Simple Disk Technique for Detection of Nitrate Reduction by Anaerobic Bacteria

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The laboratory and clinical evaluation of a potassium nitrate-saturated disk for the rapid detection of nitrate reductase production in anaerobes was investigated. The optimal disk concentration and incubation time were determined by utilizing triplicate sets of quadrant plates prepared with supplemented brucella (Difco) blood agar and swabbed with a 24-h broth (BBL; 135 C thioglycolate) suspension of the test organism. Each set of plates received one control disk and three disks of varying concentrations of potassium nitrate (1 to 8 mg) with 0.1% sodium molybdate. All sets were incubated in GasPak jars for 24, 48, or 72 h, and subsequently sulfanilic acid and 1,6-Cleve's acid were added to each disk. A pink or red color change was indicative of nitrate reductase production. Eighty-eight stock isolates, 23 American Type Culture Collection strains, and 214 fresh clinical isolates were evaluated and compared with results obtained with tubes of preduced indole-nitrite medium (BBL) incubated for 7 to 10 days. The 6-mg disk incubated for 48 h yielded an overall agreement of 89% with the conventional tube technique, and fresh clinical isolates demonstrated better disk-tube agreement (93%) than previously frozen stock strains. The simplicity and ease of this disk test suggest its value as a preliminary screening procedure for nitrate reductase production. There were no false positives. Negative results by disk should be rechecked by tube.

An article by ZoBell in the *Journal of Bacteriology* in 1932 began, "Although the ability to reduce nitrates has been used for the identification of bacteria since the extensive investigations of Maassen (1902), the literature is still replete with inconsistencies and contradictory findings" (18). Forty-four years later, this introduction to the discussion of nitrate reduction remains as appropriate as ever.

In most all reports in which a species is described, a statement is given as to whether or not it reduces nitrate. Most manuals of determinative bacteriology merely make a categorical statement, "nitrates reduced" or "nitrates not reduced," as if it were a simple, reliable determination (1). The end product possibilities of nitrate reduction are many: nitrate (NO_3), ammonia (NH_3), molecular nitrogen (N_2), nitric oxide (NO), nitrous oxide (N_2O), and hydroxylamine (R-NH-OH), just to mention a few (4, 9, 13). Some organisms reduce nitrate to nitrite only, whereas others are capable of further reduction. The reduction may be so rapid that nitrite does not accumulate. Further, assimilation of the nitrite by the bacteria may occur. Undoubtedly, many bacteria convert ni-

trate directly to cell nitrogen, whereas others reduce nitrate to nitrite as a preliminary step in its utilization.

Certainly there is no simple procedure by which the ability of an organism to reduce nitrate can be categorically determined. However, the nitrate reduction disk test described in this paper is simple and inexpensive, and it yields results consistent with the more complex tube tests.

MATERIALS AND METHODS

Disk preparation. The nitrate disks were prepared by using 0.25-inch (ca. 0.64-cm) sterile blank disks (Schleicher & Schuell, Keene, N.H.). Five, twenty, thirty, and forty percent solutions of potassium nitrate were prepared in aqueous 0.1% sodium molybdate and filter sterilized. Twenty-microliter aliquots of each of these solutions were dispensed to the sterile disks to yield 1-, 4-, 6-, and 8-mg disks. The disks were dried and stored at room temperature.

To determine the stability of the disks, freshly prepared disks were tested with control organisms and subsequently retested after 8 months of storage at room temperature, using freshly prepared disks as controls.

Test organisms. Twenty-three American Type Culture Collection (ATCC) strains of anaerobic bacteria, 88 stock cultures of well-characterized anaerobic bacteria isolated from human normal flora studies, and 214 strains of anaerobic bacteria recently isolated from clinical material, for a total of 325 strains, were included in the study.

Media. Three types of liquid media were utilized. Stock cultures were maintained in fluid thioglycolate medium (135 C; BBL, Division of Bioquest, Cockeysville, Md.) supplemented with hemin (5 μ g/ml), vitamin K₁ (0.1 μ g/ml), a marble chip, and Fildes enrichment (5%, vol/vol; BBL). Clinical isolates were also maintained in semisolid Gifu anaerobic medium (Nissui) (GAM). For comparative nitrate reduction tests, indole-nitrite medium (BBL) supplemented with yeast extract (0.2%, vol/vol), hemin (5 μ g/ml), and vitamin K₁ (10 μ g/ml) was prepared under anaerobic conditions, dispensed in 5-ml aliquots into stoppered tubes, and then autoclaved.

