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Quantification of Influenza Neuraminidase Activity by Ultra-High Performance Liquid Chromatography and Isotope Dilution Mass Spectrometry

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Abstract

Mounting evidence suggests that neuraminidase's functionality extends beyond its classical role in influenza virus infection and that antineuraminidase antibodies offer protective immunity. Therefore, a renewed interest in the development of neuraminidase (NA)-specific methods to characterize the glycoprotein and evaluate potential advantages for NA standardization in influenza vaccines has emerged. NA displays sialidase activity by cleaving off the terminal *N*-acetylneuraminic acid on α-2,3 or α-2,6 sialic acid containing receptors of host cells. The type and distribution of these sialic acid containing receptors is considered to be an important factor in transmission efficiency of influenza viruses between and among host species. Changes in hemagglutinin (HA) binding and NA specificity in reassortant viruses may be related to the emergence of new and potentially dangerous strains of influenza. Current methods to investigate neuraminidase activity use small derivatized sugars that are poor models for natural glycoprotein receptors and do not provide information on the linkage specificity. Here, a novel approach for rapid and accurate quantification of influenza neuraminidase activity is achieved utilizing ultra-high performance liquid chromatography (UPLC) and isotope dilution mass spectrometry (IDMS). Direct LC-MS/MS quantification of NA-released sialic acid provides precise measurement of influenza neuraminidase activity over a range of substrates. The method provides exceptional sensitivity and specificity with a limit of detection of 0.38 μM for sialic acid and the capacity to obtain accurate measurements of specific enzyme activity preference toward α-2,3-sialyllactose linkages, α-2,6-sialyllactose linkages, or whole glycosylated proteins such as fetuin.

Graphical Abstract

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Notes

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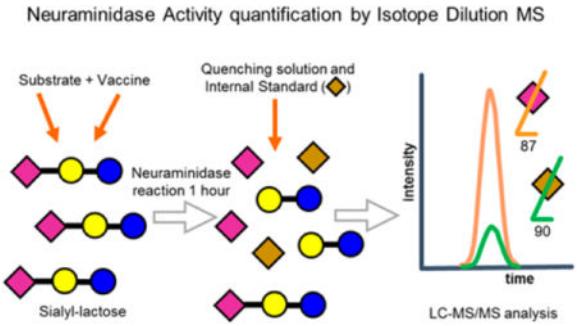

Influenza viruses are constantly changing, reassorting, and evolving leading to the possible emergence of new virulent strains with the potential to cause serious seasonal disease and influenza pandemics.[1] Hemagglutinin (HA) and neuraminidase (NA), the major viral surface glycoproteins, play critical roles in the virus infection.[2] The main function of HA is to initiate the host cell infection process by binding to terminal sialic acids in the polysaccharide chains of cellular receptors, consequently facilitating the release of the viral genome into the target host cell cytoplasm.[3,4] Conversely, NA's major function occurs during the final stages of infection: enzymatically cleaving sialic acids from both the viral envelope glycoproteins and host cell receptors to avoid aggregation of the progeny viruses and thus aid viral spread.[5–9] It is postulated that the functional balance between HA's binding ability to terminal sialic acid and NA's ability to cleave off terminal sialic acid from cell surface receptors is required for efficient replication and transmission to occur and that this balance plays a major part in determining strain virulence and a virus's ability to adapt and thrive under various influences such as foreign genetic material, target hosts, antibodies, and antivirals.[4,10–14] Mechanisms of action also include NA's ability to modulate type and magnitude of T cell responses,[15] enzymatically mediate LTGF-β activity,[16] and alter viral resistance[17] while NA's established role in aiding viral release has led to the development of NA inhibitor antiviral medications that prevent virus propagation and control infection.[4,18] More recently, NA enzymatic activity has been found to facilitate viral entry into the respiratory tract by binding and/or cleaving sialic acid containing receptors in the epithelial cells of the human upper respiratory tract, indicating that NA's role in the viral life cycle extends beyond the final stages of infection and also plays specific roles in viral attachment and entry.[7,19,20] The realization of NA's expanding functionality and complexity has resulted in interest in the development of sensitive and specific methods to characterize and quantify NA activity.

Sialic acids (*N*-acetylneuraminic acids), small acidic C-9 backbone amino α-keto sugars found as terminal α-linked residues of cell surface glycoproteins, were shown to be receptors for influenza virus.[19,21] Sialylation can vary significantly, with the types of sialic acids expressed and the types of linkages that exist between sialic acids being species dependent. While not fully understood, species-specific sialylation can influence influenza host specificity[7,22] and much research has been conducted studying sialic acid structures, linkages, and *in vivo* distributions in relation to influenza viruses.[23] In particular, the amount, density, and locations for α-2,3 and α-2,6 sialic acid to galactose linkages varies among hosts, and selectivity to this linkage is thought to play a critical role in host

determination and virulence of different influenza strains within or between hosts.[13] Sialic acids with α-2,3 glycosidic linkages predominantly found in human bronchi and alveoli[24] and the upper respiratory tract of chimpanzees[25] are the preferred receptor for avian- and equine-origin influenza viruses while α-2,6 linkages predominantly found in the human upper respiratory tract are the preferred receptor for human adapted influenza viruses.[26] It is thought that the relative absence of α-2,3 glycosidic linkages in the upper respiratory tract of humans along with the presence of respiratory human mucins serve as host barriers to avian influenza viruses.[22,27]

