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Rapid, Modified Oxidase Test for Oxidase-Variable Bacterial Isolates

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A modified oxidase reagent, 1% tetramethyl-*p*-phenylenediamine in dimethyl sulfoxide, proved superior to the routinely used 1% aqueous tetramethyl-*p*-phenylenediamine dihydrochloride in detecting weakly oxidase-positive gram-negative bacteria after 24 h of growth on agar media (40 of 40 positive versus 22 of 40 positive). The bacterial inoculum was obtained with a cotton-tipped swab instead of a loop or wooden applicator, and the reaction required less than 15 s.

The oxidase reaction test is most useful for characterizing gram-negative bacteria (1). Since the reaction is affected by the pH of the growth medium (4), the age of the culture, the reagent used, and the timing of the reaction (7), standardization of the test procedure is required. The method originally described by Kovacs in 1956 is generally recommended (5). However, some species of gram-negative rods are weakly positive or give variable results with the Kovacs method (6). Some commercial oxidase reagents based on the Kovacs method have proven to be unsuitable for the genus *Pasteurella* (3). Variable results of the oxidase reaction with some strains of *Aeromonas hydrophila* and *Aeromonas punctata* were found to be caused by a pH below 5.2 in the growth medium (4). Yet even at a neutral pH, weak oxidase reactions are seen with *Pasteurella multocida* and *Pseudomonas cepacia*.

Spurious oxidase test results after overnight growth may delay the identification of bacteria, especially if rapid biochemical systems such as the 5-h API 20E (Analytab Products, Plainview, N.Y.), the Auto Microbic system (Vitek Systems, Inc., Hazelwood, Mo.), or the Micro-ID (General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.) are used. After working with the modified oxidase test recently described by Faller and Schleifer (2) for the differentiation of staphylococci and micrococci, we tested a modification of its reagent with the weakly oxidase-positive gram-negative bacteria usually encountered in clinical laboratories.

Fresh clinical isolates identified by API 20E and other routine laboratory methods (7) and stock culture strains (College of American Pathologists and Centers for Disease Control survey and American Type Culture Collection strains) were grown on blood Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) or chocolate agar plates for 20 to 24 h at 35°C.

Our laboratory routinely uses the Cepti-Seal oxidase test reagent (1% aqueous tetramethyl-*p*-phenylenediamine dihydrochloride [TMPD-hydrochloride], Marion Scientific Corp., Kansas City, Mo.). The modified oxidase reagent was prepared as 1% (wt/vol) tetramethyl-*p*-phenylenediamine (Aldrich Chemical Co., Milwaukee, Wis., catalog no. 16020-2, not HCl salt; the chemical is a respiratory and skin irritant and should be handled in a fume hood) in certified-grade dimethyl sulfoxide (Fisher Scientific Co., Silver Spring, Md., catalog no. D128) (TMP-DMSO). The reagent is stable under refrigeration for at least a month. The oxidase test was performed as follows. Ashless filter paper (Whatman no. 40, quantitative grade) was placed in a petri dish and wetted with 0.5 ml of TMPD-DMSO or TMPD-hydrochloride (other filter paper types may cause false-positive results owing to oxidation of the reagent). Each bacterial isolate was tested with both reagents by two methods. (i) One large isolated colony was picked up with a cotton-tipped swab, and the inoculum was allowed to dry for about 5 s (swab method). (ii) Another colony was tested by the routine method with a wooden applicator stick (applicator method). The swab was tamped lightly 10 times on the wet filter paper, and the reaction was observed on the swab tip. The applicator inoculum was streaked on the wet filter paper, and the reaction was read on the paper. The color change of the positive reaction to blue-purple was recorded after 10, 15, and 30 s. All tests were performed in triplicate with both the swab and the applicator methods.