Dehydrated brucella agar (Difco Laboratories, Detroit, Mich.) was prepared according to the directions of the manufacturer and supplemented with hemin (5 μ g/ml) before autoclaving and with vitamin K₁ (10 μ g/ml) and 5% sheep blood after autoclaving. Plates were not reduced, and no specific attempt was made to use freshly poured media. Blood agar plates were stored in the refrigerator for up to 1 week prior to use.

Test reagents. Sulfanilic acid and 1,6-Cleve's acid (5-amino-2-naphthalenesulfonic acid) were used for both the disk and broth nitrate reduction determinations. Sulfanilic acid was prepared using 0.5 g of sulfanilic acid, 30 ml of glacial acetic acid, and 120 ml of distilled water. The 1,6-Cleve's acid was prepared using 0.2 g of 1,6-Cleve's acid, 30 ml of glacial acetic acid, and 120 ml of distilled water.

Procedure. The optimal disk concentration and incubation time were determined by utilizing triplicate sets of quadrant plates prepared with supplemented brucella blood agar and swabbed with a 24-h broth suspension of a stock culture. Each set of plates received one control disk and three disks of varying concentrations of potassium nitrate, from 1 to 8 mg each with 20 μ g of sodium molybdate. All sets were incubated in GasPak jars for 24, 48, and 72 h, after which 1 drop each of sulfanilic acid and 1,6-Cleve's acid was added to the disks. A pink or red color change was interpreted as positive and indicative of microbial reduction of nitrate to nitrite. Disks exhibiting no color change after 3 to 5 min were investigated for the presence of unreduced nitrate by sprinkling a small amount of zinc dust on the disk. The development of a red color was interpreted as a negative reaction. No color change was presumptive evidence for reduction beyond nitrite.

After the preliminary screening procedure, all test organisms were grown in supplemented thioglycolate or GAM for 18 to 24 h to achieve a 3+ or better turbidity (4+ equals maximum turbidity achievable) and then evenly distributed on the brucella blood agar plates by swabbing, one organism per plate. A nitrate disk was placed on each plate, and the plates were incubated in GasPak jars and subsequently tested for nitrate reduction as described

above. Concomitantly, a tube of indole-nitrite medium was inoculated with 2 to 4 drops of the fresh broth suspension and incubated. All indole-nitrite medium cultures were routinely incubated for 5 to 7 days at 35°C before testing for nitrate reduction by adding 0.2 ml of each test reagent to the culture. In addition, 211 of the 325 strains were also incubated for a 48-h period prior to testing for reduction to compare results of the disk and tube methods with equivalent incubation periods. Again, negative tests were confirmed with the addition of a small amount of zinc dust.

RESULTS

Preliminary screening of the various nitrate disk concentrations against known controls of nitrate-reducing organisms revealed the 6-mg potassium nitrate disk with sodium molybdate to be the optimum disk concentration and a 48-h incubation period to be sufficient. With a positive nitrate reduction test, the 6-mg disk yielded darker color development in a shorter length of time than the less concentrated disks. The 8-mg disk yielded results comparable to the 6-mg disk; however, preparation of this disk necessitated preparing a supersaturated solution of potassium nitrate. Since results were not superior to the 6-mg disk, the 8-mg disk was eliminated after preliminary evaluation.

Comparative indole-nitrite medium incubation periods of 48 h and 5 to 7 days were evaluated. The two incubation periods yielded 99% agreement. Therefore, further examination of the 48-h cultures was discontinued, because we were attempting to compare our routine procedure with the simplified disk technique.

