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Development of a miniaturized nitrate reduction test for the identification of oral bacteria

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Summary

A miniaturized nitrate reduction test (MNRT) for oral bacteria was developed and its reliability compared with a conventional nitrate reduction test (CNRT). In the MNRT 100 µl aliquots of freshly grown heavy suspension of various oral bacterial species, in physiological saline, were added to equal volumes of 0.1% filter-sterilized KNO_3 solution in distilled water in wells of transparent plastic plates. Duplicate plates were incubated aerobically or anaerobically at 35°C for 12–15 h. At the end of the incubation period the test was performed by adding either a trace amount of a non-liquid reagent (mixture of L-(+)-tartaric acid, sulfanilic acid and 1-naphthylenediamine dihydrochloride, 10:1:1, wt/wt) or conventional liquid reagents A and B (sulfanilic acid and *N,N*-dimethyl-1-naphthylamine). In the conventional nitrate reduction test (CNRT), tubes of a basal anaerobic broth were inoculated with the same bacterial species used for MNRT, and the nitrate reduction tests performed after anaerobic incubation of the cultures for 4–6 days. Several hundred anaerobic and facultative bacterial isolates belonging to genera *Veillonella, Bacteroides, Fusobacterium, Selenomonas, Actinomyces* and *Capnocytophaga* were characterized by MNRT and CNRT. Analysis of the data showed that MNRT and CNRT systems were comparable. In the MNRT system *Veillonella parvula* and *Selenomonas sputigena* were capable of reducing nitrate only under anaerobic conditions. Actinomycetes reduced the nitrates under aerobic and anaerobic conditions, while all black-pigmented *Bacteroides, Fusobacterium* and *Capnocytophaga* species did *not* reduce nitrate. These findings suggest that the MNRT is reliable, rapid and may be conveniently used in clinical or research laboratories with a heavy microbiological work load.

Key words: *Oral bacteria – Rapid nitrate reduction test – Reagent – Solid nitrate reduction test*

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Introduction

Milicroaerophic and anaerobic bacteria capable of reducing nitrate have been found in the human oral cavity and are frequently isolated from both healthy and disease-associated plaques [1–12]. Nitrate reduction tests requiring growth of the organisms in broth media containing nitrate have been widely used. In laboratories with a heavy bacteriological culture load, and involved in characterization of a large number of bacterial isolates from various plaque specimens, the conventional tests are rather inconvenient or not economical. Consequently, rapid identification systems have been developed which avoid additional growth of bacteria in broth media containing selected substrates [14–16].

In the present investigation a rapid miniaturized nitrate reduction test (MNRT) was developed for routine use in conjunction with other identification tests for bacteria [17, 18]. The details concerning the procedures of this test and the results obtained with various bacterial species are described in this paper.

Materials and Methods

Source of organisms and their maintenance

Fresh human oral isolates belonging to the following species were utilized in this study: *Actinomyces viscosus, A. naeslundii* and *A. odontolyticus, Streptococcus sanguis, S. mutans* and *S. salivarius, Fusobacterium nucleatum*, smooth and rough colony types of *Capnocytophaga* sp., *Bacteroides gingivalis, B. intermedius, B. melaninogenicus, B. loeschii, B. denticola, Veillonella parvula* and *Selenomonas sputigena*. Additional strains of *Capnocytophaga* sp. viz *C. gingivalis* 27, *C. ochracea* 6 and *C. sputigena* 4 were received from Dr. Ann Tanner, Forsyth Dental Center, Boston, MA. The following reference strains from the American Type Culture Collection (ATCC) were also included: *Bacteroides gingivalis* 33277, *Bacteroides intermedius* 25611, *Bacteroides melaninogenicus* 25845, *Bacteroides asaccharolyticus* 25260 and *Selenomonas sputigena* 33150. The organisms were maintained and routinely transferred on an enriched trypticase soy blood agar [19] (ETSA) in the anaerobic glove box [20], Coy Laboratory Products, Ann Arbor, MI (USA).