It is well recognized that the functional balance between the “receptor-binding” HA and “receptor-destroying” NA is central to viral transmission in humans; however, the role of NA has been studied to a lesser extent. It has been observed that pandemic viruses isolated from humans exhibited either high HA binding affinity coupled with high NA cleavage activity (A/Japan/305/1957) (A/Hong Kong/1/1968) or low HA affinity coupled with low NA activity (A/South Carolina/1/1918) (A/California/04/2009).[28] Evidence of NA’s importance in cross-species transfer was also seen in the H1N1 2009 pandemic. While swine to human transmission was observed in both precursor pH1N1 viruses containing the North American-origin NA, no secondary human cases were observed and pre-2009 isolates do not appear to be transmissible to ferrets via respiratory droplets.[28–31] Only after attaining the Eurasian-origin M segment and Eurasian-origin NA, of increased activity and similarity to HA, did the virus become transmissible.[28,32] A comprehensive study by Kobasa et al. of N2 viruses isolated from 1957 to 1987 found a time-dependent drift in substrate specificity. N2s of the earliest human isolates following avian transmission (1957) only displayed enzymatic activity toward α-2,3 sialic acids, whereas N2s in the late 1960s, while still showing primary activity to α-2,3 sialic acids, had also acquired limited activity toward α-2,6 sialic acids. By 1972, the N2 activity toward α-2,6 sialic acids had increased such that it was relatively equivalent to that of α-2,3 sialic acids, suggesting a selective advantage was obtained; however, further increases toward α-2,6 sialic acids was not observed through 1987.[33] While in general the active site of NA is well conserved across the nine NA subtypes, a subtle change in two amino acid residues located near the N2 NA active site was identified as being responsible for this change in NA activity. Further, while the majority of NA subtypes have been identified as causing human infection, only A/N1, A/N2, and B type viruses continue to circulate and cause epidemics. The ability of these subtypes to establish successful lineages suggests that specific requirements must be met before a new subtype can emerge and support influenza growth in humans; however, the requirements for NA activity and its contribution in making a virus successful in human populations is not completely understood.[33,34] Considering that even point mutations can influence NA’s adaptive mechanism, these findings alone stress the need for continued research of NA activity and specificity.[19]

Techniques including CE,[35] GC/MS,[36] and HPLC coupled to various detection systems such as PAD,[37] UV,[38] fluorescence,[39] and MS[40–44] have been used to quantify free sialic acids in different biological matrices[40] and for the diagnoses of diseases such as sialic acid storage disease.[42,43] For influenza NA activity, the traditional NA fluorometric assay, using sodium 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4-MUNANA), is typically used. This assay was first described and published by Potier et al. in 1979[45] and has since been adopted

worldwide.[18,46] The fluorogenic assay offers rapid analyses with high precision and reproducibility but has a relatively high limit of detection making it prone to false negatives and ill-suited for drug susceptibility testing. Other NA activity fluorescent[47,48] assays as well as colorimetric[49] and chemiluminescent[50] detection techniques offer simplicity of detection and inexpensive reagents and instrumentation; however, they present certain limitations including the sensitivity, high substrate background, and interferences.[45,50,51] More importantly, a majority of the conventional methods are not able to differentiate α-2,3 linked sialic acids from α-2,6 sialic acids, and thus, they are unable to yield information about the specificity of NA activity.

Here, we describe the first ultra-high performance liquid chromatography isotope dilution mass spectrometry (UPLC-IDMS) method for the direct quantification of influenza NA activity after enzymatic cleavage of sialic acid from various substrates. IDMS offers several advantages because of the use of an isotopically labeled internal standard which behaves essentially identical to the analyte but can be distinguished by mass. Because the chromatography and the ionization efficiency for the ^{13}C labeled internal standard are the same as the analyte, the method provides excellent accuracy, reproducibility, sensitivity, and specificity. In combination with IDMS techniques to quantify total NA content,[52] this method will greatly strengthen the ability to characterize the properties of influenza neuraminidases in seasonal and pandemic strains of influenza virus. The method has been developed using the small and well-characterized α-2,3-sialyllactose and α-2,6-sialyllactose sugar substrates, which can be used to obtain accurate measurements of enzyme activity and selectivity toward each type of sugar linkage.

In addition, bovine fetuin was also used as a substrate. This well-characterized complex glycoprotein contains a mixture of α-2,3- and α-2,6-linked sialic acids.[53] Although it cannot yield any information regarding linkage specificity, it may be a more representative model of the real targets of the influenza virus during human infection. A comparison between the UPLC-IDMS method with the widely accepted fluorescence assay using the linkage-agnostic 4-MUNANA substrate was performed.

EXPERIMENTAL PROCEDURES

Materials

Ammonium acetate, ammonium hydroxide, *N*-acetyl-D-neuraminic acid ($C_{11}H_{19}NO_9$, MW 309.3 Da), α-2,3-sialyllactose, α-2,6-sialyllactose, and fetuin from fetal calf serum were obtained from Sigma-Aldrich (St. Louis, MO). *N*-Acetyl-D-[1,2,3-$^{13}C_3$]-neuraminic acid internal standard ($^{13}C_3C_8H_{19}NO_9$, MW 312.3 Da) was acquired from Omicron Biochemicals, Inc. (South Bend, IN). All chemicals were of the highest purity available and used without further purification. NA-Fluor 2× Assay buffer, 66.6 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, 8 mM $CaCl_2$, pH 6.5, was from Applied Biosystems (Foster City, CA). The LC column was from Imtakt USA (Portland, OR), and influenza vaccines A–F (Table 1) were commercially available.

During the implementation of the fluorescence method, the NA-Fluor Influenza Neuraminidase Assay kit from Applied Biosystems, Cat No. 4457091, was used. Ethanol

absolute, 4-methylumbelliferone (4-MU), and sodium chloride solution were from Sigma-Aldrich. 96-well opaque black flat-bottom plates from Corning (Corning, NY) were used with a SpectraMax M5e micro plate reader from Molecular Devices LLC (Sunnyvale, CA) for fluorescence measurements.

Sample Preparation

Appropriate safety control measures, including engineering, administrative, and personal protective equipment, were used for all procedures based on a site-specific risk assessment that identified physical, health, and procedural hazards.

Sialic acid native and internal standard stock solutions were prepared at 1 mg/mL in dH_20. Working solutions of the native sialic acid were prepared at 1.62, 3.24, and 81 μM in 85:15% (v/v) ACN/dH_20, and the internal standard working solution was at 162 μM in the same solvent system. Different volumes of these stock solutions were used to prepare a nine-point calibration curve spanning 0.05–30 μM (all with constant 20 μM internal standard), bringing all final volumes to 200 μL with 85:15 ACN/dH_20. Three quality control (QC) levels, low (QCL), medium (QCM), and high (QCH), were prepared at 1.0, 5.0, and 20 μM, respectively, to monitor the method's performance over time. All vaccines were undiluted, and in most cases, a unit dose was 15 μg of HA in 0.5 mL (unless specified). Ten μL of vaccine, 10 μL of substrate, and 80 μL MES buffer were used in the timed reactions. A quenching solution was prepared at 95:5% (v/v) ACN/ammonium hydroxide. After addition of substrate and incubation at 37 °C for 1 h, the enzyme reactions were quenched. Fifteen μL of the timed NA reaction was mixed with 85 μL of quenching solution and 14.2 μL of internal standard (IS) (giving an overall 7.61-fold dilution of the reaction mixture with a final level of 85% ACN). The samples were held at 6 °C until LC-MS/MS analysis, Figure 1.

