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Nonliquid Reagent for Detecting Nitrate Reduction

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A nonliquid reagent for detecting nitrate reduction is described. The reagent contains *N*-(1-naphthyl)ethylenediamine dihydrochloride as a substitute for the carcinogenic α-naphthylamine. The reagent was tested on 135 strains and gave reliable results.

Many bacteria can produce nitrite by the reduction of nitrate, a characteristic that may be helpful for the identification of clinically isolated organisms. The Griess-Ilosvay method is usually employed to detect nitrite: in an acidic solution, nitrite ions are converted to nitrosonium ions; a diazotizing reagent (sulfanilic acid) is added to form a diazonium ion, and this diazonium ion binds with a substituted naphthyl compound to produce an azo compound, which is observable as a magenta dye (1, 6). Two solutions, reagents A and B, are usually employed for this reaction. Reagent A contains sulfanilic acid in acetic acid. Reagent B contains a naphthyl compound in acetic acid. In the past, the naphthyl compound most widely used in reagent B was α-naphthylamine (1). However, the U.S. Occupational Safety and Health Administration (8) has listed α-naphthylamine as a carcinogen, and for this reason, many laboratory manuals for microbiology now suggest using *NN*-dimethyl-1-naphthylamine as an alternative (1-3, 7). All of these reagents should have a regular quality control check and should be discarded when they demonstrate a negative or weak reaction with a known positive organism (5).

Griess-Romijn reagent has been used for many years in our laboratory, but acetic acid has been substituted for *l*-tartaric acid in this powder. The complete composition ratio is (wt/wt) *l*-tartaric acid, 90; sulfanilic acid, 10; and α-naphthylamine, 1 (4). With a spatula, a very small amount of reagent is added to a nitrate broth culture. The advantages of using this reagent in powder form are: (i) it is very stable and can be stored in a dark glass container at room temperature for at least 1 year; and (ii) only one reagent is needed.

Substitution of α-naphthylamine in the powder reagent is the subject of this investigation. Since *N*,*N*-dimethyl-1-naphthylamine is an oily substance, it is inappropriate for use as a substitute. *N*-(1-naphthyl)ethylenediamine dihydrochloride is one of the alternate compounds suggested by the Committee on Laboratory Standards and Practices of the American Pubic Health Association and is crystalline. Therefore, this chemical was chosen for the experiments.

Various compositions of *l*-tartaric acid, sulfanilic acid, and *N*-(1-naphthyl)ethylenediamine dihydrochloride (E. Merck AG, Darmstadt, Germany) were tested on five known positive and five known negative strains. The composition ratio of the mixture that gave the best results, "mixture 4," was (wt/wt): *l*-tartaric acid, 10; sulfanilic acid, 1; and *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1. To determine the sensitivity of mixture 4 in comparison with reagents A plus B (B with *N*,*N*-dimethyl-1-naphthylamine), both were added to various dilutions of $NaNO_2$ in distilled water. The comparison showed a very slight difference in favor of mixture 4. To test the properties of mixture 4 when used on a more extensive collection of species and strains, 125 strains (41 taxa) were tested with mixture 4 and reagents A plus B. The tests were done when the nitrate broth showed growth, usually after 24 h. Zinc dust was added to each tube that showed a negative reaction. The results for reagents A plus B and mixture 4 were in agreement with the data in the literature (Table 1). A difference between reagents A plus B and mixture 4 was seen for only one strain, *Alcaligenes faecalis* (clinical isolate), which gave a weak reaction with reagents A plus B and a negative one with mixture 4. The test with zinc dust was negative for both reagents for the five strains of *Pseudomonas aeruginosa*. These strains may have reduced nitrate to other products or may have converted the formed nitrate to another nitrogen compound. In every other case, the test was positive. The character of the color obtained with the reagents differed. In the tubes with reagents A plus B, the fluid stained purple-red and remained so for hours. In the tubes with mixture 4, the purple compound usu-

TABLE 1. *Comparison of tests with reagents A plus B and tests with mixture 4 (125 strains)*

