DIFFERENTIAL DIAGNOSIS OF PSEUDOMONAS-LIKE MICROORGANISMS IN THE CLINICAL LABORATORY

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Three new laboratory tests are currently available for the identification of *Pseudomonas aeruginosa* isolated from clinical specimens. None of these procedures are specific for *P. aeruginosa* and cannot be used to differentiate between species within the genus. However, since *P. aeruginosa* is the only known human pathogen it is the only member of this genus that usually will be encountered in clinical specimens. All three tests are based on the oxidative characteristics of the organisms. Haynes (1951) suggested that the ability of pseudomonads to oxidize gluconate to keto-gluconate is a major characteristic of the genus and can be used to define *P. aeruginosa* more precisely when correlated with growth temperature and slime formation. Kovacs (1956) described a method in which the sensitivity of the oxidase test was increased to the extent that all 436 strains of *P. aeruginosa* examined by him gave quick, positive reactions while other gram-negative bacteria were either oxidase negative or gave delayed reactions. Gaby and Hadley (1957) developed a laboratory procedure based on the ability of pseudomonads to oxidize *p*-aminodimethylaniline oxalate (Difco) in the presence of molecular oxygen with the subsequent formation of indophenol blue within 10 to 30 sec following the addition of α-naphthol.

The three tests were incorporated as routine procedures in our clinical laboratory and were compared not only for accuracy of results but also for simplicity of the technical procedure.

MATERIALS AND METHODS

Gluconate oxidation test (Haynes). A practical laboratory method for the determination of gluconate oxidation by pseudomonads has only recently become available. This procedure is based on the ability of keto-gluconate (oxidative product of gluconate by *Pseudomonas*) to reduce Benedict's reagent while gluconate is unable to do so. One gluconate substrate tablet (Key) is added to 1 ml of distilled water in a sterile test tube and inoculated heavily with the test organism. Following a 12 to 18 hr incubation period at 37 C, the presence of reducing sugars was determined by adding Benedict's reagent (one clinitest tablet, Ames) and comparing the resulting color with a standard chart. It should be noted that any satisfactory test for reducing sugar can be employed.

Oxidase test (Kovacs). A 6 cm square piece of Whatman's No. 1 filter paper was placed in a petri dish and 2 or 3 drops of a 1 per cent aqueous solution of tetramethylparaphenylenediamine dihydrochloride placed on the center of the paper. The test colony was removed with a platinum loop or rod and streaked onto the reagent-impregnated paper. The smeared colony turns dark purple in from 5 to 10 seconds if the reaction is positive, (i. e., *Pseudomonas*) and it is assumed to be a member of the genus *Pseudomonas.*

Cytochrome oxidase test (Gaby). To a 12 to 18 hr nutrient broth (2 to 5 ml) culture of the test organism 0.3 ml of a 1 per cent aqueous solution of *p*-aminodimethylaniline oxalate (Difco) and 0.2 ml of a 1 per cent ethanol solution of α-naphthol were added and the tube shaken vigorously to ensure thorough oxygenation of the culture. The appearance of a blue color is indicative of the presence of cytochrome oxidase. An immediate blue color indicates *Pseudomonas* while a slowly developing blue color is thought to indicate *Alcaligenes faecalis.*

Test cultures. All pseudomonas-like bacteria isolated from clinical specimens were routinely tested by the three methods described above in addition to the usual diagnostic procedures. The specimens from which these organisms were isolated included blood, urine, feces, wound, nasopharyngeal and bronchial secretions. The bacteria were isolated from the clinical specimens in the usual manner by streaking on blood agar plates and if indicated on eosin methylene blue and salmonella shigella agar plates. Representative colonies of the gram-negative organisms to be

identified were transferred to carbohydrate, urea and semisolid media in addition to a nutrient agar slant and a nutrient broth tube. All cultures whose results did not correlate were retested in an attempt to determine the reason for the discrepancy. "*Bacterium anitratum*" was identified by its morphological characteristics and its ability to oxidize 10 per cent lactose when the carbohydrate is incorporated in an agar slant medium containing an indicator (Schaub and Foley, 1952).

RESULTS

A total of 50 pseudomonas-like bacteria have been isolated over an 18 month period and examined by the three procedures. All of the cultures gave negative reactions in lactose, sucrose and urea media, 21 produced slight acid in glucose media and 26 were motile. These cultures are characterized by their weak or negative biochemical reactions. The majority of the strains were non-pigmented when grown on nutrient agar although an occasional pigmented *P. aeruginosa* isolate was included as a check on the procedure.

To accurately evaluate and compare the procedures, the results obtained from each of the tests are compared individually with each other. The results of the cytochrome oxidase (Gaby) and gluconate oxidation (Haynes) tests are compared in table 1. The agreement between the two tests was excellent. In only 4 instances was there disagreement. Three of the cultures gave a slow cytochrome oxidase reaction (indicative of *A. faecalis*) but oxidized gluconate (indicative of *P. aeruginosa*). All three of these cultures were biochemically inactive, and were motile by lophotrichous flagella. Only one culture gave a positive cytochrome oxidase but negative gluconate test without any other recognizable differences in its characteristics. This particular culture also gave a positive oxidase (Kovacs) test and has been clinically identified as *P. aeruginosa*. Table 2 compares the results of the oxidase (Kovacs) and cytochrome oxidase (Gaby) tests. It is evident from the results listed as well as from clinical impressions that the oxidase test is overly sensitive. A total of 15 cultures gave immediate positive reactions by the oxidase test (indicative of *P. aeruginosa*) but gave slow reactions by the cytochrome oxidase test (indicative of *A. faecalis*). Of these 15 cultures, 3 oxidized gluconate but, as mentioned above, were lophotrichous.