All NA reactions were carried out in 4 mM $CaCl_2$ in a 33 mM MES buffer solution at pH 6.5 and incubated at 37 °C for 1 h. Sialyl-lactose substrates were at 300 μM, while protein substrates were at 5 mg/mL in the reaction mixtures. The quenching step completely stopped the NA reactions and provided compatibility with the LC solvent system. Substrate blanks were included and prepared following the same procedure. In-house quality control (QC) samples were prepared, aliquoted, and stored at 4 °C. Microsoft Excel was used to analyze the data, characterize QC values and QC limits, and calculate the method's limit of detection (LOD).

Fluorescence Assay

The NA-Fluor Influenza Neuraminidase Assay kit protocol (Applied Biosystems) was used to quantify NA activity, using a SpectraMax M5e. The instrument was set to read at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

A 5-point 4-MU calibration curve ranging from 2.5 to 40 μM was generated in the SpectraMax M5e to identify the RFU (relative fluorescent units) value within the linear range of the fluorescence detection of the instrument. It was found that 10 μM of 4-MU gave approximately 29 000 RFU, our target value to normalize the NA activities of each vaccine or strain. From this, 50 μL of two vaccine samples was serially diluted, and a reaction with

67 μM 4-MUNANA substrate was carried out for 1 h at 37 °C. The reaction was terminated by the addition of 100 μL of NA-Fluor stopping solution, and the plate was immediately loaded in the plate reader. The dilutions at our target RFU, one for each vaccine, were determined, and the NA activity was calculated for the MUNANA fluorescent method, Table 1.

LC System

An Acquity UPLC system (Waters Corporation, Milford, MA) was interfaced to a triple quadrupole mass spectrometer for analysis. A Unisom UK-Amino (Imtakt USA) aminopropyl 100 × 1.0 mm ID, 3 μm particle size analytical column was used for all chromatographic separations. The column temperature was maintained at 60 °C; the sample injection volume was 5 μL, and the sample manager temperature was kept at 6 °C throughout all analyses. Solvents A and B were 50 mM ammonium acetate buffer in dH_20 and 100% ACN, respectively. Analytes were eluted isocratically under HILIC (hydrophilic interaction liquid chromatography) conditions at 42:58% (v/v) solvent A/solvent B at a flow rate of 75 μL/min. LC cycle time was 8 min, and retention time for sialic acid was approximately 4.5 min.

Mass Spectrometer

A 4000 QTRAP mass spectrometer (AB Sciex LLC, Framingham, MA) with a standard Turbo-V ion source operating in the negative ion electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) at unit resolution was used for all analyses. Instrument operating conditions were optimized for all individual MRM transitions. Sialic acid and $13C_3$-sialic acid data were acquired using transitions m/z 308 → 87 and 170 (quantification and confirmation ions) and m/z 311 → 90 and 173, respectively. A similar fragmentation pattern for sialic acid has been previously reported.[43]

Quantification of Total NA

Total N1 NA was quantified in commercial seasonal vaccines by isotope dilution mass spectrometry as previously described[52] where conserved peptides from neuraminidase of strains present in monovalent influenza vaccines and other virus preparations were carefully selected and precisely quantified. This method provides the specific and accurate quantification of the total amount of the main viral surface antigens, and therefore, it also allows for the precise determination of the ratio of HA to NA in any of the strains in commercial vaccines. It was found that the ratio of HA to NA varies significantly between different strains. Although the significance of these ratios in terms of protective immunity, safety, or potency of the vaccine is not clear at the moment, it provides very valuable information which in addition to our IDMS NA activity method can potentially enhance vaccine efficacy evaluation.

Method Validation

Data from 36 analytical runs acquired over a 10 week period by two analysts using one LC-MS/MS instrument were collected. Calibration curves prepared in MES buffer (as used for NA activity measurement) were analyzed by linear regression with 1/× weighting on the

native/labeled peak area ratio versus expected sialic acid concentration to construct response curves with the coefficients of determination (R^2) exceeding 0.993 for all analyses, Figure 2. These plots were used for quantification of QC samples, blanks, and vaccines. The LODs were calculated using sets of between 60 and 66 values for the blank and the four lowest standards, according to the method described by Taylor.[54]

The average accuracy for the 0.05 μM standard was 95%. The limit of detection (LOD) was 0.013 μM for the sialic acid quantification ion (m/z 308 → 87) and 0.020 μM for the sialic acid confirmation ion (m/z 308 → 170) in the LC-MS/MS analysis, which is equivalent to 0.10 and 0.15 μM in the NA reactions. However, the reportable range of 0.38 to 228 μM sialic acid in the NA reactions was set by the lowest and highest standard concentration, and the ratio of the confirmation ion to the quantification ion was evaluated as a requirement for identification and confirmation of the presence or absence of sialic acid in the samples analyzed, Figure 3. Coefficients of variation (CVs) for QCL, QCM, and QCH were 9.8%, 11.0%, and 7.2%. In addition, data were analyzed for the presence of outliers and acceptable accuracy and precision.

RESULTS AND DISCUSSION

Numerous human and animal studies have demonstrated the benefit of NA-specific antibodies,[55–59] and past challenge studies suggest even very low NA inhibition antibody (NAIs) titers protect against disease;[60,61] however, at present, only measurements of serum antibodies in response to HA are used to evaluate influenza vaccine efficacy.[62] While NA-based vaccine studies have been shown to be safe and effective for disease prevention,[63–65] currently, the U.S. Food and Drug Administration's influenza vaccine production protocols focus solely on the content and potency of HA, as reflected by single radial immunodiffusion (SRID) measurements in their seasonal formulations.[66] Similarly, while the European Pharmacopoeia protocol requires identification of the intact NA antigen to its corresponding strain in monovalent bulks, it does not regulate the amount of NA in the final formulations.[2] However, the performance of seasonal vaccines over the past decade, with an effectiveness ranging from 10% to 60%,[67] has prompted improved countermeasures. With these, the first healthy volunteer human challenge to evaluate a hemagglutination inhibition (HAI) titer of ≥1:40 for evaluating influenza vaccines was recently published.[68] The results of this study by Memoli et al. confirmed that HAI titers of ≥1:40 definitively correlate with disease protection and more importantly revealed that increasing NAI titers negatively correlate with all aspects of disease severity, suggesting that NAI titers may be more predictive of protection and reduced disease severity than HAIs.[68] Clearly, more emphasis needs to be given to the role that NA, and NA and HA collectively play in immune protection against influenza infection.

