone and Ada, 1950).

Upon this cleavage, the absence of neuraminic acid residues on the surfaces of the RBCs caused hemagglutination inhibition upon incubation of the RBCs with influenza viruses (Hirst, 1941, Hudson *et al.*, 1943). This is because during hemagglutination, the virus hemagglutinin binds to sialylated receptors on RBC. The absence of these receptors due to desialylation by neuraminidase means that the virus cannot anchor on the RBC receptors and thus hemagglutination is inhibited.

This functional assay showed that lysates derived from half of the transformed colonies caused hemagglutination inhibition indicating that functional neuraminidase was produced in 50% of the clones. This was as expected since the cloning of the NA2 fragment in the pBK vector was non-directional and statistically only half of the inserts are expected to have accepted the insert in the forward orientation. The neuraminidase activity in the lysate was low. This was shown by the fuzzy rings in those wells treated with the lysate as compared to the sharp and intense rings seen in wells that had the receptor-destroying enzyme of *V. cholerae*. The activity of the influenza-derived neuraminidase would have been enhanced by a purification step

after sonication of the pBK-NA2-transformed *E. coli*. Furthermore, the lysate enzyme preparation inevitably had cell debris and possibly whole bacterial cells as carry-over which may have interfered with the enzyme activity. However, these deleterious influences did not completely abrogate neuraminidase activity and it was possible to show that the neuraminidase activity was indeed present. The trans-sialidase assay was crucial as it would generate information on whether or not the influenza neuraminidase had the ability to transfer sialic acid residues from a donor to acceptor molecule thereby synthesizing sialo-oligosaccharides. The trans-sialidase activity was also tested using a functional assay whereby the donor of the sialic acid residues was RBCs. The acceptor molecule in this assay was PNP-Galactose.

The resolution of the products on a TLC plate and capillary electrophoresis showed that there was no transfer of sialic acid residues from RBCs to the acceptor molecule. This result suggested that the influenza-derived neuraminidase (NA2) is not able to synthesise sialo-oligosaccharides by transferring sialic residues from RBCs to an acceptor molecule such as PNP-Galactose. This apparent inability to transfer sialic acids to an acceptor may be erroneous because RBCs may not be the best donors of the transfer groups. Furthermore, the RBCs which come as a mixture with many other substances may present with inhibitors of this activity. In future, such experiments ought to be repeated with other sialic acid donors and under different reaction conditions to definitively show that the influenza enzyme is not able to carry out a trans-sialidation reaction.

Neuraminidases with trans-sialidase activity have been shown to have distinct binding site for the acceptor molecule β-galactose (Buschiazzo *et al.,* 2000). This site

has been found to be close to the sialic acid-binding site and is thought to favor the transfer reaction. *T.Cruzi* trans-sialidase has distinct donor and acceptor binding sites which accounts for the sialyltransferase activity. Tyr120 and a shallow depression formed by Pro284 and Tyr249 have been shown to be involved in binding the acceptor molecule. Pro284 has been shown to be one of the essential amino acid residues for trans-sialylation. Infact the substitution of Tyr120 by Serine in *T.Cruzi* trans-sialidase has been shown to favour hydrolase activity (Buschiazzo *et al.,* 2000). If indeed the influenza A derived neuraminidase lacks trans-sialidase activity as these experiments seem to suggest, then the lack of activity should correlate with the absence of residues in the active site of NA2 lacks that would make it an efficient trans-sialidase. This can only be investigated through the use of computer simulations of the NA2 structure. This however does not mean that the enzyme cannot be used in future as a trans-sialidase. There exists substantial knowledge on the trans-sialidase transfer mechanism this can contribute towards engineering of glycosidases such as the influenza NA2 so as to make them efficient in transferring monosaccharides (Ichikawa *et al.,* 1992).

CHAPTER SIX

6 CONCLUSION AND RECOMENDATIONS

6.1 CONCLUSION

In this work, the complete influenza A (H3N2) neuraminidase gene was amplified by reverse transcriptase – Polymerase chain reaction from a nasopharyngeal sample. Phylogenetic analysis showed close association with A/Brisbane/10/2007(H3N2) cluster. The neuraminidase gene was subsequently cloned and expressed and shown to be functional using hemagglutination inhibition assay as a proxy for neuraminidase activity. Trans-glycosylation products were however not detected by TLC and capillary electrophoresis and hence trans-sialidase activity of the neuraminidase derived from influenza A/Nairobi/2041/2006 was not significant.

