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Reactivities of *N*-acetylgalactosamine-specific lectins with human IgA1 proteins

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Abstract

Lectins are proteins with specificity of binding to certain monosaccharides or oligosaccharides. They can detect abnormal glycosylation patterns on immunoglobulins in patients with various chronic inflammatory diseases, including rheumatoid arthritis and IgA nephropathy (IgAN). However, lectins exhibit binding heterogeneity, depending on their source and methods of isolation. To characterize potential differences in recognition of terminal *N*-acetylgalactosamine (GalNAc) on IgA1, we evaluated the binding characteristics of several commercial preparations of GalNAc-specific lectins using a panel of IgA1 and, as controls, IgA2 and IgG myeloma proteins. These lectins were from snails *Helix aspersa* (HAA) and *Helix pomatia* (HPA), and the plant *Vicia villosa* (VV). Only HAA and HPA bound exclusively to IgA1, with its *O*-linked glycans composed of GalNAc, galactose, and sialic acid. In contrast, VV reacted with sugars of both IgA subclasses and IgG, indicating that it also recognized *N*-linked glycans without GalNAc. Furthermore, HAA and HPA from several manufacturers differed in their ability to bind various IgA1 myeloma proteins and other GalNAc-containing glycoproteins in ELISA and western blot. For serum samples from IgAN patients, HAA was the optimal lectin to study IgA1 glycosylation in ELISA and western blot assays, including identification of the sites of attachment of the aberrant glycans. The Gal-deficient glycans were site-specific, localized mostly at Thr228 and/or Ser230. Because of the heterogeneity of GalNAc-specific lectins, they should be carefully characterized with appropriate substrates before undertaking any study.

Keywords

IgA nephropathy; IgA; lectins; glycosylation; *N*-acetylgalactosamine

1. Introduction

Lectins are proteins of plant or animal origin with specificity for a certain monosaccharide or oligosaccharide and have been employed in different areas of cell biology, immunology, and pathology (Carpenter *et al.*, 1997; Mestecky *et al.*, 1993; Mody *et al.*, 1995; Tomana *et al.*,

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1999; Rudd *et al.*, 2001; Tabares *et al.*, 2005; Wold *et al.*, 1994). Lectin-based assays can be used to diagnose certain types of cancer and also to evaluate progression and treatment of these malignancies (Brooks, 2000; Mody *et al.*, 1995; Tomana, 1996). In addition, lectins were used to detect markers of various chronic inflammatory diseases, including immunoglobulins (Ig) in diseases of an autoimmune (Mullinax and Mullinax, 1975; Ezaki *et al.*, 2001; Mackiewicz and Mackiewicz, 1995; Malhotra *et al.*, 1995; Novak *et al.*, 2001; Tomana, 1996) or infectious nature (Moore *et al.*, 2005).

Lectin-based assays have been extensively used to study the glycosylation of IgA1 in patients with IgA nephropathy (IgAN), the most common form of primary glomerulonephritis in the world (Julian *et al.*, 1988). In the hinge region of human serum IgA1, 3–5 Ser/Thr residues are occupied by *O*-glycans of variable composition; the prevailing forms include Gal-GalNAc disaccharide, and its mono- and di-sialylated forms (Mattu *et al.*, 1998; Novak *et al.*, 2000). A variant with terminal GalNAc or sialylated GalNAc in serum IgA1 is more common in patients with IgAN (Allen *et al.*, 2001; Allen *et al.*, 1995; Hiki *et al.*, 1995; Julian and Novak, 2004; Mestecky *et al.*, 1993; Novak *et al.*, 2001; Smith and Feehally, 2003; Tomana *et al.*, 1997; Tomana *et al.*, 1999) than in normal individuals (Field *et al.*, 1989; Mattu *et al.*, 1998). Although many GalNAc-specific lectins are available, those from the snails *Helix aspersa* (HAA) and *Helix pomatia* (HPA), and from the plant *Vicia villosa* (VV), have been most commonly used to evaluate the galactosylation of *O*-linked glycans on IgA1 of IgAN patients (Allen *et al.*, 2001; Allen *et al.*, 1995; Novak *et al.*, 2001; Tomana *et al.*, 1997; Tomana *et al.*, 1999).