Miniaturized nitrate reduction test (MNRT) procedure

Heavy bacterial suspensions (McFarland 2 to 3) of 2–4-day-old cultures were prepared in 1.5 ml sterile physiological saline (pH 6.4) in 4 ml capacity screw-capped glass vials with glass beads. Duplicate 100 µl aliquots of each bacterial suspension were added to equal volumes of filter-sterilized (membrane filter 0.22 µm pore size, Millipore, Bedford, MA, USA) 0.1% KNO_3 solution in glass-distilled water in sterile Minitek plate wells (BBL Microbiology System, Baltimore, MD, USA). Suspensions of bacterial cultures with known nitrate test reactions and uninoculated saline-nitrate solutions were included as controls for each set. One set of inoculated Minitek plates was incubated aerobically and the other anaerobically at 35°C for 12–15 h. The nitrate reduction test was performed using a non-liquid

reagent [21] which contained a mixture of L-(+)-tartaric acid, 1-naphthalenediamine dihydrochloride and sulfanilic acid (10:1:1 wt/wt, Sigma, St. Louis, MO, USA). In a separate series the conventional liquid reagents, sulfanilic acid and *N,N*-dimethyl-1-naphthylamine [22], were used to evaluate the reliability of the results. The development of red or purple color within 5 min was considered a positive reaction for nitrate reduction to nitrite. Negative tests were further confirmed with the addition of trace amounts of zinc dust. Failure of color development was then recorded as positive test for nitrate reduction beyond nitrite.

Additional tests

Bacterial cultures of *Actinomyces* sp., *Bacteroides* sp., *Capnocytophaga* sp. and *Veillonella* sp. were grown as described above and suspensions were prepared in duplicate sets in 0.15 M phosphate buffers at pH 6, 7 and 8. Following aerobic or anaerobic incubation the cultures were tested for nitrate reduction by the liquid and non-liquid reagents. The results were compared with those obtained from bacterial suspensions prepared in saline (pH 6.4). The experiment was repeated several times to confirm the reproducibility of results with the same test strains. In another experiment suspensions of selected strains of *Actinomyces* and other bacterial species were freshly prepared in saline. The nitrate reduction test was performed within 5–10 min after the bacterial suspensions were mixed with 0.1% KNO_3 solution in Minitek plate wells. Also, strains of *Actinomyces viscosus* and *A. naeslundii* were subcultured on nitrate-free ETSA for at least three consecutive growth cycles. Saline suspensions of these bacterial subspecies were tested in the MNRT.

Conventional nitrate reduction test (CNRT)

Each bacterial strain tested in the MNRT system was also inoculated into 5 ml anaerobic glucose broth [6] in screw-capped tubes, and incubated in the anaerobic glove box for 4–7 days. Uninoculated medium and strains with known biochemical characteristics were included as controls. The nitrate reduction test was performed on each test culture using non-liquid CNRT reagents. The results were recorded and compared with those of the MNRT system.

Results

All bacterial isolates belonging to the genus *Actinomyces* reduced nitrate in the MNRT system (Table 1). There was no effect of aerobic or anaerobic environment on the nitrate-reducing ability of these organisms. *V. parvula* and *S. sputigena* failed to reduce nitrate under aerobic incubation environment. However, these organisms reduced nitrate when the cultures were incubated in the anaerobic conditions. The remaining bacterial species failed to reduce nitrate irrespective of the incubation environment (Table 1). Phosphate buffer or saline at pH 6, 7 and 8, when used as the suspending medium, did not affect the results of the nitrate reduction test.

