JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1989, p. 182–184
0095-1137/89/010182-03$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 27, No. 1

Detection of Sialidase (Neuraminidase) Activity in *Actinomyces* Species by Using 2′-(4-Methylumbelliferyl)α-D-*N*-Acetylneuraminic Acid in a Filter Paper Spot Test

BERNARD J. MONCLA* AND PAMELA BRAHAM

Departments of Oral Biology and Periodontics, University of Washington, Seattle, Washington 98195

Received 11 July 1988/Accepted 19 September 1988

A rapid method for the detection of acetylneuraminyl hydrolase, EC 3.2.1.18 (sialidase or neuraminidase), was developed by using 2′-(4-methylumbelliferyl)α-D-*N*-acetylneuraminic acid as substrate in a filter paper spot test. The method was compared to conventional assays that use 2′-(4-methylumbelliferyl)α-D-*N*-acetylneuraminic acid and bovine submaxillary mucin and was found to be in excellent agreement. Organisms with >10 U of enzyme activity (in nanomoles per minute per milligram of cell protein) gave positive reactions, while those with 2.7 to 9.0 U gave only weak reactions. Isolates with <2.7 U of activity were detected upon prolonged incubation. Sialidase activity was detected in 79% of 71 clinical isolates representing five species of *Actinomyces*. The percentage of sialidase-producing isolates of each species varied considerably: *Actinomyces israelii*, 63%; *A. meyeri*, 73%; *A. naeslundii*, 85%; *A. odontolyticus*, 73%; and *A. viscosus*, 100%.

Sialidases (acetylneuraminyl hydrolases; EC 3.2.1.18) cleave α-ketosidic linkages between sialic acids and the glycosyl residues of glycoproteins, glycolipids, or colominic acids. Although these enzymes are often referred to as neuraminidases, sialidase is the preferred term, as neuraminic acid itself is not the substrate whereas glycosyl-linked sialic acids are (4, 19, 20).

Bacterial sialidases have long been considered virulence factors in many pathogenic organisms, including *Corynebacterium diphtheriae*, *Vibrio cholerae* (19), *Streptococcus pneumoniae* (7), group B streptococci (2, 14), *Clostridium perfringens* (12), *Pasteurella multocida* (11), *Pseudomonas aeruginosa* (8), and *Klebsiella aerogenes* (16). The role of sialidase in pathogenesis has been extensively reviewed elsewhere (4, 18, 19). Sialidase activity can be measured by release of *N*-acetylneuraminic acid from various biological substrates as detected by thiobarbituric acid assay and by the release of radio-labeled *N*-acetylneuraminic acid from gangliosides (22, 23) or other substrates followed by chromatographic separation of substrate and products. Simpler spectrophotometric assays are available which use various phenylketosides of *N*-acetylneuraminic acid as substrates; the liberated phenols are then measured (10, 15, 17, 24). Potier et al. (17) have described a more sensitive fluorometric sialidase assay which uses 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MUN) as substrate. Upon hydrolysis of MUN by sialidase, free *N*-acetylneuraminic acid and 4-methylumbelliferone are formed with a shift in the fluorescence spectra (excitation maximum/fluorescence maximum) from 315/374 nm (substrate) to 365/450 nm (product). Enzyme activity is than measured by fluorescence of 4-methylumbelliferone at 450 nm. This assay is now considered the most sensitive and specific assay for sialidase (20). 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. We therefore developed a rapid filter paper spot test, using MUN.

Actinomyces species are known to produce sialidase (3). We therefore used these organisms to test and define the spot sialidase assay. Reference organisms were obtained from the American Type Culture Collection: *Actinomyces bovis* ATCC 19009, *A. humiferus* ATCC 25174, *A. israelii* ATCC 12103, *A. meyeri* ATCC 33569, *A. naeslundii* ATCC 12104, *A. odontolyticus* ATCC 17982, and *A. suis* ATCC 27412. Human clinical isolates were obtained from our studies of the microflora of periodontal diseases, from B. L. Williams, University of Washington, Seattle, and from P. E. Kolenbrander, National Institute of Dental Research, Bethesda, Md. Organisms were maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) at 37°C in anaerobe jars (Oxoid, Ltd., London, England), except for *A. humiferus* which was cultured at 26°C aerobically on tryptic soy agar (Difco).