In the past, we have found that lectins are useful and informative for the analyses of glycans; however, the results may be assay-specific. Moreover, the specificity of some lectins (including jacalin, commonly used for the isolation of IgA1 because it binds to the hinge-region *O*-linked glycans containing a GalNAc-Gal disaccharide) may vary depending on their geographical origin, the part of the plant or animal from which the compound is extracted, and the method of preparation (Bourne *et al.*, 2002; Jeyaprakash *et al.*, 2003; Kabir, 1998; Kobayashi *et al.*, 1988; Raval *et al.*, 2004; Van Damme *et al.*, 2002; Wu *et al.*, 2003). Recently, we encountered such a problem with a new batch of HAA lectin from our usual supplier that did not recognize Gal-deficient IgA1 myeloma proteins, unlike previous preparations from the same company (Tomana *et al.*, 1997; Tomana *et al.*, 1999). This prompted us to evaluate several lectins with specificity for GalNAc to identify the best candidate for the analysis of samples from patients with IgAN.

To the best of our knowledge, a study comparing the specificity and the binding characteristics of HAA, HPA, and VV in different assays has not been published. Because these lectins are used quite extensively by many researchers in immunology, nephrology, and oncology, we undertook this study to determine the binding characteristics of these GalNAc-specific lectins using supplies purchased from different manufacturers. Our study demonstrated that the specificities varied substantially. Therefore, we recommend that investigators not rely on the declared specificity of the lectin, but rather determine the precise reactivity in the particular assay of interest. Furthermore, we demonstrated the utility of the best-performing lectin, HAA, for the analysis of glycosylation of IgA1 from IgAN patients in ELISA and western blot analyses.

2. Materials and methods

2.1. Myeloma Proteins

Table 1 lists the IgA1, IgA2, and IgG myeloma proteins with their molecular properties that were isolated from serum or plasma of patients with multiple myeloma, as described previously (Mestecky and Kilian, 1985). Briefly, serum or plasma samples were precipitated with

ammonium sulfate (50% saturation), dissolved in phosphate buffer and the dialyzed precipitates were fractionated by ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Jacalin-agarose to capture or remove IgA1 (Sigma Chemical Company, St. Louis, MO) (Tomana *et al.*, 1997). The final purification step included size-exclusion chromatography on columns of Sephadex G-200 or Ultrogel AcA22 (Pharmacia, now Amersham Biosciences, Piscataway, NJ). For the IgA myeloma proteins, the potential contaminant, IgG, was removed by affinity chromatography on staphylococcal protein G immobilized on agarose (Sigma). The purity of the IgA1 preparations was assessed by SDS-PAGE, western blots (using IgA1-specific and IgA2-specific monoclonal antibodies) (Mestecky *et al.*, 1996), and immunoelectrophoresis using polyvalent reagents against IgA, IgG, and human serum (Jackson ImmunoResearch Laboratories, West Grove, PA). The molecular forms of the IgA1 proteins (Table 1) were assessed by size-exclusion chromatography, nonreducing SDS-PAGE, and western blots developed with anti-IgA antibody.

2.2. Lectin sources

HAA was purchased from Sigma, ICN (Aurora, OH), and EY (San Mateo, CA); HPA, from ICN and EY; and VV, from EY and Vector (Burlingame, CA).

2.3. ELISA

For capture ELISA, Costar 96-well U-bottom plates (Corning Inc., Corning, NY) were coated overnight at 4°C with F(ab')₂ fragment of goat anti-human IgA (BioSource International, Camarillo, CA) at a concentration of 5 µg/ml. Plates were blocked with 1% bovine serum albumin (BSA; Sigma) in PBS containing 0.05% Tween 20 (v/v) for 1 h at 37°C. Ig-containing samples diluted in blocking buffer were added to each well and incubated overnight at 4°C. In some instances, the captured IgA was subsequently desialylated by treatment for 3 h at 37°C with 10 mU/ml neuraminidase from *Vibrio cholerae* (Roche, Indianapolis, IN) in 100 mM sodium acetate buffer, pH = 5 (Tomana *et al.*, 1997). Samples were then incubated for 2 h at 37°C with biotinylated lectins diluted 1:500 in blocking buffer (Tomana *et al.*, 1997). The bound lectins were measured by the addition of avidin-alkaline phosphatase (Sigma) diluted 1:8,000 and incubated for 1 h at 37°C followed by a chromogenic substrate *p*-nitrophenyl phosphate (Sigma; one tablet per 5 ml substrate). Absorbance at 405 nm was measured with an EL808 microplate reader (Bio-Tek Instruments, Winooski, VT). Alternatively, avidin-horseradish peroxidase conjugate (ExtrAvidin; Sigma) was used and the reaction developed with the peroxidase chromogenic substrate O-phenylenediamine (OPD)-H₂O₂ (Sigma). The color reaction was stopped with 1 M sulphuric acid and the absorbance at 490 nm was measured.