Analysis of sialic acid by LC-MS/MS was a rather challenging task due to the highly polar nature of the analyte. Several LC columns and buffer solvent systems were evaluated before achieving a simple, robust, and reproducible analytical method. The Imtakt aminopropyl column gave optimal chromatographic peak shape and utilized a buffer system that was easily compatible with LC-MS/MS analysis. Release of sialic acid by NA from the substrates α-2,3-sialyllactose, α-2,6-sialyllactose, and fetuin was monitored by LC-MS/MS,

under conditions where the enzyme activity remained linear with time (less than 7% substrate consumption), Figure 4.

NA activity at 60 min was measured on six available commercial vaccines A through F using α-2,3-sialyllactose and α-2,6-sialyllactose substrates and fetuin. Enzyme activity was calculated and reported as moles of sialic acid released per minute per μL of neat vaccine, Table 1. Vaccine B is a subunit vaccine preparation, and the others are all split vaccines. The results obtained were also calculated as moles of sialic acid released per minute per vaccine unit dose (0.5 mL in all cases except for vaccine E which was 0.25 mL) and compared to those of total NA amount (μg of NA protein) in moles per dose quantified in the same vaccines by IDMS in our laboratory.[52]

Analysis of commercial vaccines A and B was conducted in parallel with our LC-MS/MS method using the MUNANA fluorescence method, and a comparison of the results is shown in Table 2. Although the results for NA total protein content by IDMS for vaccines A and B are similar, commercial vaccine A displayed a higher NA activity toward the substrates used by both the MUNANA fluorescent and our LC-MS/MS methods. Also, our method reveals that the greatest contribution to the total NA activity comes from the α-2,3 sialic acid to galactose isomer linkage rather than the α-2,6 isomer linkage. This is also supported by the data shown in Figure 5, where a range of six different H1N1 pandemic monovalent commercial vaccines A–F show that the same NA subtype is consistently more specific toward the α-2,3 sialic acid to galactose linkage although significantly different levels of activity were measured across the different commercial vaccines. This raises questions about the clinical relevance and possible impact of residual NA activities in different vaccine preparations. The LC-MS/MS results for sialic acid released were qualitatively similar to those from the fluorescence NA activities toward 4-MUNANA substrate for vaccines A and B, Table 1.These activity comparisons were all obtained at substrate levels of 300 μM for the sialyllactoses, 5 mg/mL for fetuin (approximately 100 μM protein with an estimated 800–1300 μM total sialic acids assuming 5–8% by weight), and 67 μM for 4MUNANA. Since the kinetic parameters K_M and V_{max} for the NAs in the vaccine preparations were unknown, it is not possible to predict how the relative activities may vary with the substrate concentration.

The mass spectrometry-based method demonstrates that these differences can be observed and they can be selectively and accurately quantified, allowing it to become a powerful tool to explore NA activity, in historic and current influenza strains and in vaccines manufacturing. The mass spectrometry-based method appeared to provide 5–10 times more sialic acid release than the fluorescent method in our studies, Table 2, when considering the α-2,6 and fetuin substrates. The MS-based method is also clearly more specific since the MUNANA method does not provide any linkage specificity. Figure 5 shows more detail of the relative α-2,3 and α-2,6 activities in relation to the amounts of NA present. Although the amounts of NA protein per dose vary by around a factor of 3, the NA activities toward the linkage-specific substrates vary greatly, in some cases as much as 30- to 100-fold, between the different vaccine preparations. The monovalent vaccines available at the time of this work were all H1N1 A/California/07/2009 commercial vaccines from different manufacturers as shown in Table 1. However, the method is applicable to other vaccine strains and is not limited to only H1N1 strains.

CONCLUSIONS

It has been well-defined that a shift in HA's receptor-binding preference from α-2,3 to α-2,6 linkages plays a role in strain adaptation to human pandemic viruses.[69–72] Comparatively, a shift in NA enzymatic activity from an α-2,3 linkage preference to an α-2,6 linkage preference has also been observed to occur over time following avian to human transmission,[33] but complete insight into NA's adaptive mechanism is still in its infancy. Working toward this goal, we have developed a rapid and sensitive method to assess NA's receptor-cleaving specificity and differentiate NA's preferential binding activity to α-2,3 and α-2,6 profiles. The ability to quantify influenza NA activities toward α-2,3-linked and α-2,6-linked sialic acids leads the way toward achieving a more comprehensive understanding of NA's importance in viral adaptation, transmission, and infection that cannot easily be achieved using classical approaches. Further, as NA's importance as a key constituent of influenza vaccines is increasingly recognized, our LC-MS/MS method has been developed as an accurate, specific, and sensitive analytical tool to investigate the properties and characteristics of NA and support consideration of NA immunity as a pandemic and seasonal strategy.[59]

In comparison to traditional methods, mass spectrometry provides both analytical sensitivity, the ability to quantify small amounts accurately, and analytical specificity, the ability to distinguish α-2,3 from α-2,6 linked sialic acids cleaved by NA enzymatic activity directly, without interferences. Importantly, it allows for direct measurements on release of unmodified native sialic acids in various substrates, in contrast to fluorescence-tagged sialic acids necessary for many conventional methods. This work represents the first time accurate and precise quantification of influenza NA activity by LC-MS/MS that has been achieved and evaluated from a range of substrates and, most relevantly, identifies and quantifies NA's different cleaving affinities for a specific sialic acid to galactose linkage.

Quantification of both NA protein and NA activity by IDMS provides a new and powerful ability to track emerging influenza viruses' shifts from avian α-2,3 sialic acid-containing receptors to human upper respiratory tract (URT) α-2,6 sialic acid-containing receptors. The presence of α-2,3 sialic acid linkages in the lower respiratory tract (LRT) of humans may explain why viral replication of highly pathogenic avian influenza A virus H5N1 causes lower respiratory tract infection and severe pneumonia in humans.[73,74] Although attachment of influenza viruses to the URT of humans is speculated to be determinant for the efficiency of human-to-human transmission, there is no proof of a link between attachment and infection. While a highly pathogenic avian influenza A virus H5N1 is not transmitted efficiently, attaching rarely, among humans, it has a high mortality rate. As well as being used to study the wide range of historical, current, and emerging influenza strains, our method may be useful for characterizing susceptibility to NA inhibitors. Moreover, quantification of influenza NA activity in vaccines enhances our knowledge of the role of NA activity in influenza vaccines, has the potential to improve current licensed vaccine standardization, and may benefit vaccine potency and effectiveness. While many challenges still exist, this work has the potential to fill critical gaps required to prepare for and assist in pandemic and seasonal influenza.