TABLE 1

A comparison of results of cytochrome oxidase and gluconate oxidation tests

Number of Cultures	Cytochrome Oxidase (Gaby)	Gluconate Oxidation (Haynes)
21	+*	+
19	+ (Slow)†	−
6	−	−
3‡	+ (Slow)	+
1	+	−

* Blue color in 10 to 30 sec.
† Blue color in 2 to 5 min.
‡ These organisms were lophotrichous.

TABLE 2

A comparison of the results of the cytochrome oxidase and the oxidase tests

Number of Cultures	Cytochrome Oxidase (Gaby)	Oxidase (Kovacs)
21	+*	+
4	+ (Slow)†	+ (Slow)
15	+ (Slow)	+
3	+ (Slow)	−
1	+	+ (Slow)
6	−	−

* Blue color in 10 to 30 sec.
† Blue color in 2 to 5 min.

TABLE 3

A comparison of the results of the oxidase and gluconate oxidation tests

Number of Cultures	Oxidase (Kovacs)	Gluconate Oxidation (Haynes)
21	+*	+
15	+	−
5	+ (Slow)†	−
9	−	−

* Blue Color in 10 to 30 sec.
† Blue Color in 2 to 5 min.

Three cultures gave slow cytochrome oxidase tests but were negative by the Kovacs test. These three cultures did not oxidize gluconate. One culture gave a positive cytochrome oxidase test (also oxidized gluconate) but gave a negative oxidase test. Comparing the results obtained from the oxidase and gluconate oxidation tests in table 3, it is again obvious that the oxidase test is more sensitive than gluconate oxidation. The fifteen positive oxidase but negative gluconate cultures

gave a slow cytochrome oxidase reaction. Only 5 cultures gave a slow oxidase positive reaction by the Kovacs test.

DISCUSSION

The routine procedures generally carried out in the clinical laboratory are not always of sufficient specificity for the identification of all cultures of *P. aeruginosa* or for the differentiation of this species from *A. faecalis*. The *P. aeruginosa* cultures giving characteristic biochemical reactions (Gaby and Free, 1953) can be identified regardless of pigment. However, cultures are frequently encountered whose biochemical reactions are not characteristic and thus fall into the Pseudomonas-Alcaligenes-Mimeae clinical group of microorganisms. *"Bacterium antitratum" (Mimeae)* is identified by the production of acid on 10 per cent lactose agar and its morphological characteristics on staining. The cytochrome oxidase test has proven to be a reliable procedure not only for the identification of *P. aeruginosa* but also for the identification of *A. faecalis* from clinical samples. The cytochrome oxidase procedure was technically simpler than the gluconate oxidation reaction and more accurate than the oxidase test described by Kovacs.

Several cultures gave false negative gluconate oxidation reactions due to the failure of inoculating the gluconate tube with a heavy enough suspension of the test organism. When these cultures were retested using sufficient inocula the results were satisfactory.

The oxidase test as described by Kovacs does have the technical advantage that several colonies can be picked from the original plate and tested immediately. However, the extreme sensitivity of the tetramethylparaphenylenediamine dihydrochloride reaction should be kept in mind. On the other hand, Elek (1958, *personal communication*) found in preliminary tests that a mixture of equal amounts of *p*-aminodimethylaniline oxalate (Difco) and α-naphthol reagents (Gaby and Hadley, 1957), used to moisten the center of filter paper discs gave satisfactory results when colonies from the original plate were streaked on the saturated paper. This method has not been compared with those reported in the present study, thus comment on the specificity of the results is impossible at this time.

The over-all impressions of these three procedures as to accuracy and technical simplicity when used with organisms from clinical material are: (1) the gluconase oxidation and cytochrome oxidase tests parallel one another in accuracy; (2) the gluconate oxidation test is technically more complicated than either of the other two procedures; (3) the gluconate oxidation test may give false negative results if a heavy inoculum is not used; (4) in our hands the oxidase test as described by Kovacs was overly sensitive; (5) both the cytochrome oxidase and oxidase tests can be used for the identification of *A. faecalis* in addition to *P. aeruginosa* whereas the gluconate oxidation test is useful only for the identification of pseudomonads; (6) The cytochrome oxidase test was technically superior in our diagnostic laboratory. It is recognized, however, that the reagent saturated filter paper method may be more adaptable in many laboratory routines.

SUMMARY

Following a thorough and impartial comparison of three laboratory procedures for the identification of *Pseudomonas aeruginosa* from clinical specimens the cytochrome oxidase test or Gaby test appeared to be technically simpler and of equal or superior accuracy. The oxidase test of Kovacs was found to be overly sensitive in that several cultures of *Alcaligenes faecalis* gave reactions similar to *P. aeruginosa*. The gluconate oxidation test of Haynes was technically more complicated but the accuracy paralleled that of the cytochrome oxidase test.

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