For some experiments, ELISA microtiter plates were directly coated with IgA myeloma proteins (2.5 µg/ml) overnight at 4°C. Plates were then blocked, treated with neuraminidase, and developed as described above. The amount of coated IgA was verified by ELISA with anti-IgA antibody.

2.4. Western blot

Lectin binding was further confirmed by SDS-PAGE separation of reduced Ig proteins followed by western blot analysis (Tsuchiya *et al.*, 1993). Briefly, 1-µg aliquots of purified Ig proteins were dissolved in reducing buffer containing 1% (v/v; final concentration) 2-mercaptoethanol, and then electrophoretically separated by SDS-PAGE. These proteins were transferred to PVDF membranes by electroblotting. After blocking with Superblock (Pierce, Rockford, IL) supplemented with 0.05% Tween 20 for 1 h at room temperature, the membranes were incubated for 3 h at 37°C with 10 mU/ml neuraminidase in blocking buffer adjusted to pH 6.0, to remove sialic acid from Ig glycans. The removal of sialic acid was verified by gas-liquid chromatography. After washing, the membranes were incubated overnight at 4°C with

biotinylated lectin at a 1:500 dilution. For detection of the IgA heavy chain, a duplicate membrane was incubated overnight at 4°C with biotinylated goat anti-human IgA (BioSource) at a 1:30,000 dilution. After washing, the membranes were incubated with horseradish peroxidase-conjugated NeutrAvidin (Pierce). After addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 10 min at room temperature, the membranes were developed on Biomax MS Film (Eastman Kodak Company, Rochester, NY).

IgA proteins were also digested with IgA proteases from *Clostridium ramosum* AK183, *Streptococcus pneumoniae* TIGR 4, and *Haemophilus influenzae* HK50 (Kilian *et al.*, 1996) before SDS-PAGE separation and blotting on PVDF. The digestions were conducted for 20 h at 37°C in PBS, pH 7.4. Proteins were desialylated on the blot by incubating for 3 h at 37°C with 10 mU/ml neuraminidase in blocking buffer adjusted to pH 6.0. The blots were then probed with HAA and developed following the protocol described above.

2.5. Gas-liquid chromatography

The monosaccharides from purified IgA myeloma proteins were determined as trifluoroacetates of methyl-glycosides by gas-liquid chromatography (Tomana *et al.*, 1976; Tomana *et al.*, 1984). A Hewlett-Packard model 5890 series II gas chromatograph was used with a 25-m fused silica (0.22-mm inner diameter) OV-1701 WCOT column and electron capture detector; GC ChemStation software (Agilent Technologies, Palo Alto, CA) was used for acquisition of chromatographic data and peak integration. About 20 µg of each IgA was used for analyses.

3. Results

3.1. HAA, HPA, and VV from various manufacturers differ in their binding to IgA

The fact that HAA recently purchased from EY did not reproduce our previous results with an earlier batch of this lectin prompted us to investigate the heterogeneity of lectins purchased from various manufacturers. We screened three lectins purportedly specific for GalNAc (HAA, HPA, VV) for their binding to a panel of IgA1 and IgA2 myeloma proteins (Table 1). The ELISA results for each lectin purchased from two different suppliers are shown in Fig. 1. HAA from Sigma exhibited a broader binding capacity for IgA1 than did HAA from ICN. In addition, HPA and VV from EY bound to the IgA1 myeloma proteins more effectively than did the lectins purchased from ICN and Vector. Based on these results, we selected HAA from Sigma and HPA and VV from EY for further experiments in this study.

3.2. Binding specificity of HAA, HPA, and VV for Gal-deficient IgA1

To further examine the binding specificity of these lectins, additional IgA1 and IgA2 myeloma proteins were used. These myeloma proteins were analyzed for molecular form by size-exclusion chromatography and SDS-PAGE and for monosaccharide composition by gas-liquid chromatography; the results confirmed the absence of GalNAc in the IgA2 and IgG preparations. Furthermore, western blot analysis with IgA subclass-specific monoclonal antibodies confirmed the immunochemical purity of the preparations. In addition to IgA1 proteins, VV bound IgA2 in ELISA and western blot (Fig. 2A and B). Surprisingly, VV also bound to an IgG myeloma protein. Thus, this lectin was not specific for GalNAc.

