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Production of ^{14}C-Labeled Gas in BACTEC *Neisseria* Differentiation Kits by *Neisseria cinerea*

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Six strains of *Neisseria cinerea* were tested in BACTEC *Neisseria* Differentiation kits (Johnston Laboratories, Inc., Towson, Md.), and all yielded positive glucose growth indices and negative maltose and fructose growth indices. These results were similar to those achieved with *Neisseria gonorrhoeae*. However, most of the *N. cinerea* isolates tested yielded 3-h glucose growth indices that were lower than those obtained with gonococci. ^{14}C-labeled gas was produced significantly faster ($P < 0.02$) by *N. gonorrhoeae* than by *N. cinerea*. Additional studies suggested that the ^{14}C-labeled gas produced by *N. cinerea* was carbon dioxide. *N. cinerea* strains were similar to *Branhamella catarrhalis* strains because both species failed to produce detectable acid from glucose, maltose, sucrose, fructose, and lactose in cysteine-tryptic agar media. However, in contrast to *N. cinerea* strains, *B. catarrhalis* strains did not metabolize glucose in BACTEC *Neisseria* Differentiation kits.

Neisseria cinerea strains are opportunistic neisseriae which may colonize the human oropharynx and less commonly the genital tract (3, 5, 6, 8). In a recent survey that identified all isolates recovered on a selective-differential medium for commensal *Neisseria* spp., *N. cinerea* strains were recovered from 21% of posterior pharyngeal cultures and 3% of genital tract cultures (J. S. Knapp, P. A. Totten, B. H. Minshew, and E. W. Hook III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C26, p. 316). Although *N. cinerea* has seldom been identified in secretions from patients, the apparent rarity of these organisms may reflect (i) difficulties in differentiating these strains from other commensal neisseriae, *Neisseria gonorrhoeae*, and *Branhamella catarrhalis* strains and (ii) the common use of selective media (e.g., Thayer-Martin) that can inhibit the growth of *N. cinerea*, rather than a failure of these organisms to colonize mucosal surfaces.

In many clinical microbiology laboratories, *Neisseria* spp. and *B. catarrhalis* isolates are differentiated on the basis of colony morphology, acid production from carbohydrates in cysteine-tryptic agar media, and nitrate reduction reactions. BACTEC *Neisseria* Differentiation kits (Johnston Laboratories, Inc., Towson, Md.) may be used in place of reactions in cysteine-tryptic agar medium and have the advantage of requiring only 3 h of incubation. Because the ability of *N. gonorrhoeae* to produce detectable acid from glucose is one of the major criteria for differentiating *N. gonorrhoeae* from *N. cinerea*, the ability of *N. cinerea* to yield positive glucose reactions in BACTEC *Neisseria* Differentiation kits represents a source of possible confusion for laboratories that use BACTEC *Neisseria* Differentiation kits for identifying pathogenic neisseriae (2). This paper describes studies of ^{14}C-labeled gas production by *N. cinerea* in BACTEC *Neisseria* Differentiation kits.

MATERIALS AND METHODS

Strains. *N. cinerea* UMC 2768, ATCC 14685, and CDC F4340 and three other *N. cinerea* isolates were included in the study. Four *N. gonorrhoeae* isolates, two *Neisseria meningitidis* isolates, and six *B. catarrhalis* isolates were also used in some experiments.

BACTEC *Neisseria* Differentiation kits. All *N. cinerea* isolates and several of the other study strains were tested in model ND-1 BACTEC *Neisseria* Differentiation kits. Isolates were streaked onto chocolate agar and incubated for 18 to 24 h. A swab was used to harvest a heavy inoculum which was then suspended in BACTEC Infusion Broth. Then, 0.3 ml of the suspension was transferred to each of three vials which contained U-^{14}C-labeled glucose, maltose, and fructose. Standard 3-h readings were obtained with the BACTEC model 460 device. Several *N. cinerea* strains were tested on multiple occasions to determine if the results were reproducible.