The 88 oxidase-positive and oxidase-negative strains are listed in Table 1. Of these strains, 40

TABLE 1. Oxidase reactions of 24-h cultures of known oxidase-positive and oxidase-negative gram-negative bacteria

Bacterium	Oxidase reaction	No. of strains tested
Achromobacter xylosoxidans	+	1
Aeromonas hydrophila	+	1
Bordetella bronchiseptica	+	3
Flavobacterium meningosepticum	+	1
Moraxella osloensis	+	3
Moraxella phenylpyruvica	+	1
Moraxella sp.	+	1
Neisseria flavescens	+	1
Neisseria gonorrhoeae	+	1
Neisseria meningitidis	+	1
Neisseria sicca	+	1
Plesiomonas shigelloides	+	2
Pseudomonas aeruginosa	+	2
Pseudomonas fluorescens	+	1
Vibrio alginolyticus	+	1
Vibrio parahaemolyticus	+	2
Vibrio spp.	+	2
Acinetobacter calcoaceticus	−	4
Citrobacter freundii	−	1
Enterobacter aerogenes	−	6
Enterobacter cloacae	−	14
Enterobacter sakazakii	−	1
Escherichia coli	−	16
Haemophilus aphrophilus	−	1
Klebsiella oxytoca	−	2
Klebsiella pneumoniae	−	10
Proteus vulgaris	−	1
Salmonella enteritidis	−	1
Salmonella typhi	−	1
Serratia marcescens	−	5

weakly or irregularly oxidase-positive organisms or dysgonic growers showed discrepancies between the two reagents and the two inoculation methods after 24 h of growth (Table 2). With TMPD-DMSO, all 40 strains were clearly positive within 15 s. With TMPD-hydrochloride, only 5 of 40 strains were positive within 15 s by the swab method, and only 3 were positive by the applicator method. After 30 s, TMPD-hydrochloride indicated a positive reaction in more than one-half (22 of 40) of the strains by the swab method but in only 15 of 40 strains by the applicator technique. TMPD-DMSO showed no false-positive results with the oxidase-negative organisms tested; however, higher concentrations (3 and 6% TMPD) did give false-positive reactions. It is important that only a 1% reagent and not the 6% TMPD-DMSO of Fuller and Schleifer be used with gram-negative bacteria. With both reagents, the test accuracy was better with the cotton swab than with the applicator method and could be increased by allowing the bacterial inoculum on the swab to dry for 5 s before exposure to the enzyme substrate. We assume that during the drying period moisture will be reduced by absorption and evaporation. Therefore, the reagent absorbed on the swab will not be diluted with water. The performance of the oxidase reagent (TMPD-hydrochloride) of Marion Scientific Corp. could not be altered significantly by adjusting the pH of 2.1 to 7.0 immediately before testing; only 4 of 23 discrepancies could be corrected. Of 10 strains of *Pasteurella multocida* grown on MacConkey agar for 24 h, all were positive with TMPD-DMSO, and none was positive with TMPD-hydrochloride. It was of interest that three different strains of *Pseudomonas maltophilia* were oxidase positive with TMPD-DMSO and oxidase negative with TMPD-hydrochloride. Further studies of this species with the modified reagent are required.

We recommend the modified oxidase reagent-swab method as a rapid and reliable alternative to the Kovacs method presently recommended for clinical laboratories.

TABLE 2. Comparison of routine method (1% aqueous TMPD-hydrochloride with wood applicator inoculation) with modified method (1% TMPD-DMSO with swab inoculation) in testing oxidase-variable gram-negative bacteria after 24 h of growth

Bacterium	(No. tested)	No. of strains positive with:		
		TMPD-DMSO at reaction time of <15 s	TMPD-hydrochloride at indicated reaction time	
			<15 s	>15 s
Cardiobacterium hominis	(1)	1	0	0
Eikenella corrodens	(1)	1	0	0
Haemophilus influenzae	(11)	11	0	6
Haemophilus parainfluenzae	(2)	2	1	2
Kingella kingae	(1)	1	0	0
Legionella micdadei	(1)	1	0	0
Pasteurella multocida	(10)	10	0	0
Pseudomonas cepacia	(10)	10	2	7
Pseudomonas maltophilia	(3)	3	0	0

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