Stock solutions of MUN (Sigma Chemical Co., St. Louis, Mo.) were prepared in distilled water at a concentration of 110 mM and stored frozen in 180-μl aliquots. For use, an aliquot was thawed, and 20 μl of a 1.0 M sodium acetate buffer (pH 4.6) was added, mixed, and used to soak filter paper strips (approximately 90 by 6 mm; Whatman no. 2; Whatman, Inc., Clifton, N.J.). One or two isolated bacterial colonies were smeared onto the filter papers, which were held at 37°C in a plastic petri dish. Samples were observed under a long-wavelength, hand-held, mineral lamp after 5 and 15 min. Sialidase activity was observed as a bright blue fluorescence. Observable fluorescence at 5 min was scored as a positive reaction. A positive reaction at 15 min but not at 5 min was recorded as a weak positive. No special precautions were taken to prevent drying of the filters during the short incubation periods; however, when samples were incubated for ≥1 h, a water-soaked cotton ball was placed in the petri dish to prevent drying.

For determination of specific activity, cells were removed from the surface of agar plates, washed three times in sterile distilled water, suspended in distilled water to a density of 200 Klett units, and held on ice until assayed. Sialidase activity with MUN was determined as described by Potier et al. (17) with a FOCI manual spectrophotometer (Farrand Optical Co., New York, N.Y.), using excitation light at 365 nm and measuring the emission of 450 nm. 4-Methylumbel-

* Corresponding author.

TABLE 1. Comparison of spot sialidase assay with conventional assays

Bacterium	Spot test[a]	Sialidase activity (nmol released/min per mg of cell protein), using	
		MUN	BSM
A. humiferus ATCC 25174	−	0.9	ND[b]
A. israelii ATCC 12103	−	0.9	ND
A. israelii X523	−	1.7	2.4
A. suis ATCC 27412	−	0.9	0.1
A. meyeri ATCC 33569	w+	8.9	ND
A. naeslundii ATCC 12104	w+	3.9	ND
A. naeslundii X735	w+	2.7	2.7
A. bovis ATCC 19009	+	20.2	33.0
A. odontolyticus ATCC 17982	+	23.0	22.0
A. odontolyticus W2998	+	11.5	ND
S. aureus	−	0.0	0.0
E. coli	−	0.0	0.0

[a] −, No reaction detected after 15 min; +, positive reaction observable after 5 min; w+, positive reaction observable after 15 min but not after 5 min.
[b] ND, No determination made.

liferone was used as a standard. The release of sialic acid from bovine submaxillary mucin (BSM; Sigma) was measured by the periodic acid-thiobarbituric acid assay method of Aminoff (1) as modified by Skoza and Mohos (21), using authentic sialic acid (Sigma) as standard. Thin-layer chromatography was used to confirm the release of sialic acid from all substrates as described previously (6). Protein concentrations were determined by the method of Lowry et al. (9). Average values of duplicate assays are reported. Organisms were assayed on two separate occasions, but only the results from one series of experiments are presented.

The test organisms were characterized as negative, weak positive, or positive by the spot sialidase test (Table 1). Organisms characterized as negative had between 0 and 1.74 U of enzyme activity, using MUN as the substrate in the quantitative assay. *A. humiferus*, *A. israelii*, and *A. suis* gave negative reactions in the spot sialidase test but had low but measurable activity, ranging from 0.90 to 1.74 U, in the quantitative MUN assays (Table 1). When these organisms were incubated for 30 min in the spot sialidase test, a positive reaction was observed, whereas organisms which demonstrated no activity in the MUN quantitative assay (*Escherichia coli* and *Staphylococcus aureus*) did not result in visible blue fluorescence even when incubated for up to 3 h. Thus, the spot test could differentiate truly negative organisms from very weak sialidase-producing microorganisms. Organisms characterized as weak positives possessed between 2.7 and 8.9 U of enzyme activity, and positive organisms had >10 U of activity. To confirm the enzyme activity, the specific activity was measured, using BSM as substrate. Specific activities for *A. israelii*, *A. naeslundii*, and *A. odontolyticus* were comparable to those observed with a MUN substrate (Table 1); however, the *A. bovis* enzyme appeared to be more active against BSM than MUN. The release of sialic acid from BSM (as a result of exposure to the bacteria) was confirmed by thin-layer chromatography and by examining the absorption spectra of the reaction products in the periodic acid-thiobarbituric acid assay (data not shown).