References

1. Manceur AP, Kamen AA. Vaccine. 2015; 33:5913–5919. [PubMed: 26271833]
2. Bright RA, Neuzil KM, Pervikov Y, Palkonyay L. Vaccine. 2009; 27:6366–6369. [PubMed: 19840675]
3. Colacino JM, Staschke KA, Laver WG. Antiviral Chem Chemother. 1999; 10:155–185.
4. Tisoncik JR, Guo Y, Cordero KS, Yu J, Wang J, Cao Y, Rong L. Virol J. 2011; 8:14. [PubMed: 21232128]
5. Palese P, Tobita K, Ueda M, Compans RW. Virology. 1974; 61:397–410. [PubMed: 4472498]
6. Palese P, Compans RW. J Gen Virol. 1976; 33:159–163. [PubMed: 978183]
7. Cohen M, Zhang XQ, Senaati HP, Chen HW, Varki NM, Schooley RT, Gagneux P. Virol J. 2013; 10:321. [PubMed: 24261589]
8. Griffin JA, Compans RW. J Exp Med. 1979; 150:379–391. [PubMed: 222875]
9. Nayak DP, Hui EK, Barman S. Virus Res. 2004; 106:147–165. [PubMed: 15567494]
10. Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, Kawaoka Y. Journal of virology. 2000; 74:6015–6020. [PubMed: 10846083]
11. Gubareva LV, Nedyalkova MS, Novikov DV, Murti KG, Hoffmann E, Hayden FG. J Gen Virol. 2002; 83:2683–2692. [PubMed: 12388803]
12. Nedyalkova MS, Hayden FG, Webster RG, Gubareva LV. J Infect Dis. 2002; 185:591–598. [PubMed: 11865415]
13. Shtyrya Y, Mochalova L, Voznova G, Rudneva I, Shilov A, Kaverin N, Bovin N. Glycoconjugate J. 2009; 26:99–109.
14. Wagner R, Matrosovich M, Klenk HD. Rev Med Virol. 2002; 12:159–166. [PubMed: 11987141]
15. Oh S, Eichelberger MC. Virology. 1999; 264:427–435. [PubMed: 10562504]
16. Carlson CM, Turpin EA, Moser LA, O'Brien KB, Cline TD, Jones JC, Tumpey TM, Katz JM, Kelley LA, Gauldie J, Schultz-Cherry S. PLoS Pathog. 2010; 6:e1001136. [PubMed: 20949074]
17. Gimsa U, Grotzinger I, Gimsa J. Virus Res. 1996; 42:127–135. [PubMed: 8806180]
18. Gubareva LV, Webster RG, Hayden FG. Antiviral Res. 2002; 53:47–61. [PubMed: 11684315]
19. Yang J, Liu S, Du L, Jiang S. Rev Med Virol. 2016; 26:242. [PubMed: 27061123]
20. Wohlbold TJ, Krammer F. Viruses. 2014; 6:2465–2494. [PubMed: 24960271]
21. Varki, A., Schauer, R. Essentials of Glycobiology. 2nd. Varki, A.Cummings, RD., Esko, JD., editors. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.
22. Zanin M, Baviskar P, Webster R, Webby R. Cell Host Microbe. 2016; 19:159–168. [PubMed: 26867175]
23. Jia N, Barclay WS, Roberts K, Yen HL, Chan RW, Lam AK, Air G, Peiris JS, Dell A, Nicholls JM, Haslam SM. J Biol Chem. 2014; 289:28489–28504. [PubMed: 25135641]
24. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JS. Respir Res. 2007; 8:73. [PubMed: 17961210]
25. Gagneux P, Cheriyan M, Hurtado-Ziola N, van der Linden EC, Anderson D, McClure H, Varki A, Varki NM. J Biol Chem. 2003; 278:48245–48250. [PubMed: 14500706]
26. Garcia-Sastre A. Am J Pathol. 2010; 176:1584–1585. [PubMed: 20203283]
27. Imai M, Kawaoka Y. Curr Opin Virol. 2012; 2:160–167. [PubMed: 22445963]
28. Zanin M, Marathe B, Wong SS, Yoon SW, Collin E, Oshansky C, Jones J, Hause B, Webby R. J Virol. 2015; 89:5935–5948. [PubMed: 25810540]
29. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, Lindstrom S, Gubareva LV, Deyde V, Garten RJ, Harris M, Gerber S, Vagasky S, Smith F, Pascoe N, Martin K, Dufficy D, Ritger K, Conover C, Quinlisk P, Klimov A, Bresee JS, Finelli L. N Engl J Med. 2009; 360:2616–2625. [PubMed: 19423871]
30. Komadina N, Roque V, Thawatsupha P, Rimando-Magalong J, Waicharoen S, Bomasang E, Sawanpanyalert P, Rivera M, Iannello P, Hurt AC, Barr IG. Virus Genes. 2007; 35:161–165. [PubMed: 17429716]
31. Yen HL, Liang CH, Wu CY, Forrest HL, Ferguson A, Choy KT, Jones J, Wong DD, Cheung PP, Hsu CH, Li OT, Yuen KM, Chan RW, Poon LL, Chan MC, Nicholls JM, Krauss S, Wong CH,

Guan Y, Webster RG, Webby RJ, Peiris M. Proc Natl Acad Sci U S A. 2011; 108:14264–14269. [PubMed: 21825167]

32. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. Journal of virology. 2014; 88:3802–3814. [PubMed: 24429367]
33. Kobasa D, Kodihalli S, Luo M, Castrucci MR, Donatelli I, Suzuki Y, Suzuki T, Kawaoka Y. Journal of virology. 1999; 73:6743–6751. [PubMed: 10400772]
34. Neumann G, Kawaoka Y. Virology. 2015; 479–480:234–246.
35. Taga A, Sugimura M, Suzuki S, Honda S. Journal of chromatography A. 2002; 954:259–266. [PubMed: 12058910]
36. Zanetta JP, Pons A, Iwersen M, Mariller C, Leroy Y, Timmerman P, Schauer R. Glycobiology. 2001; 11:663–676. [PubMed: 11479277]
37. Rohrer JS. Anal Biochem. 2000; 283:3–9. [PubMed: 10929801]
38. Siskos PA, Spyridaki MH. J Chromatogr, Biomed Appl. 1999; 724:205–212.
39. Martin MJ, Vazquez E, Rueda R. Anal Bioanal Chem. 2007; 387:2943–2949. [PubMed: 17333152]
40. Allevi P, Femia EA, Costa ML, Cazzola R, Anastasia M. Journal of chromatography A. 2008; 1212:98–105. [PubMed: 18952219]
41. Hashii N, Kawasaki N, Nakajima Y, Toyoda M, Katagiri Y, Itoh S, Harazono A, Umezawa A, Yamaguchi T. Journal of chromatography A. 2007; 1160:263–269. [PubMed: 17570377]
42. van der Ham M, Prinsen BH, Huijmans JG, Abeling NG, Dorland B, Berger R, de Koning TJ, de Sain-van der Velden MG. J Chromatogr B: Anal Technol Biomed Life Sci. 2007; 848:251–257.
43. Valianpour F, Abeling NG, Duran M, Huijmans JG, Kulik W. Clin Chem. 2004; 50:403–409. [PubMed: 14684624]
44. Shaw CJ, Chao H, Xiao B. Journal of chromatography A. 2001; 913:365–370. [PubMed: 11355834]
45. Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Anal Biochem. 1979; 94:287–296. [PubMed: 464297]
46. Eichelberger MC, Hassantoufighi A, Wu M, Li M. Virol J. 2008; 5:109. [PubMed: 18822145]
47. Mochalova LV, Korchagina EY, Kurova VS, Shtyria JA, Gambaryan AS, Bovin NV. Anal Biochem. 2005; 341:190–193. [PubMed: 15866544]
48. Mochalova L, Kurova V, Shtyrya Y, Korchagina E, Gambaryan A, Belyanchikov I, Bovin N. Arch Virol. 2007; 152:2047–2057. [PubMed: 17680329]
49. Warren L. J Biol Chem. 1959; 234:1971–1975. [PubMed: 13672998]
50. Buxton RC, Edwards B, Juo RR, Voyta JC, Tisdale M, Bethell RC. Anal Biochem. 2000; 280:291–300. [PubMed: 10790313]
51. Nayak DP, Reichl U. J Virol Methods. 2004; 122:9–15. [PubMed: 15488615]
52. Williams TL, Pirkle JL, Barr JR. Vaccine. 2012; 30:2475–2482. [PubMed: 22197963]
53. Green ED, Adelt G, Baenziger JU, Wilson S, Van Halbeek H. J Biol Chem. 1988; 263:18253–18268. [PubMed: 2461366]
54. Taylor, JK. Quality assurance of chemical measurements. Lewis Publishers; Chelsea, Michigan: 1987. p. 328
55. Gerentes L, Kessler N, Aymard M. Dev Biol Stand. 1999; 98:189–196. (discussion 197). [PubMed: 10494973]
56. Webster RG, Reay PA, Laver WG. Virology. 1988; 164:230–237. [PubMed: 2452514]
57. Schild GC, Newman RW. Bull World Health Organ. 1969; 41:437–445. [PubMed: 5309453]
58. Monto AS, Kendal AP. Lancet. 1973; 301:623–625.
59. Eichelberger MC, Wan H. Curr Top Microbiol Immunol. 2014; 386:275–299.
60. Murphy BR, Kasel JA, Chanock RM. N Engl J Med. 1972; 286:1329–1332. [PubMed: 5027388]
61. Clements ML, Betts RF, Tierney EL, Murphy BR. J Clin Microbiol. 1986; 24:157–160. [PubMed: 3722363]
62. Cox RJ. Hum Vaccines Immunother. 2013; 9:405–408.
63. Couch RB, Kasel JA, Gerin JL, Schulman JL, Kilbourne ED. J Infect Dis. 1974; 129:411–420. [PubMed: 4593871]

64. Kilbourne ED. J Infect Dis. 1976; 134:384–394. [PubMed: 789791]
65. Kilbourne ED, Couch RB, Kasel JA, Keitel WA, Cate TR, Quarles JH, Grajower B, Pokorny BA, Johansson BE. Vaccine. 1995; 13:1799–1803. [PubMed: 8701596]
66. Air GM, Laver WG. Proteins: Struct, Funct Genet. 1989; 6:341–356. [PubMed: 2482974]
67. Seasonal Influenza Vaccine Effectiveness. Centers for Disease Control and Prevention; 2005–2016. https://www.cdc.gov/flu/professionals/vaccination/effectiveness-studies.htm, accessed May 17, 2016.
68. Memoli MJ, Shaw PA, Han A, Czajkowski L, Reed S, Athota R, Bristol T, Fargis S, Risos K, Powers JH, Davey RT, Taubenberger JK. mBio. 2016; 7:e00417–16. [PubMed: 27094330]
69. Childs RA, Palma AS, Wharton S, Matrosovich T, Liu Y, Chai W, Campanero-Rhodes MA, Zhang Y, Eickmann M, Kiso M, Hay A, Matrosovich M, Feizi T. Nat Biotechnol. 2009; 27:797–799. [PubMed: 19741625]
70. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y. Journal of virology. 2000; 74:8502–8512. [PubMed: 10954551]
71. Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson JC, Wilson IA. J Mol Biol. 2006; 355:1143–1155. [PubMed: 16343533]
72. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A. Science. 2007; 315:655–659. [PubMed: 17272724]
73. van Riel D, den Bakker MA, Leijten LM, Chutinimitkul S, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. Am J Pathol. 2010; 176:1614–1618. [PubMed: 20167867]
74. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. Am J Pathol. 2007; 171:1215–1223. [PubMed: 17717141]