Organism (total no. of strains)	No. of clinical isolates	Source of non-clinical isolate	No. of strains with reaction combination:[a]				Reaction reported in literature[b]
			++	WW	W−	−−	
Achromobacter xylosoxidans (1)		G. L. Gilardi	1				Positive
Acinetobacter calcoaceticus (10)	9	G. L. Gilardi				10	Negative
Alcaligenes faecalis (2)	1	NCTC 415			1	1	Variable
Bacillus subtilis (1)		NCTC 8236	1				Positive
Bacteroids fragilis (3)	2	NCTC 9344				3	Negative
Bordetella bronchiseptica (1)		NCTC 452				1	Variable
Branhamella catarrhalis (3)	3		3				Variable
Campylobacter fetus subsp. *jejuni* (3)	3		3				Positive
Citrobacter species (6)	6		6				Positive
Clostridium difficile (1)		NCTC 11223				1	Negative
Clostridium perfringens (3)	3		2	1			Positive
Eikenella corrodens (3)	3		3				Positive
Enterobacter cloacae (3)	2	ATCC 23355	3				Positive
Escherichia coli (3)	3		3				Positive
Flavobacterium meningosepticum (1)		G. L. Gilardi				1	Negative
Fusobacterium necrophorum (1)		NCTC 10575				1	Negative
Klebsiella pneumoniae (3)	2	ATCC 13883	3				Positive
Micrococcus luteus (1)		ATCC 9341				1	Negative
Moraxella osloensis (1)		NCTC 10465				1	Variable
Mycobacterium chelonei (2)	2					2	Negative
Mycobacterium gordonae (2)	2					2	Negative
Mycobacterium kansasii (1)	1		1				Positive
Mycobacterium tuberculosis (3)	3		3				Positive
Propionibacterium acnes (3)	3		3				Variable
Proteus species (6)	5	ATCC 13315	6				Positive
Pasteurella multocida (3)	3		3				Positive
Pseudomonas acidovorans (2)	1	ATCC 15668	1	1			Variable
Pseudomonas aeruginosa (6)	5	ATCC 27853		1		5[c]	Variable
Pseudomonas cepacia (5)	4	NCTC 10661	4			1	Variable
Pseudomonas fluorescens (5)	4	ATCC 13525				5	Variable
Pseudomonas maltophilia (6)	5	ATCC 13637	4			2	Variable
Pseudomonas putida (5)	5					5	Negative
Pseudomonas putrefaciens (3)	2	G. L. Gilardi	3				Positive
Pseudomonas stutzeri (2)	1	NCTC 10475				2	Variable
Salmonella species (3)	2	ATCC 14028	3				Positive
Serratia marcescens (3)	3		3				Positive
Shigella species (3)	3		3				Positive
Staphylococcus aureus (3)	3		3				Positive
Staphylococcus epidermidis (5)	5		4			1	Variable
Streptococcus pyogenes (3)	3					3	Negative
Yersinia enterocolitica (1)	1		1				Positive

[a] ++, Positive with A plus B and mixture 4; WW, weak with A + B and mixture 4; W−, weak with A + B, negative with mixture 4; −−, negative with A plus B and mixture 4.

[b] Based on *Cowan and Steel's Manual* (2) and Gilardi's *Glucose Nonfermenting Gram-Negative Bacteria in Clinical Microbiology* (3).

[c] Reaction with zinc negative.

ally sank to the bottom within half an hour, leaving an orange-red supernatant fluid with a purple precipitate at the bottom. When zinc dust was added to the tubes with mixture 4, the color reaction developed rapidly, starting at the bottom and spreading upwards. In the tubes with reagents A and B, there was a more gradual development of color from pink through purple, distributed evenly throughout the contents.

The results show that mixture 4 is a reliable reagent. The slight difference seen in one strain of *Alcaligenes faecalis* seems unessential because for this subspecies, nitrate reduction is stated in the literature as variable. Miller and Neville (6) evaluated four naphthyl compounds as replacements for α-naphthylamine in the liquid reagent. They found no difference in sensitivity between *N*,*N*-dimethyl-1-naphthylamine and *N*-(1-naphthyl)ethylenediamine dihydrochloride. Nevertheless, they preferred to use *N*,*N*-1-dimethyl-1-naphthylamine because the solution with *N*-(1-naphthyl)ethylenediamine dihydrochloride turned dark brown after 60 days of storage. Our preparation of this reagent in powder form remained colorless for at least 6 months. It is possible that the liquid form of this reagent is less stable.

We conclude that mixture 4 is a useful reagent for the detection of nitrate reduction. The reagent showed at least the same sensitivity as the

commonly used liquid reagents. Stored in a dark glass container at room temperature, it will be stable for at least 6 months. It has the advantage of requiring only one reagent. Although the naphthyl compound used is not listed as a carcinogen, it seems prudent to use the reagent with care.

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