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A rapid assay of the sialidase activity in species of the *Bacteroides fragilis* group by using peanut lectin hemagglutination

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Abstract

In this study, a novel, simple and rapid hemagglutination assay by using a peanut lectin to detect a neuraminidase activity in strains of the *Bacteroides fragilis* group was developed. One hundred and fourteen species of the *B. fragilis* group isolated from children with and without diarrhea and 15 reference strains were evaluated. Neuraminidase production was determined by using the method above described and its inhibition was observed by using galactose. The neuraminidase production was observed in 54 (84.37%) diarrhea and in 43 (86%) non-diarrhea strains. HA titers were ranged from 2 to 32. This neuraminidase assays based on PNA hemagglutination is highly sensitive, reproducible and could be used as a tool to detect the sialidase activity in anaerobic bacteria, particularly, in species of the *B. fragilis* group.

Keywords: Neuraminidase; *Bacteroides fragilis* group; Hemagglutination; Peanut lectin; *Arachis hypogaea*

1. Introduction

Sialidases or neuraminidases (NANase) catalyze the hydrolysis of the sialic acid (NANA-*N*-acetyl neuraminic acid) from glycoproteins, glycolipids, and oligo and polysaccharides, whether the enzyme or the substrates are in solution or on cell membrane [1]. Sialidase treatment (desialylated) exposes the β-galactosyl determinant, which acts as ligand in the adherence among bacteria and host's mucosa [2,3].

Sialic acids are involved in the binding and in the transport of positively charged compounds and in the cells aggregation (via Ca^{2+} bridges) or disaggregation (by repulsive effects, such as in erythrocytes). Moreover, they play a role in the glycoproteins conformation, and they are important in the arrangement of molecules in cell membranes, in the glycoprotein enzymes activity and to the glycoproteins resistance against proteases. Neuraminic acids have been found to prevent the recognition of receptors by corresponding ligands or vice versa and the recognition of antigenic sites (polypeptide or carbohydrate chains) by components of the host' immune-defense system. This masking of the specific recognition sites on molecules and cells is possibly the most important role of sialic acids [2].

Bacterial sialidases have been considered virulence factors in many pathogenic organisms which colonize mucosal surfaces [4].

Neuraminidase production is mediated by the *nanH* gene, which is present in several pathogens including *Streptococcus pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium, Corynebacterium diphtheriae, Vibrio cholerae, Porphyromonas* spp., *Prevotella* spp., *Clostridium* spp., *Actinomyces viscosus* and *Bacteroides fragilis* [5–7].

Several *B. fragilis* strains have neuraminidase activity and it has been suggested that this activity plays a role in the bacterial attachment to animal cells and to the hemagglutination. However, this conclusion was based on indirect experiments in which *B. fragilis* was found to attach more efficiently to erythrocytes previously treated with the *V. cholerae* or the clostridial neuraminidase enzyme [3,8]. Sialidase activity can be measured by release of *N*-acetylneuraminic acid from various biological

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substrates as detected by thiobarbituric acid assay and by the release of radio-labeled *N*-acetylneuraminic acid from gangliosides [9] or other substrates followed by chromatographic separation of substrate and products. Other spectrophotometric assays are available which use various phenylketosides of *N*-acetylneumraminic acid as substrates; the liberated phenols are then measured [10,11].

Potier et al. [12] have described a sensitive fluorometric sialidase assay which uses 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MUN) as substrate. Moncla and Braham [6] developed a rapid filter paper spot test using MUN. This assay is considered as a rapid method for the detection of acetylneuraminyl hydrolase. Because all of the available assays have some limitation in sensitivity or time and may require some specialized equipment, detection of sialidase is not practical for routine clinical work. In this report we developed a quantitative, novel, simple and rapid technique to detect the sialidase activity in members of the *B. fragilis* group and in other organisms.