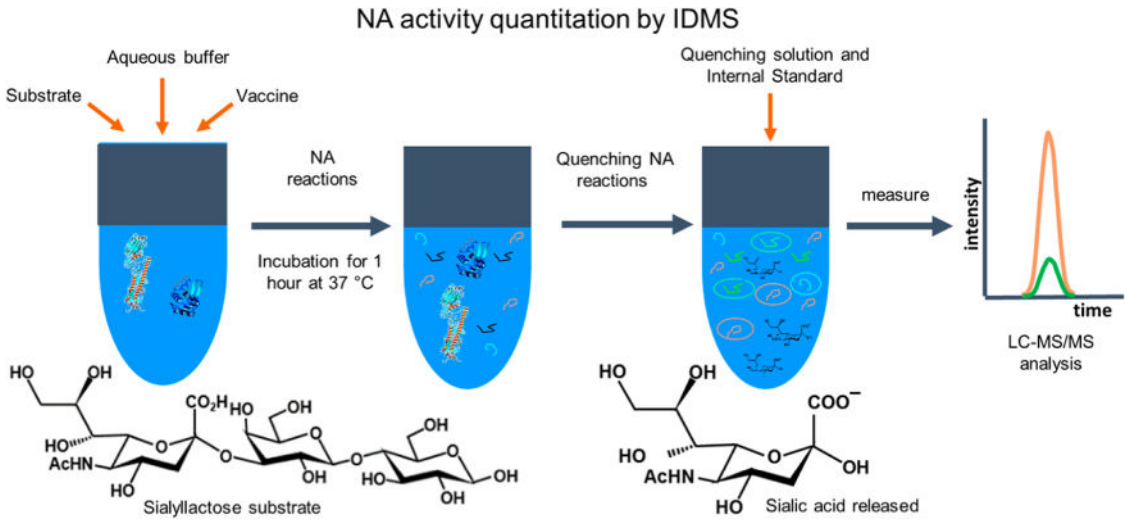

Figure 1.

Schematic of the enzymatic reactions and quantitative measurement of released sialic acid by isotope dilution LC-MS/MS.

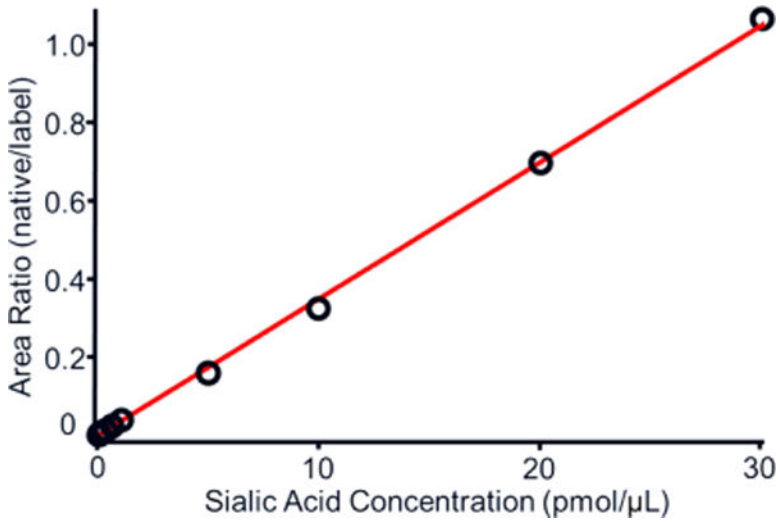

Figure 2.

A set of standards comprises 9 different levels of native sialic acid with concentrations ranging from 0.05 to 30 pmol/μL and a fixed level (30 pmol/μL) of $13C_3$-labeled sialic acid internal standard. Injections were 5 μL.

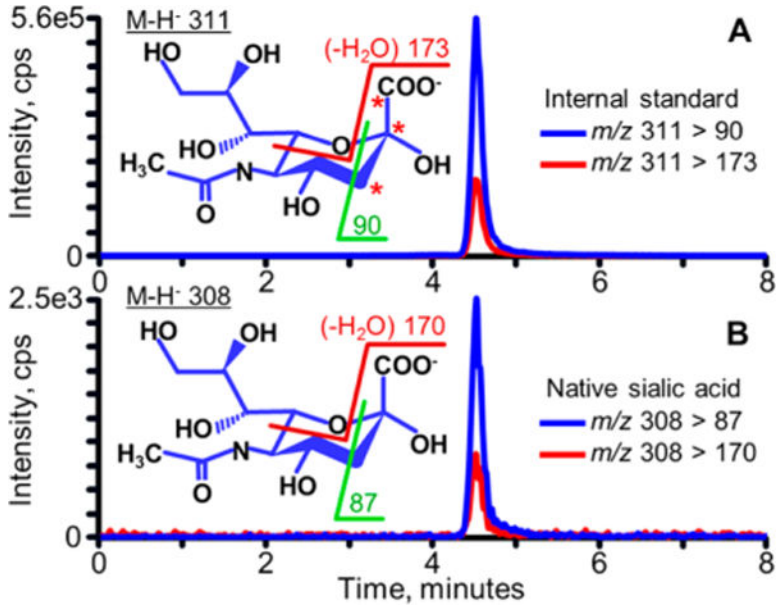

Figure 3.

LC-MS/MS chromatographic profile by isocratic elution for (A) 30 μM $^{13}C_3$-labeled (∗) internal standard sialic acid and (B) 0.05 μM native sialic acid, equivalent to 0.38 μM sialic acid in the NA reactions. The legends indicate the precursor > fragment *m*/*z* values.

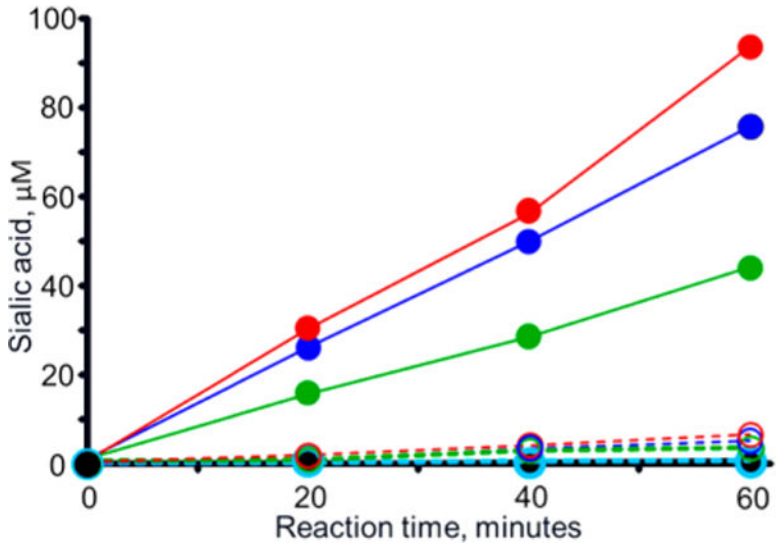

Figure 4.

NA activity toward α-2,3- and α-2,6-sialyllactose measured by LC-MS/MS at 0, 20, 40, and 60 min time points in commercial vaccines. Dotted lines represent vaccine A (in blue), vaccine B (in turquoise), vaccine E (in green), and vaccine F (in red) NA activity toward α-2,6-SL and solid lines represent vaccine A (in blue), vaccine B (in black), vaccine E (in green), and vaccine F (in red) NA activity toward α-2,3-SL.