We compared the binding efficiency of HAA and HPA by ELISA and western blot analysis (Fig. 3A, B, and C). Although these two lectins purportedly have similar binding specificities (Dahr *et al.*, 1975; Ishiyama and Uhlenbruck, 1972), we found disparate characteristics (Figs. 1 and 3). Results from ELISA showed that although both lectins bound all IgA1 myeloma proteins except Kni, HPA bound some IgA1 proteins more effectively than did HAA. In contrast, in the neuraminidase-treated western blots, HPA bound only four of the eight tested

proteins whereas HAA bound to all. Using biotinylated jacalin, we confirmed the presence of *O*-linked GalNAc-Gal in all IgA1 myeloma proteins by western blot analysis (Fig. 3C).

The difference in binding HAA to NA-treated and untreated IgA1 proteins in the western blot analysis demonstrated heterogeneity of sialylation of their GalNAc moieties (Fig. 3C). After treatment with neuraminidase to remove sialic acid and thereby increase the number of exposed GalNAc moieties, the number of IgA1 myeloma proteins binding to HAA increased from two to eight, irrespective of the molecular form (see Table 1). For instance, after neuraminidase treatment, HPA bound IgA1 polymer (Mce) and IgA1 monomer (Kni) to the same degree (Fig. 3). In summary, the removal of sialic acid improved the accuracy of the measurement of total GalNAc content in the hinge-region IgA1 *O*-linked glycans and reduced the assay-dependent variability.

3.3 HAA and HPA binding to Gal-deficient IgA1: differences between ELISA and western blot results

In addition to lectin-dependent differences in binding to various IgA1 myeloma proteins, we also found marked assay-dependent differences. For example, HPA bound only to myeloma proteins Mce, Gou, and neuraminidase-treated Kni on the western blot. In contrast, in ELISA, HPA did not significantly bind IgA1 (Kni) protein although it bound other IgA1 myeloma proteins, such as Sin and Ber1, at high levels. We speculated that the discrepancies in the results of the ELISA and the western blot may have been due to a difference in sample preparation (reduction of disulphide bonds in samples for western blot analysis) and/or different three-dimensional positioning and exposure of the binding sites (GalNAc). To test whether reduction of IgA1 accounts for the observed differences, we used these samples in ELISA. We observed lower levels of lectin binding to the reduced IgA1 proteins, particularly Gou, compared to the unreduced samples (Table 2). Although in ELISA the pattern of binding was similar, there was no correlation with the pattern on western blots. We propose that the discrepancy was caused by differences in the exposure of hinge-region glycans in ELISA and western blot rather than by the reduction of disulphide bridges.

To better understand the possible effect of three-dimensional conformation on the lectin-IgA1 interactions, we compared the binding of HAA and HPA to several IgA1 myeloma proteins, either directly bound to ELISA plates or captured by anti-IgA antibody (Table 2). For most of the IgA1 proteins, direct coating resulted in better overall binding; similar results were observed with native and reduced samples. The pattern of HAA and HPA binding was generally the same as that obtained by capture ELISA (Fig. 1, Table 2) and did not correlate with the results of western blots. We postulate that the three-dimensional conformation of hinge-region GalNAc plays an important role in the lectin binding and that the accessibility of the glycans differs between assays (ELISA vs. western blot).

3.4. HAA binding to IgA1 from serum samples of IgAN patients and healthy controls

Due to its ability to bind seven of eight IgA1 myeloma proteins in both assays, we concluded that HAA from Sigma was the best lectin to detect Gal-deficient IgA1. We tested the HAA binding to IgA1 from serum samples of IgAN patients and healthy controls using ELISA with neuraminidase treatment of captured IgA1 (see Methods 2.3) (Fig. 4). HAA bound more to IgA1 from IgAN patients than to IgA1 from healthy controls ($p = 0.0003$). Western blot analysis confirmed the ELISA results (Fig. 5A).