To determine if acid was produced in the BACTEC kits, we measured the pH in the vials containing glucose after the vials were inoculated and incubated for 3 h. pH papers with ranges of 0.1 to 14 and 4.5 to 7.5 were used to estimate the pH.

Rate of ^{14}C-labeled gas production by *N. cinerea*. To compare the rates of ^{14}C-labeled gas production by *N. cinerea* UMC 2768, *N. cinerea* CDC F4340, and an *N. gonorrhoeae* isolate, we inoculated each organism into BACTEC Infusion Broth and then inoculated 0.3-ml portions of each suspension into seven BACTEC *Neisseria* Differentiation kit vials containing ^{14}C-labeled glucose. Glucose growth indices were determined after 15 min, 30 min, and 1, 1.5, 2, 2.5, and 3 h of incubation. Each vial was read only once in this experiment. The growth indices obtained with *N. cinerea* UMC 2768 and CDC F4340 were compared with those obtained with the *N. gonorrhoeae* isolate by using the sign test (10).

$^{14}CO_2$-trapping studies. To establish if the ^{14}C-labeled gas produced by *N. cinerea* in the BACTEC system was CO_2, we conducted the following experiment. Strain UMC 2768 was inoculated into duplicate BACTEC *Neisseria* Differentiation kits (kits A and B) and incubated for 3 h. A 10-ml amount of gas was then withdrawn from the glucose vial of kit A and injected into an evacuated sidearm Erlenmeyer

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TABLE 1. BACTEC *Neisseria* Differentiation kit glucose growth indices obtained with *N. cinerea* and *B. catarrahalis*

Strain[a]	BACTEC glucose growth index at:	
	3 h[b]	30 min[c]
N. cinerea		
UMC 2768	647 (315–758)	269
CDC F4340	678 (593–721)	75
ATCC 14685	194 (160–240)	12
NRL 32165	603	51
NRL 32828	356	ND[d]
NRL 32824	362	27
N. gonorrhoeae		
UMC GC-A	999	815
UMC GC-B	999	667
MSBH GC-1	ND	705
MSBH FG-98	ND	778
N. meningitidis		
MSBH *N. meningitidis*	ND	586
ATCC 13090	ND	797
B. catarrhalis		
UMC TM	0	ND
UMC JN	2	ND
CDC KC646	4	ND
CDC 5228	3	ND
CDC 4402	4	ND
CDC 4453	6	ND

[a] UMC, University of Mississippi Medical Center; CDC, Centers for Disease Control; ATCC, American Type Culture Collection; NRL, Neisseria Reference Laboratory; MSBH, Mississippi State Board of Health.
[b] The median (range) value is given if multiple determinations were performed.
[c] The highest value is given if more than one determination was performed.
[d] ND, Not done.

flask containing 0.5 ml of Hyamine (Rohm & Haas Co., Philadelphia, Pa.) hydroxide (Sigma Chemical Co., St. Louis, Mo.), a CO_2-trapping agent (7, 9). The flask was placed on a rotary shaker for 20 min at 20°C, and then all the Hyamine was removed and added to 10 ml of liquid scintillation fluid containing toluene and 2,5-diphenyloxazole. The amount of radiolabeled $^{14}CO_2$ trapped in the Hyamine was determined with a liquid scintillation counter. This procedure was also performed with gas removed from the BACTEC kit A vials containing maltose and fructose. For comparison, vials from kit B were read on the BACTEC model 460 device.

Base-line studies with (i) scintillation fluid alone, (ii) scintillation fluid plus [^{14}C]toluene (internal standard), and (iii) scintillation fluid plus [^{14}C]toluene plus 0.5 ml of Hyamine determined that the addition of Hyamine to the internal standard permitted an 88% recovery rate (i.e., 12% quenching). Controls included scintillation fluid plus Hyamine which had been exposed to 10 ml of gas from a glucose vial inoculated with sterile BACTEC Infusion Broth and scintillation fluid plus Hyamine which had been exposed to 10 ml of gas from a glucose vial inoculated with *N. gonorrhoeae*.