2. Material and methods

2.1. Detection of the neuraminidase

Neuraminidase production was determined in microtiter plates by using fresh normal human erythrocytes drawn from healthy volunteers. The erythrocytes were collected in Alsever's solution (10%), and washed three times in 0.01 M of phosphate buffered saline buffer (PBS, Na_2HPO_4, KH_2PO_4, NaCl, pH 7.4) and kept at 4 °C, during 1 week. Bacterial cells from 114 strains of the *B. fragilis* group isolated from children with (64) and without (50) diarrhea and 15 reference strains (Table 1) were grown in BHI broth (anaerobiosis, at 37 °C, for 24 h) and ressuspended to 1.5×10^8 bacteria/mL. Bacterial suspension was three times washed by centrifugation ($12{,}000 \times g$, 10 min) in PBS. Then, 1 mL of bacteria was added to 10 μL of the washed erythrocytes achieving a final concentration of 1% and carefully homogenized and incubated in anaerobiosis, at 37 °C, overnight, except for *Prevotella* and *Porphyromonas* strains, incubated for 4 h. Serial dilutions of the lectin from peanut (2.5 μg/μL) (*Arachis hypogaea;* PNA, Sigma Chemical Co.) in PBS, were performed, and 20 μL of each dilution was added to the mixture bacteria erythrocytes, and then homogenized and incubated at room temperature for 1 h followed by an additional incubation at 4 °C, overnight. The presence of the neuraminidase was noticed when an agglutination (bacteria-erythrocytes-lectin) was observed. A mixture of bacteria-erythrocytes without lectin was used as negative control (precipitation). The agglutination titer was defined as the reciprocal of the end point dilution.

The inhibition of the neuraminidase production was verified in neuraminidase-positive strains. Briefly, bacteria were treated with 1 mL of 1 mM galactose, and incubated at room temperature, for 30 min. Samples were washed and harvested by centrifugation ($12{,}000 \times g$, 10 min) in PBS. Then, 2 μL of diluted lectin were mixed with 18 μL of bacteria (1.5×10^8 cells/mL) treated with 1 mM galactose and added to 20 μL of erythrocytes (1% final concentration). Plates were lightly homogenized and incubated at 37 °C, for 1 h, and the enzyme inhibition was noticed by a precipitation of erythrocytes. A mixture of bacteria–erythrocytes–lectin was used as negative control.

The neuraminidase production was observed in 54 (84.37%) members of the *B. fragilis* group isolated from diarrhea stool and in 43 (86%) isolated normal stools (Table 1). Agglutination titer values for members of the *B. fragilis* group were ranged from 2 to 32, and HA titer values of 256 for *A. viscosus* ATCC 91014, *P. gingivalis* ATCC 33277 and *P. intermedia* ATCC 33563. Additionally, five *B. ovatus* isolated from diarrhea, *C. difficille* VPI 10468, *A. actinomycetemcomitans* JP2, *A. actinomycetemcomitans* SUNY 465, *F. nucleatum* ATCC10953, *P. micros* ATCC 33270, *E. faecalis* ATCC 2912, *E. coli* J53pACYc 184 and *S. mutans* ATCC 25175 did not produce neuraminidase. Moreover, the neuraminidase production was inhibited by galactose in all the neuraminidase-positive organisms. On the other hand, the HA assay of the black-pigmented anaerobic rods such as *Prevotella* and *Porphyromonas* strains was performed by modifying the incubation time to 4 h due to the erythrocyte were lysed when it was overnight incubated.

3. Results and discussion

Sialidase activity has been detected in anaerobic rods such as *Prevotella* spp., *Porphyromonas* spp. and *B. fragilis* group species. *B. fragilis* strains produce several enzymes and some of them are known to play a significant role in infections, such as neuraminidase. Sialidase activity has been observed in material taken from abscess of which *B. fragilis* was recovered. Moreover, studies have shown that *B. fragilis* recovered from pathological specimens produce significantly higher levels of sialidase activity than isolates from non-pathological specimens [13]. Additionally, many studies have demonstrated that bacterial sialidases have the capacity to modify the ability of the host to respond to the infection [14,15].