3.5. HAA reveals Gal-deficient sites in IgA1 from IgAN patients

We isolated serum IgA1 from 3 patients with IgAN and digested the proteins with three IgA proteases. These enzymes cleaved after Pro 221, 227, and 231 (Kilian *et al.*, 1996). The fragments generated, Fc and Fd, were separated by SDS-PAGE, blotted and after desialylation

probed with HAA. The lectin reacted with the Fc fragment after digestion with proteases from AK183 or TIGR 4 (see Methods) but with Fd fragment after digestion with protease from HK50; this finding localized the Gal-deficient sites to Thr228 and/or Ser230 (Fig. 5B). One IgA1 protein also had additional Gal-deficient sites at or after Ser 232.

4. Discussion

Lectins are important tools to isolate and characterize glycan moieties on glycoproteins (Baenziger and Kornfeld, 1974; Frangione and Wolfenstein-Todel, 1972; Mattu *et al.*, 1998; Rudd *et al.*, 2001; Tabares *et al.*, 2005). Because some glycoproteins or glycolipids are available in only small quantities, they may not be amenable to direct carbohydrate analyses. Therefore, we had previously developed a lectin-based ELISA to determine glycosylation abnormalities on IgA1 from IgAN patients (Tomana *et al.*, 1997; Tomana *et al.*, 1999). In this assay, IgA1 from some patients with IgAN reacted more effectively with GalNAc-specific lectins than did IgA1 from healthy controls (Tomana *et al.*, 1997; Tomana *et al.*, 1999). This finding indicated structural differences in GalNAc-containing glycans in the hinge region of IgA1 (Baenziger and Kornfeld, 1974; Frangione and Wolfenstein-Todel, 1972; Mattu *et al.*, 1998). Specifically, our data showed that IgA1 from IgAN patients contained fewer terminal Gal residues, thus exposing the underlying GalNAc. Animal and plant lectins are usually available from several sources and each manufacturer uses its own proprietary purification process. Recently, we observed unexpected discrepancies in the binding of different batches of HAA from the same manufacturer to Gal-deficient IgA1 and asialo ovine submaxillary mucin. Upon inquiry, we discovered that the manufacturer had changed the supplier of the snails.

We compared the binding capabilities of three lectins purportedly specific for GalNAc, using well-defined standard IgA1 and control IgA2 and IgG myeloma proteins. HAA, HPA, and VV from different suppliers displayed variable specificities in the binding. Furthermore, HAA and HPA recognized terminal GalNAc in IgA1 while VV, with its previously claimed specificity for GalNAc (Tollefsen and Kornfeld, 1983), recognized other glycans in addition to GalNAc. The type of assay used in lectin binding analyses, in our case ELISA and western blot, profoundly influenced the outcome of measurements. When we compared the binding of HAA and HPA to IgA1 in these two assays, the reactivity of HAA and HPA varied substantially. In western blot analysis, HAA reacted with all IgA1 myeloma proteins tested whereas HPA reacted with only a few. However, in ELISA, HPA bound the IgA1 proteins more effectively than did HAA. The differential binding by HAA and HPA may have been a result of a variation in content or exposure of GalNAc on the IgA1 myeloma proteins.

Using HAA, we demonstrated by western blotting that a well characterized lectin can be useful not only in generally confirming the ELISA data, but also in providing site-specific information about the Gal-deficiency of IgA1 in patients with IgAN (Fig. 5B). If verified, this information would imply that the Gal deficiency in IgAN is not random, but rather is site-specific.

In summary, these results demonstrated a variability in the binding characteristics of several, purportedly GalNAc-specific, lectins based on their source and the assay used. Therefore, when lectins are used to analyze disease-associated glycans, we recommend that their binding properties be evaluated by using standard substrates in the assay of interest.

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Fig. 1.

Capture ELISA of lectin binding to a neuraminidase-treated IgA2 myeloma protein, Fel (triangles), and a neuraminidase-treated IgA1 myeloma protein, Mce (circles). (A) Binding of VV purchased from Vector (open symbols) and EY (closed symbols). (B) Binding of HPA purchased from ICN (open symbols) and EY (closed symbols). (C) Binding of HAA purchased from ICN (open symbols) and Sigma (closed symbols).

Figure 2A

Figure 2B

Fig. 2. VV binding to IgA1 and IgA2 myeloma proteins using ELISA (A) and western blot analysis (B). The numbers identify the same proteins in both figures

A) VV binding to myeloma proteins (0.625 μ g/ml) determined by capture ELISA. Protein were native (black bars) or treated with neuraminidase to remove sialic acid (light bars).