TABLE 1

EFFECT OF GAS ENVIRONMENT ON NITRATE REDUCTION BY PLAQUE BACTERIA IN MINIATURIZED NITRATE REDUCTION TEST SYSTEM (MNRT)

Organism	No. tested	NO_3 reduction after		
		Aerobic incubation	Anaerobic incubation	
		MNRT	MNRT	CNRT
A. viscosus	160	+	+	+
A. naeslundii	130	+	+	+
A. odontolyticus	29	+	+	+
Veillonella parvula[a]	25	−	+	+
Selenomonas sputigena[a]	40	−	+	+
Bacteroides urealyticus[a]	1	−	+	+
B. gingivalis	50	−	−	−
B. asaccharolyticus	1	−	−	−
B. intermedius	105	−	−	−
B. loeschii & denticola	50	−	−	−
B. melaninogenicus	6	−	−	−
F. nucleatum	135	−	−	−
Capnocytophaga sp.	38	−	−	−
S. sanguis	5	−	−	−
S. mutans	5	−	−	−
S. salivarius	2	−	−	−

[a] Organisms reduced nitrated only when incubated anaerobically.

Some *Actinomyces* strains gave a positive nitrate reduction after only 1–2 h of incubation, while *V. parvula* and *S. sputigena* required 12–15 h before they were positive for this test. The actinomycetes, repeatedly grown on nitrate-free ETSA, retained the capacity to reduce nitrate when tested by MNRT system. Comparison of the CNRT and the MNRT data obtained with anaerobically incubated cultures showed complete agreement between the two test systems (Table 1).

Discussion

Nitrate reduction test has been widely used in bacterial taxonomy for biochemical characterization and differentiation of bacteria. The results obtained in this investigation demonstrate that the MNRT is as reliable and reproducible as the CNRT system. When introduced in routine work the MNRT system will be more economical and time saving than the CNRT system as it will avoid additional growth of cultures in nitrate-supplemented media. The MNRT system may be successfully used without additional effort in conjunction with other rapid enzyme profile tests [17, 18] which are currently used in the laboratories for the characterization of bacteria.

Two obligately anaerobic bacterial species, i.e., *V. parvula* and *S. sputigena* were positive for nitrate reduction test only after anaerobic incubation of the bacte-

rial cell suspensions. This would suggest that these bacteria reduce nitrate via an oxygen-sensitive nitrate reductase. This finding has an additional diagnostic value and can be used as a criterion for some anaerobes, e.g., *Veillonella* sp. or *Selenomonas* sp. Actinomycetes gave positive reaction for nitrate reduction irrespective of gas environment during the incubation period, which suggests that the nitrate reductase system of this group of organisms is functional under aerobic and anaerobic conditions.

If it is desirable to differentiate anaerobic and microaerophilic nitrate-reducing bacteria, then the test should be performed on duplicate sets of cell suspensions incubated in both aerobic and anaerobic conditions. Alternatively, the MNRT system can be used to identify all nitrate reducers (both aerobes and anaerobes) if the bacterial suspensions are incubated in oxygen-free environment. In this regard the test will be useful for discriminating nitrate-reducing organisms from non-nitrate reducers. The MNRT system, as compared to CNRT system does not require growth of the organism. Therefore, the possibility of bacterial contamination during incubation, and possibly false positive results, are eliminated. The efficiency of the solid reagent in nitrate reduction test for clinically important bacteria was also reported by Lampe [21] and for myobacteria by Warren et al. [23].

The MNRT should be performed with heavy bacterial suspensions made from freshly grown agar cultures. The use of bacterial suspensions prepared from old cultures may lead to false negative tests. Nitrite-free culture media, glassware and pure chemical reagents (analytical grade) should be used to avoid false-positive reactions. Since a prolonged contact of the reagents with the Minitek plate material results in gradual development of light or dark pink color, it is recommended that the test-reactions should be recorded within 5 min after the addition of the nitrite-detecting solid reagent. Any source of contamination of the solid reagent with zinc dust should be avoided. Similarly, the prolonged exposure of solid reagents to moisture seems to shorten the shelf life of the reagents. Therefore, it should be kept in a dry place. Only a small quantity of the reagent should be used and breathing of its dust should be avoided. Uninoculated suspending fluid controls should always be included with each batch of test plates to monitor the quality of nitrate solution and solid reagent used in the MNRT system. In our studies we found that a 12 h incubation of test cultures was adequate for all the organisms used in MNRT system. Prolonged incubation beyond 12 h had no additional effect on the number of positive tests in this system.

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