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Rapid Identification of *Branhamella catarrhalis* with 4-Methylumbelliferyl Butyrate

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***Branhamella catarrhalis* can be distinguished from *Neisseria* spp. by the presence of butyrate esterase. This enzyme can be rapidly detected when 4-methylumbelliferyl butyrate is used as the substrate. All *B. catarrhalis* strains tested gave a positive fluorescence reaction within 5 min, while *Neisseria* spp. remained negative, even after 18 h of incubation.**

Branhamella catarrhalis can be distinguished from *Neisseria* spp. by its ability to hydrolyze ester-linked butyrate groups (1). The activity of the responsible enzyme, butyrate esterase, is detected with tributyrin as a substrate in the presence of a phenol red indicator (Rosco Diagnostica, Taastrup, Denmark) or with naphthyl butyrate (API, Montalieu-Vercieu, France).

The possibility of rapid detection of bacterial enzymes by 4-methylumbelliferyl substrates was first reported in 1975 (5). Other authors used these substrates to study the enzyme activities of mycobacteria (4), mycoplasmata (3), and yeasts (2) and for rapid isolation of *Escherichia coli* (6).

Because 4-methylumbelliferyl butyrate (MUB) (Sigma Chemical Co., St. Louis, Mo.) can serve as a substrate for butyrate esterase, its use in a rapid test for the identification of *B. catarrhalis* was obvious. Enzyme activity can be detected because the 4-methylumbelliferone moiety, released from the hydrolyzed substrate, is fluorescent when viewed at 366 nm with an intensive UV source.

A selection of 10 *B. catarrhalis* and 30 *Neisseria* strains, chosen from the strains listed in Table 1, was used to find the optimal test conditions, i.e., rapid positive reactions and maximal delay of spontaneous degradation of the substrate. *B. catarrhalis* strains were identified as gram-negative diplococci, which were oxidase, DNase, and tributyrin positive and glucose negative.

A stock solution was made by dissolving 100 mg of MUB in 10 ml of dimethyl sulfoxide (Sigma) to which 100 μl of Triton X-100 (Sigma) was added, according to the instructions of the manufacturer. The stock solution was stored at −70°C. Heavy flocculation and precipitation occurred upon further dilution in aqueous buffers when Triton X-100 was omitted. Replacing dimethyl sulfoxide by *N*,*N*-dimethylformamide (Sigma) as proposed by Bobey and Ederer (2) gave finer emulsions. The difference between positive and negative reactions, however, was more difficult to read. The influence of pH was tested with 1/25 dilutions of the stock solution in 0.1 M Tris, previously adapted to pHs 7.0, 6.0, 5.0, 4.0, and 3.0 by adding HCl. Samples (250 μl each) of the different emulsions were inoculated with 2 to 3 colonies grown on solid medium (blood agar or gonococcal culture medium) for 18 h at 37°C in 5% CO_2, and the inoculated samples were incubated at room temperature. At pHs 3.0 and 4.0, reactions were slow and often remained false-negative. At pH 7.0, the uninoculated control tube showed fluorescence within 30 min.

To find the optimal substrate dilution, the stock solution was diluted 1/10, 1/25, 1/50, and 1/100 in Tris (pH 5.0) and Tris (pH 6.0). The 1/10 dilutions gave immediate positive reactions (within 1 to 5 min) for *B. catarrhalis* strains, while the tubes inoculated with *Neisseria* strains did not fluoresce for at least 2 h when Tris (pH 5.0) was used as a diluent. The positive reaction was weaker at 1/25 dilution, while 1/50 and 1/100 dilutions did not allow distinct differentiation. The optimal combination was found to be a 1/10 dilution of the stock solution, i.e., a final concentration of 4 mM MUB in 0.1 M Tris (pH 5.0). Since the Tris buffer cannot perform at this pH, its suitability as a diluent was compared with that of 0.1 M citrate (0.1 M citric acid, 0.05 M $Na_2HPO_4 \cdot 12H_2O$) at pH 5.0. Positive reactions occurred within 5 min, while false-positive reactions, caused by the instability of the substrate after prolonged incubation, were delayed with citrate buffer for at least 18 h.