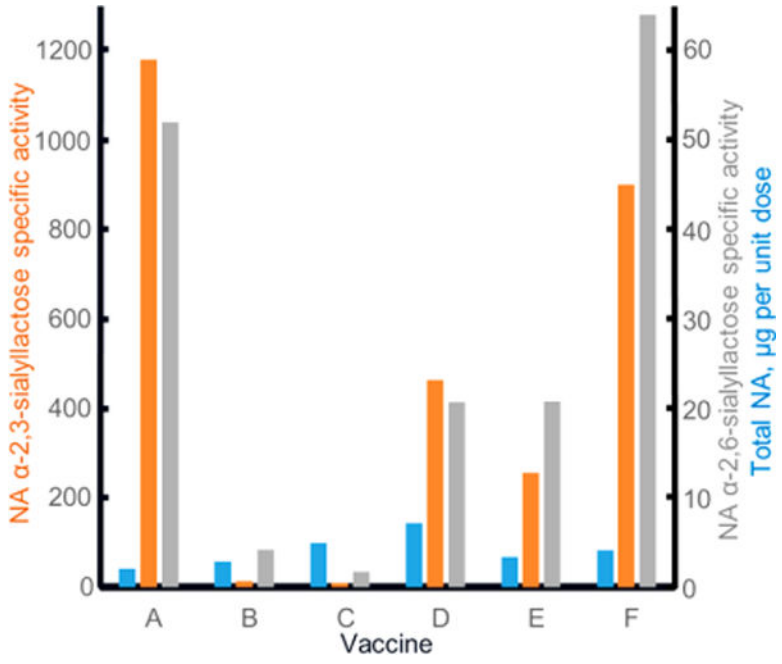

Figure 5.

N1 NA specific activity toward α-2,3 sialyllactose (orange; left-hand axis) and α-2,6 sialyllactose (gray; right-hand axis) substrates using six different H1N1 2009 monovalent split and subunit commercial vaccines (A–F). Specific activity units are in moles of sialic acid released per minute per mole of NA protein in 1 unit dose (Table 1). Total NA protein content is shown in blue (right-hand axis).

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Table 1

N1 NA Specific Activity Towards a Range of Different Substrates in H1N1 2009 Monovalent Commercial Vaccines A–F[a]

vaccine	type	substrate	Pmoles SA released in 60 min per unit dose	moles SA released in 60 min per unit dose	NA activity (moles SA released/min) per unit dose	total NA protein (μg) per unit dose	moles NA per unit dose	NA specific activity (NA activity/ moles NA protein)	α-2,3/α-2,6 ratio	total HA in moles	total HA (μg) per dose
A	split	α-2,3-SL	2 360 394	2.4×10^{-6}	3.9×10^{-8}	2.1	3.3×10^{-11}	1180	22.9	1.6×10^{-10}	12.5
		α-2,6-SL	103 116	1.0×10^{-7}	1.7×10^{-9}	2.1	3.3×10^{-11}	52			
B	subunit	α-2,3-SL	37 784	3.8×10^{-8}	6.3×10^{-10}	2.9	4.6×10^{-11}	13.7	3.2	2.4×10^{-10}	18.3
		α-2,6-SL	11 719	1.2×10^{-8}	2.0×10^{-10}	2.9	4.6×10^{-11}	4.2			
C	split	α-2,3-SL	47 900	4.8×10^{-8}	8.0×10^{-10}	5.05	8.0×10^{-11}	10.0	5.7	3.4×10^{-10}	25.6
		α-2,6-SL	8333	8.3×10^{-9}	1.4×10^{-10}	5.05	8.0×10^{-11}	1.7			
D	split	α-2,3-SL	3 180 980	3.2×10^{-6}	5.3×10^{-8}	7.2	1.1×10^{-10}	464	22.4	3.6×10^{-10}	27.2
		α-2,6-SL	141 927	1.4×10^{-7}	2.4×10^{-9}	7.2	1.1×10^{-10}	20.7			
E	split	α-2,3-SL	841 858	8.4×10^{-7}	1.4×10^{-8}	3.45	5.5×10^{-11}	256	12.3	1.2×10^{-10}	8.9
		α-2,6-SL	68 490	6.8×10^{-8}	1.1×10^{-9}	3.45	5.5×10^{-11}	20.8			
F	split	α-2,3-SL	3 561 480	3.6×10^{-6}	5.9×10^{-8}	4.15	6.6×10^{-11}	901	14.2	1.3×10^{-10}	9.9
		α-2,6-SL	251 511	2.5×10^{-7}	4.2×10^{-9}	4.15	6.6×10^{-11}	64			
A	split	fetuin	1 085 567	1.1×10^{-6}	1.8×10^{-8}	2.1	3.3×10^{-11}	543	N/A	1.6×10^{-10}	12.5
B	subunit	fetuin	27 662	2.8×10^{-8}	4.6×10^{-10}	2.9	4.6×10^{-11}	10.0	N/A	2.4×10^{-10}	18.3
C	split	fetuin	7191	7.2×10^{-9}	1.2×10^{-10}	5.05	8.0×10^{-11}	1.5	N/A	3.4×10^{-10}	25.6
D	split	fetuin	1 921 145	1.9×10^{-6}	3.2×10^{-8}	7.2	1.1×10^{-10}	280	N/A	3.6×10^{-10}	27.2
A	split	4-MUNANA	242 718	2.4×10^{-7}	4.0×10^{-9}	2.1	3.3×10^{-11}	121	N/A	1.6×10^{-10}	12.5
B	subunit	4-MUNANA	5556	5.6×10^{-9}	9.3×10^{-11}	2.9	4.6×10^{-11}	2.0	N/A	2.4×10^{-10}	18.3

[a]Reactions were carried out under the same conditions, and N1 NA activity was measured at $t = 60$ min for each. Sialyllactoses were present at 300 μM in the reactions, while fetuin was at 5 mg/mL and MUNANA was at 67 μM.

Table 2

Comparison of Specific Activities of Two Vaccines, A and B, with Four Substrates

		NA specific activity with various substrates			
vaccine	**total NA (μg per unit dose)**	**α-2,3-sialyl lactose**	**α-2,6-sialyl lactose**	**fetuin**	**4-MUNANA**
A	2.1	1180	52	543	121
B	2.9	13.7	4.2	10	2
ratio, A/B	0.72	86.1	12.4	54.3	60.5