6.2 RECOMENDATIONS

Further analysis of the trans-glycosylation reaction of the influenza NA2 neuraminidase should be carried out using other approaches such as nuclear magnetic resonance or mass spectroscopy. These approaches are be able to detect trace amounts of glycoproducts in this case (sialo-oligosaccharides) that are otherwise undetectable by the TLC and Capillary electrophoresis.

Since the influenza neuraminidase (NA2) did not show any significant trans-sialidase activity, it is recommended that structural and bioinformatic studies be carried out to compare the neuraminidase structure with the structure of other known trans-sialidases. This will give insight as to why the influenza neuraminidase may lack the trans activity. Once the answers as to why the NA2 lacks a trans-sialidase

activity are available, molecular evolution engineering modifications should performed on the enzyme to transform it into a trans-sialidase. This may involve incorporation of particular amino acid residues that are known to be essential in the trans-sialidase activity through site directed mutagenesis. This process may be costly but worthwhile considering that synthesis by chemical means is also costly and has many other limitations.

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APPENDICES

Appendix 1:

Oligonucleotide primer sequences

Primer	**Primers to be paired**	**Expected Amplicon size**	**Application of primer**
NA-Forward 5'ATG AAT CCA AAT CAA AAG ATA ATA ACR-3'	NA-Forward and NA-Reverse	1500bp	Amplification of neuraminidase (NA2) from cDNA
NA-Reverse 5'-TTA TAT AGG CAT GAG ATT GAT GTC C-3'	NA-Forward and NA-Reverse	1500bp	Amplification of neuraminidase (NA2) from cDNA
NA-Forward-KpnI 5'-TTT TGG TAC CAT GAA TCC AAA TCA AAA GAT AAT AAC R-3'	NA-Forward-KpnI and NA-Reverse HindIII	1500bp	Amplification of neuraminidase (NA2) from pGEMT clone for sub-cloning
NA-Reverse-HindIII -TTT TAA GCT TTT ATA TAG GCA TGA GAT TGA TGT CC-3'	NA-Forward-KpnI and NA-Reverse HindIII	1500bp	Amplification of neuraminidase (NA2) from pGEMT clone for sub-cloning

Appendix II:

Antibiotic stock solutions

Ampicillin.1 g of Ampicillin sodium salt (Sigma Aldrich St. Louis USA) was dissolved in 10 ml of deionized water to give a stock solution of 100mg/ml ampicillin.

Tetracycline**.** 1 vial containing 10mg of tetracycline hydrochloride (Sigma Aldrich St. Louis USA) was dissolved by adding 1ml of deionized water to give a stock solution of 10mg/ml.

Kanamycin. Kanamycin sulfate salt (Sigma Aldrich St. Louis USA)

10mg/ml Kanamycin in deionized water

Appendix III: Culture media

LB medium

10g Bacto-tryptone, 5g Bacto-yeast extract and 5g NaCl were dissolved in 900ml of distilled water and the pH adjusted to 7.5 using 10N NaOH the solution was topped up to 1litre using distilled water and autoclaved.

LB plates with ampicilli**n**

15g of agar was added to 1 liter of LB medium. The solution was autoclaved and allowed to cool to 50°C before adding ampicillin to give a final concentration of 100µg/ml. 30–35ml of the medium was poured onto 85mm Petri dishes and stored at 4°C.

LB plates with tetracycline

15 grams of agar was added to 1 liter of LB medium and the solution autoclaved and allowed to cool to 50°C before adding tetracycline hydrochloride to give a final concentration of 10µg/ml. 30–35ml of the medium was poured onto 85mm Petri and stored at 4°C.

LB plates with ampicillin and IPTG/X-Gal

100µl of 0.1M IPTG (Sigma Aldrich St. Louis USA) and 100µl of 20mg/ml X-Gal was spread on LB plates with ampicillin and allowed to absorb for 30 minutes at 37°C.