To determine whether the spot sialidase test is useful in differentiating species of *Actinomyces*, we examined 71 clinical isolates of *Actinomyces* species for the presence of this characteristic (Table 2). Overall, 79% of these isolates were positive. With the exception of those isolates listed in Table 1, no clinical isolates were characterized as weak positives. This is probably a reflection of decreased sialidase production upon extensive subculture (4). Although this test does not appear to differentiate species within this genus, these data suggest that a negative test would rule out *A. viscosus*. A positive sialidase test may be useful for differentiating *Actinomyces* species from other filamentous or diphtheroidal organisms. For example, morphological differentiation of *A. israelii* from *Eubacterium nodatum* may be very difficult (5). In our preliminary studies, sialidase activity was not detected in three *Propionibacterium* isolates or nine clinical isolates of *Eubacterium* species, including three *E. nodatum* isolates. Additional studies will be required to confirm this trend in these groups of organisms.

TABLE 2. Detection of sialidase activity in clinical isolates of *Actinomyces* species

Species	No. of isolates tested	No. positive[a]	% Isolates positive
A. israelii	16	10	63
A. meyeri	11	8	73
A. naeslundii	13	11	85
A. odontolyticus	15	11	73
A. viscosus	16	16	100

[a] Organisms were tested for sialidase activity with the spot sialidase test as described in the text.

The reagents used in the spot test are stable, up to 6 months in our hands, if stored in distilled water at −20°C until used. Potier et al. (17) reported the slow hydrolysis of MUN under the acidic conditions of the assay (pH 4.6); however, we have observed that the substrate is suitable for use in the spot test for up to 6 h even at pH 4.6 if held at 4°C. The reagents are also quite inexpensive, and a single strip could be used to test up to 10 or 12 isolates; therefore, the cost is only a few cents per test. This test is quicker than conventional assays, resulting in a considerable saving of time. Further studies will be required to determine whether or not the spot sialidase test will be useful for identification of organisms from clinical material. We have used the spot test in our identification scheme for black-pigmented saccharolytic and nonsaccharolytic *Bacteroides* species and find it a very useful differential test (B. J. Moncla and P. Braham, submitted for publication).

Recently, numerous 4-methylumbelliferyl derivatives have become available for use in the assay of specific enzymes (*N*-acetyl-β-D-glucosaminidase, α-D-galactosidase, β-D-galactosidase, α-glucosidase, β-D-glucuronidase, phosphatases, lysozyme, α- and β-trypsin, α-chymotrypsin, β-xylosidase, and α-L-fucosidase). Many of these enzymes have proven valuable in the identification of many different groups of bacteria and are included as part of the various API rapid identification systems. Since the use of 4-methylumbelliferyl derivatives has now been shown for both sialidase and chitinase filter paper spot assays (13), it seems likely that they could be used in rapid spot tests for these other enzymes as well.

A rapid filter paper spot test for the detection of sialidase activity was developed. The method is rapid (<15 min), inexpensive, and easy to prepare and use. The method can discriminate the relative amounts of enzyme activity, using one or two isolated bacterial colonies. The results suggest that similar tests for other enzymes are possible with other derivatives of 4-methylumbelliferone.

This work was supported by Public Health Service grant DE 02600 from the National Institutes of Health.

We thank M. B. Coyle and S. L. Hillier for help in reviewing the manuscript.