B) One μ g IgA1 and IgA2 protein were loaded per well. Selected western blot membranes were treated with neuraminidase (NA), then developed with VV. IgA myeloma proteins used: (1) Mce, (2) Ham, (3) Ste, (4) Gou, (5) Lat, (6) Ber1, (7) Sin, (8) Kni, (9) Fel, (10) Ber2, (11)

Hum, (12) Kur, (13) Cur, and (14) Cob. An IgG myeloma protein (Mat) was used as a negative control. Anti-IgA staining was performed as a loading control.

Figure 3A

Figure 3B

Figure 3C

Fig. 3. Comparative binding of HPA and HAA binding in ELISA (A and B) and western blot analysis (C)

A) and B). Binding of HAA and HPA, respectively, to myeloma proteins (0.625 $\mu\text{g}/\text{ml}$) determined by capture ELISA. Proteins were native (black bars) or treated with neuraminidase to remove sialic acid (light bars).

C) One μg myeloma IgA1 and IgA2 proteins loaded per well. Membranes were not treated (-) or treated (+) with neuraminidase. Anti-IgA and jacalin were used as controls for loading and presence of GalNAc-Gal disaccharides, respectively. The same numbers identify the same proteins in A through C. IgA myeloma proteins used: (1) Mce, (2) Ham, (3) Ste, (4) Gou, (5) Lat, (6) Ber1, (7) Sin, (8) Kni, (9) Fel, (10) Ber2, and (11) Cob.

Fig. 4. HAA (Sigma) binding to serum IgA1 from IgAN patients (n = 17) and healthy controls (n = 16) measured by capture ELISA with neuraminidase treatment of IgA1. Concentration of IgA1 was normalized to 1 μ g per well. Samples were treated with neuraminidase after capture.

Figure 5A

Figure 5B

Fig. 5.

A) Western blot of serum samples from patients with IgAN and healthy controls developed with HAA after neuraminidase treatment on the membranes. Load of serum samples was normalized to IgA concentration of 0.25 µg/well for separation by SDS-PAGE under reducing conditions.

B) Western blot of purified IgA1 isolated from serum of an IgAN patient and digested with 3 different IgA proteases (1, *Clostridium ramosum* AK183; 2, *Streptococcus pneumoniae* TIGR 4; 3, *Haemophilus influenzae* HK50), desialylated and probed with HAA. The amino acid(s) with a Gal-deficient *O*-glycans was localized to a site between the cleavage sites 2 and 3.

Table 1

Myeloma proteins used in this study.

Myeloma protein	Ig Isotype	Molecular form
Mce	IgA1	Polymer
Ham	IgA1	Polymer
Ste	IgA1	Polymer
Gou	IgA1	Polymer
Lat	IgA1	Dimer
Ber1	IgA1	Dimer
Sin	IgA1	Monomer
Kni	IgA1	Monomer
Fel	IgA2	Polymer
Ber2	IgA2	Polymer
Cob	IgA2	Polymer
Hum	IgA2	Polymer
Kur	IgA2	Polymer
Cur	IgA2	Monomer
Mat	IgG	

Table 2

HPA binding to reduced and native IgA myeloma proteins in ELISA.

Myeloma protein	Reduced (Capture)	Reduced (Direct)	Native (Capture)	Native (Direct)
NA-treated				
Mce	1.055 ^a	1.175	2.407	2.719
Ham	0.196	0.375	1.017	1.434
Ste	0.101	0.398	1.050	1.972
Gou	0.689	0.602	3.326	3.771
Lat	0.182	0.294	0.754	1.604
Ber1	0.984	0.629	2.065	2.462
Sin	0.404	0.622	1.026	2.108
Kni	0.034	0.013	0.074	0.064
Fel	0.000	0.003	0.000	0.000
Ber2	0.000	0.000	0.000	0.000
Cob	0.000	0.001	0.000	0.000
Without NA				
Mce	0.523	0.380	1.888	2.001
Ham	0.092	0.059	0.447	0.679
Ste	0.079	0.043	0.395	0.723
Gou	0.867	0.343	3.388	3.667
Lat	0.105	0.091	0.301	0.984
Ber1	0.341	0.089	0.803	0.720
Sin	0.383	0.251	0.740	1.643
Kni	0.080	0.020	0.000	0.029
Fel	0.000	0.004	0.003	0.003
Ber2	0.000	0.007	0.000	0.000
Cob	0.009	0.002	0.000	0.000

^a Absorbance at 405 nm.

NA, neuraminidase