SOC medium (100ml)

2.0g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl and 250µl 1M KCl, were dissolved in 97ml of distilled water and autoclaved. On cooling 1ml of 2M filter-sterilized glucose and 1ml of 2M Mg2+ stock were added and the final volume made up to 100ml using sterile distilled water.

Appendix IV:

Plasmid extraction solutions

Resuspension solution **(**50mM Glucose, 25mM Tris-Hcl, 10mM EDTA pH8) This was prepared by mixing, 40% Sterile glucose 2.27 ml, 25Mm Tris-HCL pH 8.0 2.0 ml, 10Mm EDTA pH 8.0 2.5ml,and Sterile distilled H_20 93.3ml giving a total volume of 100ml.

Lysis solution (0.2M NaoH, 1% SDS**)** This was prepared by mixing**,** 1 N NaOH freshly prepared 2.0ml, 10% SDS 1.0ml and Sterile distilled H_20 7.0ml giving a total volume of 10ml.

Potassium Acetate Solution (pH 4.8) This was prepared by mixing,5M potassium acetate 60ml,Glacial Acetic Acid 11.5ml and Sterile distilled H_20 28.5ml giving a total volume of 100ml.

TE Buffer This was prepared by mixing 10mM Tris-Cl pH 7.5 and 1mM EDTA

Appendix V:

Competent cell preparation solutions

0.1 M $Cacl_2$ 1.47 grams of $cacl_2$ (Fwt 147) was dissolved in 100ml of distilled water and filter sterilized.

$MgCl_2$ –$Cacl_2$ (80mM $MgCl_2$, 20mM $Cacl_2$) 1.6246 grams of $MgCl_2$ and 2.94 grams of $Cacl_2$ were dissolved in 100ml of distilled H_20 and filter sterilized.

2M Mg2+ stock 20.33g MgCl2 • $6H_2O$ and 24.65g of MgSO4 • $7H_2O$ were dissolved in 100 ml of distilled water and Filter sterilize.

0.2M $MgSO_4$ stock
49.3 grams of $MgSO_4$ was dissolved in 100ml of distilled H_20

Appendix VI:

Other Solutions

1X TBE buffer

89mM Tris base (TRIZMA base), 89mM boric acid and 2mM EDTA (pH 8.0)

Phosphate buffered saline pH 7.4

8.5 gm NaCl, 0.316 gm Na phosphate monobasic, 1.096 gm of Na phosphate dibasic containing 0.25% trypsin dissolved in l litre distilled water

Revelation solution

The revelation solution was made up of, (3.48% (w/v) ammonium molybdate [(NH4)6 Mo7 O24)], 1.66% (w/v) cerium sulphate and 19% (v/v) concentrated sulphuric acid)

Appendix VII: NA2 Partial sequences

>T7 Forward primer

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   1 TTTTATAATG TAAATTCCAT CCAGCTCCGG CGCCAGGCGG CCGCGGGATT CGATTAGCAA
  61 AAGCAGGAGT GAAGATGAAT CCAAATCAAA AGATAATAAC GATTGGCTCT GTTTCTCTCA
 121 CCATTTCCAC AATATGCTTC TTCATGCAAA TTGCCATCTT GATAACTACT GTAACATTGC
 181 ATTTCAAGCA ATATGAATTC AACTCCCCCC CAAACAACCA AGTGATGCTG TGTGAACCAA
 241 CAATAATAGA AAGAAACATA ACAGAGATAG TGTATCTGAC CAACACCACC ATAGAGAAGG
 301 AAATATGCCC CAAACTAGCA GAATACAGAA ATTGGTCAAA GCCGCAATGT GACATTACAG
 361 GATTTGCACC TTTTTCTAAG GACAATTCGA TTAGGCTTTC CGCTGGTGGG GACATCTGGG
 421 TGACAAGAGA ACCTTATGTG TCATGCGATC CTGACAAGTG TTATCAATTT GCCCTTGGAC
 481 AGGGAACAAC ACTAAACAAC GTGCATTCAA ATGACACAGT ACGTGATAGG ACCCCTTATC
 541 GGACCCTATT GATGAATGAG TTAGGTGTTC CTTTTCATCT GGGGACCAAG CAAGTGTGCA
 601 TAGCATGGTC CAGCTCAAGT TGTCACGATG GAAAAGCATG GCTGCATGTT TGTATAACGG
 661 GGGATGATAA AAATGCAACT GCTAGCTTTC ATTTACAATG GGAGGCTTGT AGATAGTATT
 721 GTTTCATGGT CAAAAGAAAT CCTCAGGACC CAGGAGTCAG ATGCGTTTTG TTATCAATGG
 781 AACTTTGTAC AGTAGTAATG ACTGATGGGG AGTGCTTTCA GAAAAGCTGA TACTAAATAC
 841 TATTCATGGA GGAGGGAAAT CGTTCATACC TAGCACATTT TCAGGAAGGC CTCAGCATTT
 901 TCAGATGGCC TGCTATCTCG ATTCCTGATG TCAGAGTGTT CTGGCGAGAA CACTTG
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>SP6 Reverse primer