By the end of the 8th-month study period, a total of 325 anaerobes had been investigated for their ability to reduce nitrate (Table 1). Results of the 6-mg nitrate disk tests have been com-

TABLE 1. Nitrate reductase test organisms

Genus	No. of isolates		
	ATCC ^a	Stock	Clinical
<i>Actinomyces</i>	1	19	6
<i>Arachnia</i>	1	2	0
<i>Bacteroides</i>	2	7	66
<i>Bifidobacterium</i>	0	1	4
<i>Clostridium</i>	5	10	22
<i>Eubacterium</i>	4	25	16
<i>Fusobacterium</i>	0	0	16
<i>Lachnospira</i>	1	0	0
<i>Lactobacillus</i>	1	2	4
<i>Peptococcus</i>	5	3	30
<i>Peptostreptococcus</i>	1	1	23
<i>Propionibacterium</i>	2	4	12
<i>Streptococcus</i>	1	5	5
<i>Veillonella</i>	2	9	10

^a Isolate source.

pared with those obtained with the indole-nitrite medium incubated for 5 to 7 days (Table 2). Eighteen of the 24 ATCC strains demonstrated similar results with the disk and tube tests. Among the 88 stock cultures investigated, 71 organisms showed similar results, and 199 of the 214 fresh clinical isolates gave the same reactions with the disk and tube methods. Overall, 288 of 325 organisms tested for nitrate reductase gave the same results with both tests. This represents an 89% test agreement.

Of the 37 discrepant test results, 7 were too variable to classify (i.e., repeated tests gave inconsistent results). The remaining 30 discrepant test reactions and organisms have been grouped in Table 3. Five organisms had posi-

tive disk tests and negative tube tests. Four organisms had negative disk tests and positive tube tests. Twelve organisms showed no reaction (i.e., the disk remained colorless after the zinc was added) or gave variable results versus a positive tube test. Finally, nine organisms showed no reaction or variable reactions with the disk test versus a negative tube test.

There were no false positive disk tests, and comparative tests at the beginning and end of the study established that the nitrate disks are stable for at least 8 months when stored at room temperature.

DISCUSSION

The 6-mg potassium nitrate disk with sodium molybdate investigated in this study for the detection of nitrate reductase production was shown to be comparable (89% test agreement) to the more conventional and time-consuming indole-nitrite medium assay.

Comparative indole-nitrite medium incubation periods were evaluated for 48 h, which is the same incubation period as for the disk, and 5 to 7 days, which is the routine incubation period for all clinical isolates processed at the Wadsworth Anaerobic Bacteriology Laboratory. A 99% agreement resulted between the two incubation periods. However, because we are comparing our routine nitrate reduction test with the disk test, only the 5- to 7-day broth incubation period results are discussed.

This disk test is very easy to perform and read; however, a few points should be mentioned concerning the interpretation of results. A rapidly growing organism may turn the disk a tan color during the 48-h incubation period as a result of hemolysis and/or metabolism. When the test reagents are then added, occasionally only a very subtle color change will be discernible or no color change will occur at all, even with the addition of zinc dust. When this occurs, a tube test or some other means of nitrate reduction evaluation is suggested. Nearly two-thirds of the test discrepancies in Table 3 fell into this category.

Second, the quantity of nitrate reductase formed is directly related to the rate of growth of the test organism (13). Only fresh cultures should be used to swab the plates and, if after a 48-h incubation period little or no growth is observed, the plates should be reincubated before the test reagents are added. Not only are young cultures necessary for this assay, but recent isolates, yet to be frozen, demonstrate better test agreement between the disk and tube tests than previously frozen cultures (93 versus 80%). This is evident in the comparative test results between clinical isolates and ATCC

TABLE 2. Nitrate reductase tests: comparative results

Isolate source	No. of strains tested	Disk-tube agreement (no. of strains)	% Agreement
ATCC	23	18	78
Stock	88	71	81
Clinical	214	199	93

TABLE 3. Nitrate reductase tests: discrepant test reactions and organisms

Test reaction	Organisms (no.)
Disk (+)	<i>Bacteroides corrodens</i> (1)
Tube (-)	<i>Bacteroides</i> sp. (1) <i>Eubacterium</i> sp. (1) <i>Peptococcus prevotii</i> (2)
Disk (-)	<i>Actinomyces viscosus</i> (1)
Tube (+)	<i>B. corrodens</i> (1) <i>Clostridium paraputrificum</i> (1) <i>C. perfringens</i> (1)
Disk (NR/V) ^a	<i>A. israelii</i> (1)
Tube (+)	<i>A. viscosus</i> (1) <i>B. corrodens</i> (1) <i>B. ruminicola</i> subsp. <i>brevis</i> (1) <i>C. perfringens</i> (1) <i>E. lentum</i> (1) <i>E. moniliforme</i> (1) <i>E. nitritogenes</i> (1) <i>Propionibacterium acnes</i> (1) <i>Veillonella parvula</i> (3)
Disk (NR/V)	<i>A. israelii</i> (1)
Tube (-)	<i>C. perfringens</i> (3) <i>C. sordellii</i> (1) <i>Clostridium</i> sp. (1) <i>P. prevotii</i> (2) <i>P. acnes</i> (1)