After optimal conditions for showing butyrate esterase activity with MUB as the substrate were settled, 250-μl samples of MUB diluted from the stock solution to a final concentration of 4 mM in 0.1 M citrate buffer (pH 5.0) were frozen in 5-ml glass tubes at −20 and −70°C to test for storage requirements. After thawing, the reactions of the stored samples were compared with those of freshly prepared working solutions for 10 *B. catarrhalis* and 30 *Neisseria* strains. At −70°C, solutions could be stored for at least 1 month. Storage at −20°C for longer than 14 days gave spontaneously fluorescent tubes whether a positive or a negative strain was used.

In summary, the test reagent is prepared and stored as follows. A stock solution of the substrate, made by dissolving 100 mg of MUB in 10 ml of dimethyl sulfoxide and 100 μl of Triton X-100, is diluted 1/10 in 0.1 M citrate buffer (pH 5.0). It can be stored in 250-μl portions in transparent tubes at −70°C for at least 1 month.

The MUB test, as described, was further used to test additional species from the family *Neisseriaceae*, as well as some gram-negative rods, staphylococci, and yeasts. The origins, numbers, and reaction times of the different strains are listed in Table 1. None of the *Neisseria* spp. was fluorescent within 18 h. All *B. catarrhalis* strains were positive within 5 min. Also, other genera of the *Neisseriaceae* and all non-*Neisseriaceae* except *E. coli* gave positive reactions. Therefore, it is necessary to ensure that the

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TABLE 1. Origin and number of the strains used and time needed to give a positive reaction with the MUB test

Organism	No. tested	No. giving blue fluorescence at 336 nm within:				
		5 min	15 min	30 min	120 min	>18 h
Neisseriaceae						
Acinetobacter calcoaceticus[a]	5	1	1	1	2	
Branhamella catarrhalis[a,b,c,d,e,f]	62	62				
B. caviae[b]	2	2				
B. cuniculi[b]	1					1
B. ovis[b]	2	2				
Eikenella corrodens[a]	5					5
Moraxella osloensis[a,g]	3		3			
Neisseria canis[b]	1					1
N. cinerea[c]	6					6
N. flavescens[b]	4					4
N. gonorrhoeae[a]	12					12
N. lactamica[d]	3					3
N. meningitidis[d]						
A	2					2
B	2					2
C	2					2
29E	2					2
W135	2					2
X	2					2
Y	2					2
Z	2					2
N. mucosa[a,d]	3					3
N. polysaccharea[d]	1					1
N. sicca[d]	3					3
N. subflava subsp. *perflava*[a,d]	14					14
Pasteurella multocida[a]	3					3
Various						
Escherichia coli[a]	11					11
Pseudomonas aeruginosa[a]	10	9	1			
Staphylococcus aureus[a]	10	9	1			
S. epidermidis[a]	10	4	1		5	
Candida albicans[a]	10			10		

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[b] W. Van Dyck, Tropical Institute, Antwerp, Belgium.
[c] U. Berger, Hygiene Institute of the University, Heidelberg, Federal Republic of Germany.
[d] C. Gilquin, Institute for Hygiene and Epidemiology, Brussels, Belgium.
[e] E. Falsen, Culture Collection of the University, Goteborg, Sweden.
[f] M. Denef, O.L.V. Ziekenhuis, Mechelen, Belgium.
[g] K. Kersters, Laboratory for General Microbiology, Ghent, Belgium.

strains tested are oxidase-positive, gram-negative diplococci.

The test is inexpensive when recuperable glass tubes are used, even when a negative and a positive control are included. Handling time is limited, since only thawed tubes have to be inoculated and inspected almost immediately under UV light. Only two large colonies are required for inoculation. Thus, an inexpensive, reliable, rapid test using light inocula for the differentiation between *B. catarrhalis* and *Neisseria* spp. has been established.

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