30-min BACTEC results. To determine if a shorter incubation period might facilitate the differentiation of *N. cinerea* from other neisseriae in the BACTEC system, *Neisseria* study isolates were inoculated into BACTEC *Neisseria* Differentiation kits, and readings were taken after 30 min of incubation.

RESULTS

All *N. cinerea* strains tested yielded positive 3-h glucose growth indices that ranged from 160 to 758 (Table 1). All isolates were maltose- and fructose-negative. The glucose growth indices obtained when *N. cinerea* UMC 2768, CDC F4340, and ATCC 14685 were tested on multiple occasions varied from one run to another (Table 1). Two fresh clinical isolates of *N. gonorrhoeae* each produced glucose growth indices of 999. Both were maltose- and fructose-negative. All six *B. catarrhalis* isolates tested yielded negative glucose, maltose, and fructose growth indices.

pH readings taken from a BACTEC glucose vial after *N. cinerea* UMC 2768 had been allowed to incubate for 3 h revealed a pH of ca. 7.0. The pH in glucose vials was also tested with one strain each of *N. meningitidis*, *N. gonorrhoeae*, and *B. catarrhalis* and was ca. 7.0 with each strain. The failure to detect acid production by *N. meningitidis* or *N. gonorrhoeae* was presumably due to a lack of sensitivity of the pH paper used or to the short incubation period used. Studies dealing with the rate of glucose metabolism in BACTEC *Neisseria* Differentiation kits revealed that *N. gonorrhoeae* produced ^{14}C-labeled gas significantly faster than strains UMC 2768 ($P < 0.02$) and CDC F4340 ($P < 0.02$) (Fig. 1).

Because differences between the glucose growth index curves produced by *N. gonorrhoeae* and *N. cinerea* were more striking during the early part of the incubation period, *N. cinerea* strains and the other *Neisseria* strains were tested again in BACTEC *Neisseria* Differentiation kits with a short incubation period. *N. cinerea* strains yielded 30-min glucose growth indices of <300, whereas all four gonococcal strains had 30-min readings of >600 (Table 1). Both species yielded negative maltose and fructose growth indices. The two meningococcal strains were both positive for glucose and maltose at 30 min.

In the CO_2-trapping experiments, gas removed from a BACTEC vial containing [^{14}C]glucose and uninoculated BACTEC Infusion Broth yielded 170 cpm. When gas from a glucose vial inoculated with *N. gonorrhoeae* was exposed to Hyamine, 51,928 cpm was detected. Comparable amounts of

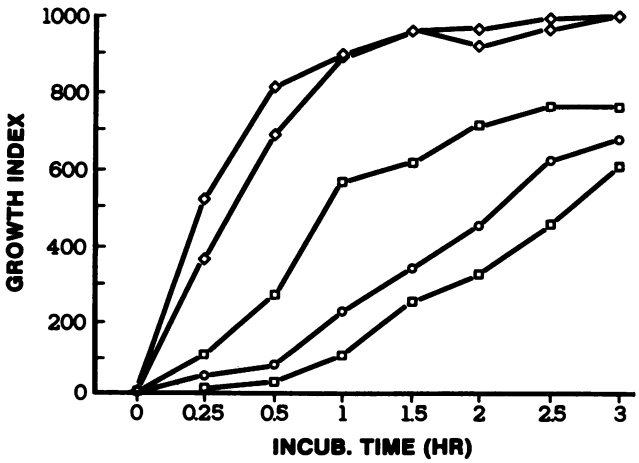

FIG. 1. BACTEC glucose growth indices after 15 min, 30 min, and 1, 1.5, 2.5, and 3 h of incubation (INCUB). Symbols: ◇, *N. gonorrhoeae* UMC-A (two runs); □, *N. cinerea* UMC 2768 (two runs); ○, *N. cinerea* CDC F4340. Each of the seven vials used for each curve was read only once.

gas from the glucose, maltose, and fructose vials of kit A inoculated with strain UMC 2768 yielded 67,954, 132, and 859 cpm, respectively. The duplicate BACTEC kit inoculated with strain UMC 2768 (kit B) was read on the BACTEC model 460 device and yielded the following growth indices: glucose (833), maltose (5), and fructose (15).