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   1 TTAATTTCTC CACGCGTTTG GGAGCTCTCC CATATGGTCG ACCTGCAGGC GGCCGCGAAT
  61 TCACTAGTGA TTAGTAGAAA CAAGGAGTTT TTTCTAAAAT TGCGAAAGCT TATATAGGCA
 121 TGAGATTGAT GTCCGCCCCA TCAGGCCATG AGCCTGTTCC ATATGTACCT GAGGTGCCAC
 181 AAAACACAAC AATACTGTTT GAGGTCCACA AGACTTCAGT TTCCTCTTTT CTTCCCCTTA
 241 TCAACTCCAC ATAAAAGCAC CGATTGATGC AGCTTTTGCC TTCAACAGAG AAAATACCAG
 301 AATAACCGGA CCTATTACCT CTGTCAACTA TGACTTGCCT ATTTATCTGC AATTTGGACT
 361 TAGGGTTGGA CCAGCCTTCA ATGACTTTGA AGGTTTCATA CCCTAAGCGT GACTTTTCGC
 421 TGATCGTTCT TCCCATCCAC ACGTCATTTC CATCATCAAA GGCCCAGCCT TTCACTCCAT
 481 GACCACCTTC TTCATTGTTA GGATCCAAAC AATGGCTACT GCTGGAGCTG TCGTTTTTCT
 541 GGGTGTGTCT CCAACAAGTC CTGAACACAC ATAACTGGAA ACAATGCTAT GATCCTTTAT
 601 GTTTATATCT ACGATGGGCC TATTGGAGCC TTTCCAGTTG TCTCTGCAGA CACATCTGAC
 661 ACCAGGGTAT CGAGGATAGC AGGGCACTCC CTCGACATGC TGAGCACTTC CTGACATGTG
 721 CTAGTATGAA CGATTTTCCC CCTCCTCAAT GAATAGTATT TTTAGTATCA GCTTTTCCTG
 781 AGCACCTCCC ATCAGTCATT ACTACTGTAC AGTCCATGAT ACAAACGCCA TCTGACTCCT
 841 GGGTCCTGAG ATTTCTTTGG ACCATGAACA ATACTATCTA CCAGCCCTCC CATTGTAATG
 901 AGCTAGCAGT GCATTTTTAT CATCCCCGGT AATAACAAAA CATGCAGCCA TGCTTCCAAT
 961 CGTGACCAAC CTGGACCTGG ACATGCCTAT GCCACACTGG CTGCCAATGG AAAGAACCCC
1021 TA
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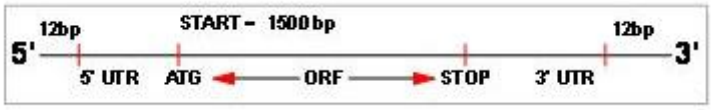
Nucleotide sequences obtained using T7 and SP6 primers annealing to theT7 and Sp6 region of the pGEMT Easy vector. The sequences are presented in a FASTA format. The name of the Sequence corresponds to the name of the primer used to obtain it. The raw sequences were formatted and grouped using the "Group DNA"algorithm of the Sequence Manipulation Suite (SMS) program (Stothard, 2000).

Appendix VIII: Neuraminidase gene structure



The neuraminidase gene structure is such that at the 3’ end and the 5’ end there is a sequence of 12 nucleotides that are complementary and form a pan handle structure. After which there is an untrans . The open reading frame ORF is ~1500bp but this varies from strain to strain

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