^a NR, No reaction; no color change before or after zinc dust added. V, Variable reactions.

and stock isolates shown in Table 2.

Nitrate reductases are reported to be particle bound (13). However, *Spirillum itersonii* (5) and one strain of *Propionibacterium acnes* investigated in this study exhibited a readily diffusible enzyme. Therefore, only one organism per plate should be tested unless sectioned plates are used.

Molybdenum has been identified as the metal component of the enzyme nitrate reductase (4, 8-11). As determined by activation analysis, this enzyme in *Escherichia coli* contains 4 mol of molybdenum per mol of enzyme (8). In *Neurospora*, increased nitrate reductase activity in various protein fractions was accompanied by an increased molybdenum concentration, and of all the micronutrient element deficiencies investigated, only molybdenum deficiency resulted in decreased nitrate reductase activity (11). Also, the addition of molybdate to media has been shown to stimulate synthesis of nitrate reductase (3). However, tungsten and vanadium are competitive inhibitors of molybdenum utilization in plants and bacteria (6, 7, 12, 17), and as a result they are potent inhibitors of the *in vivo* formation of nitrate reductase. These inhibitory effects can be prevented entirely by the addition of molybdate (13).

One possible disadvantage of incorporating molybdate in the nitrate disk is that it has been shown to inhibit the production of active nitrate reductase by *Veillonella parvula*. At concentrations of 5 μ g of molybdate per g, greater than 50% of the activity of the enzyme was inhibited in one study (2). Subsequent increases in the molybdate concentration produced some recovery in enzyme activity, but the final level was still less than half that found in the absence of the metal. Eighteen of the 325 isolates evaluated in this study were *V. parvula*. Twelve isolates demonstrated nitrate reduction by the disk and the tube methods. Three isolates did not reduce nitrate by either method, and three showed variable reactions with the disk test compared with positive tube tests. This represents a 15% test discrepancy among the *V. parvula* isolates, which is just slightly greater than the overall test discrepancy of 11%. Certainly, the significance of the incorporation of molybdate in the disk for the reasons mentioned above offsets this decrease in test sensitivity.

The reduction of nitrate to nitrite via nitrate reductase is a pH-dependent reaction (15), with optimal reduction occurring at pH 7.0 and higher depending on the organism being investigated (14). The brucella blood agar plates utilized in this experiment contain 0.1% dextrose. They have been monitored in an anaerobic at-

mosphere both with and without metabolizing organisms and have consistently shown a final pH range of 6.5 to 6.8 (unpublished data, Wadsworth Anaerobe Laboratory). Whereas this pH range is slightly lower than described as optimal (14), nitrate reduction does not appear to be inhibited significantly.

This simple nitrate reduction disk test has a practical application for the routine clinical laboratory that is endeavoring to get more involved with the isolation and identification of anaerobes. Compared with conventional nitrate tests, this test obviates the need for prereduced media, special gassing, and inoculation devices, and the nitrate disk is tested directly on a blood agar plate as opposed to removing a sample of the broth for the conventional nitrate test. The nitrate disk can be used effectively and efficiently at either of two points in a preliminary identification scheme. It can be placed on a purity plate at the time of initial isolation and, after a 48-h incubation period, nitrate reduction can be evaluated after all appropriate subcultures from that plate have been made. Preferably, however, if the antibiotic disk identification procedure described by Sutter et al. (16) is being used for the preliminary grouping of anaerobes, the nitrate disk can be added to that regimen.

Nitrate reduction can now be added to the ever increasing list of rapid, simple diagnostic tests that are slowly bringing the field of clinical anaerobic bacteriology within the reach of almost every hospital and clinical laboratory facility.

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