Literature shows the filter paper spot technique using 2′-(4-methylumbelliferyl) α-D-*N*-acetylneuraminic acid or 5-bromo-4-chloro-3indolyl-α-D-*N*-acetylneuraminic acid in the phenotypic detection of sialidase in several bacteria [4,16]. However, we developed a novel technique by using a lectin from peanuts (*Arachis hypongaea*—PNA) in a hemagglutination assay. Then, lectin agglutinating neuraminidase-treated human erythrocytes was observed. Although this hemagglutination method does provide an estimation of neuraminidase activity in biological material, it does not quantify the enzyme in an exact fashion because of the problems inherent in cell agglutination. However, several methods which quantify agglutination such as those using aggregometer and they could be adapted to the PNA assay in according to Pereira [17].

Table 1
Sialidase activity and hemagglutination (HA) titers of 114 members of the *B. fragilis* group isolated from children with and without diarrhea, and from other 15 organisms

Source of species (*n*)	No. of isolates		HA titer range
	Tested	Positive (%)	
Children with diarrhea (64)			
B. fragilis	39	37 (94.8)	2–16
B. vulgatus	8	7 (87.5)	8–16
B. uniformis	6	6 (100)	4–32
B. distasonis	6	4 (66.6)	2–8
B. ovatus	5	0 (0)	0
Children without diarrhea (50)			
B. fragilis	27	25 (92.5)	4–16
B. distasonis	10	6 (60)	2–8
B. vulgatus	6	5 (83.3)	4–16
B. eggerthii	2	2 (100)	8
B. ovatus	2	2 (100)	4–8
B. thetaiotaomicron	2	2 (100)	4–8
B. uniformis	1	1 (100)	8
Other microorganisms (15)			
Actinomyces viscosus ATCC 91014	1	1 (100)	256
Fusobacterium nucleatum ATCC 10953	1	0 (0)	0
Porphyromonas gingivalis ATCC 33277	1	1 (100)	256
Prevotella nigrescens ATCC 33563	1	1 (100)	256
B. fragilis ATCC 43858	1	1 (100)	8
B. fragilis ATCC 25285	1	1 (100)	8
B. fragilis GAI 97124	1	1 (100)	16
Clostridium difficile VPI 10468	1	0 (0)	0
Actinobacillus actinomycetemcomitans JP2	1	0 (0)	0
Actinobacillus actinomycetemcomitans SUNY 465	1	0 (0)	0
Peptostreptococcus micros ATCC 33270	1	0 (0)	0
Enterococcus faecalis ATCC 29212	1	0 (0)	0
Escherichia coli pBR 322	1	0 (0)	0
Escherichia coli J53 pACYC 184	1	0 (0)	0
Streptococcus mutans ATCC 25175	1	0 (0)	0

PNA or others lectins have already been used to study sialidase activity. Peanut lectin is highly specific for the disaccharide DGal-β-1$\rightarrow$3dGalNAc but also bind DGal-β-1$\rightarrow$4dGlcNAc to a lesser extent. These structures are commonly found in the complex carbohydrates located on cell surfaces. However, in mature fully differentiated cells, the disaccharides are usually sialylated and are not available for lectin binding, unless the cells are treated with neuraminidase to expose the lectin receptor sites [17].

In this study, the lack of sialidase activity in *F. nucleatum, A. actinomycetemcomitans, C. difficile, P. micros, E. faecalis*, *E. coli* and *S. mutans* was observed according to Popoff and Dodin [18], Moncla et al. [4] and Russo et al. [19]. The presence of sialidase in *Porphyromonas* spp. and *Prevotella* spp. suggests that sialidase activity is involved with the pathogenesis of *P. gingivalis* and *P. intermedia* or that it may be important to the abilities of these organisms to colonize mucosal surfaces.

To assure the specificity of the assay its inhibition was observed by using galactose as peanuts lectin is highly specific for the disaccharide GalNAc [20]. Thus, the neuraminidase assay based in PNA hemagglutination is quick and reproducible, simple to perform and highly sensitive.

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