LITERATURE CITED

1. **Aminoff, D.** 1961. Methods for quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **193:**165–275.
2. **Brown, J. G., and D. C. Straus.** 1978. Characterization of neuraminidases produced by various serotypes of group B streptococci. Infect. Immun. **55:**1–6.
3. **Costello, A. H., J. O. Cisar, P. E. Kolenbrander, and O. Gabriel.** 1979. Neuraminidase-dependent hemagglutination of human erythrocytes by human strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. Infect. Immun. **26:**563–572.
4. **Drzeniek, R.** 1972. Viral and bacterial neuraminidases. Curr. Top. Microbiol. Immunol. **59:**35–74.
5. **Hill, G. B., O. M. Ayers, and A. P. Kohan.** 1987. Characteristics and sites of infection of *Eubacterium nodatum*, *Eubacterium timidum*, *Eubacterium brachy*, and other asaccharolytic eubacteria. J. Clin. Microbiol. **25:**1540–1545.
6. **Jones, A. H., C. C. Lee, B. J. Moncla, M. R. Robinovitch, and D. C. Birdsell.** 1986. Surface localization of sialic acid on *Actinomyces viscosis*. J. Gen. Microbiol. **132:**3381–3391.
7. **Kelly, R. T., S. Farmer, and D. Greiff.** 1967. Neuraminidase activities of clinical isolates of *Diplococcus pneumoniae*. J. Bacteriol. **94:**272–273.
8. **Leprate, R., and Y. Michel-Briand.** 1980. Extracellular neuraminidase production by a strain of *Pseudomonas aeruginosa* isolated from cystic fibrosis. Ann. Microbiol. (Paris) **131:**209–222.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
10. **Meindl, P., and H. Tuppy.** 1967. Synthetic ketosides of N-acetylneuraminic acid. Monatsh. Chem. **98:**53–60.
11. **Muller, H. E.** 1971. Untersuchen *in vivo* uber die Neuraminidase der *Pasturella multocida*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **217:**326–344.
12. **Nees, S., and R. Schauer.** 1974. Induction of neuraminidase from *Clostridium perfringens* and the cooperation of this enzyme with acetylneuraminate pyruvate-lyase. Behring Inst. Mitt. **55:** 68–78.
13. **O'Brien, M., and R. R. Colwell.** 1987. A rapid test for chitinase activity that uses 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide. Appl. Environ. Microbiol. **53:**1718–1720.
14. **O'Toole, R. D., L. Goode, and C. Howe.** 1971. Neuraminidase activity in bacterial meningitis. J. Clin. Invest. **50:**979–985.
15. **Palese, P., P. Bucher, and E. D. Kilbourne.** 1973. Application of a synthetic neuraminidase substrate. Appl. Microbiol. **25:**195–201.
16. **Pardoe, G. I.** 1970. The inducible neuraminidase CN-acylneuraminyl hydrolase (EC 3.2.18) of *Klebsiella aerogenes* NCID9479. Pathol. Microbiol. **35:**361–376.
17. **Potier, M., L. Mameli, M. Belisle, L. Dallaire, and S. B. Melancon.** 1979. Fluorometric assay of neuraminidase with sodium (4-methylumbelliferyl-α-D-N-acetylneuraminate) substrate. Anal. Biochem. **94:**287–296.
18. **Reutter, W., E. Kottgen, C. Bauer, and W. Gerok.** 1982. Biological significance of sialic acids, p. 263–305. *In* R. Schauer (ed.), Sialic acids. Chemistry, metabolism and function. Cell biology monographs, vol. 10. Springer-Verlag, New York.
19. **Rosenberg, A., and C.-L. Schengrund.** 1976. Sialidases, p. 295–359. *In* A. Rosenberg and C.-L. Schengrund (ed.), Biological roles of sialic acids. Plenum Publishing Corp., New York.
20. **Schauer, R.** 1982. Chemistry, metabolism, and biological functions of sialic acids. Adv. Carbohyd. Chem. Biochem. **40:**131–234.
21. **Skoza, L., and S. Mohos.** 1976. Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Biochem. J. **159:**457–462.
22. **Tallman, J. F., and R. O. Brady.** 1973. The purification and properties of a mammalian neuraminidase (sialidase). Biochim. Biophys. Acta **293:**434–443.
23. **Tallman, J. F., P. H. Fishman, and R. C. Henneberry.** 1977. Determination of sialidase activities in HeLa cells using gangliosides specifically labeled in N-acetylneuraminic acid. Arch. Biochem. Biophys. **182:**556–562.
24. **Tuppy, H., and P. Palese.** 1969. A chromogenic substrate for the investigation of neuraminidases. FEBS Lett. **3:**72–75.