DISCUSSION

In the past, *N. cinerea* strains recovered from infected patients have been misidentified as *B. catarrhalis*, *Neisseria flavescens*, or *N. gonorrhoeae* (1, 3, 6). *N. cinerea* may be confused with *N. gonorrhoeae* because the two organisms have similar colony morphology and because both can occur in oropharyngeal and genital tract secretions (3, 8). However, because *N. cinerea* is susceptible to colistin, it has seldom been identified in genital tract secretions that have been plated on colistin-containing media such as Thayer-Martin, Martin-Lewis, or New York City media. Confusion is most likely to occur when *N. cinerea* recovered on chocolate agar is subsequently tested in BACTEC *Neisseria* Differentiation kits, because both *N. cinerea* and *N. gonorrhoeae* yield positive glucose growth indices in this system (2). However, the manufacturer of the kits states that all upper-respiratory-tract isolates should be screened for growth on a selective medium such as Thayer-Martin or Martin-Lewis medium before they are inoculated into BACTEC *Neisseria* Differentiation kits. Observing this precaution should reduce the risks of confusing *N. cinerea* with *N. gonorrhoeae* because *N. cinerea* seldom grows on such selective media. It should be noted that vancomycin-susceptible *N. gonorrhoeae* strains, which also grow poorly on Thayer-Martin medium, might be confused with *N. cinerea* if tested in BACTEC kits.

Occasionally, *N. cinerea* will grow on Martin-Lewis medium (5). If isolates that are initially recovered on Martin-Lewis medium give results suggestive of *N. gonorrhoeae* in BACTEC *Neisseria* Differentiation kits but possess other properties that are not characteristic for *N. gonorrhoeae*, they should be subcultured on chocolate agar with a 10-μg colistin disk (5). *N. gonorrhoeae* strains will grow right up to the disk, whereas *N. cinerea* strains will show a zone of inhibition of at least 10 mm around the colistin disk.

When *N. cinerea* is recovered from respiratory tract secretions with nonselective media, it may also be confused with *B. catarrhalis*. *N. cinerea* and *B. catarrhalis* may have similar colony morphology, and both organisms fail to produce detectable acid from sugars in cysteine-tryptic agar media and in modified oxidation-fermentation medium (5). Tests for nitrate reduction and production of DNase can be used to differentiate between *N. cinerea* (nitrate negative, DNase negative) and *B. catarrhalis* (nitrate positive, DNase positive). Our studies suggest that BACTEC *Neisseria* Differentiation kits could also be used as a supplemental test for differentiating these two species.

The results of our CO_2-trapping experiments suggest that the ^{14}C-labeled gas produced by *N. cinerea* in BACTEC glucose vials is $^{14}CO_2$. Surprisingly, the amount of $^{14}CO_2$ produced by strain UMC 2768 in BACTEC kit A vials and trapped in Hyamine was similar to the amount produced by the control gonococcal isolate tested. The glucose growth index obtained with UMC 2768 in kit B vials (inoculated with the same suspension used to inoculate kit A vials) was 833, which was higher than any previous glucose growth index recorded for this organism. We have no good explanation for why the organism produced so much $^{14}CO_2$ on the day the CO_2-trapping studies were done. Because preparation of the inoculum used in BACTEC *Neisseria* Differentiation kits is not standardized, it is possible that the large amount of $^{14}CO_2$ produced by strain UMC 2768 reflected an unusually large inoculum. Differences in inoculum size may also have been responsible for some of the day-to-day variability in the observed BACTEC glucose growth indices obtained with UMC 2768. However, we feel that it is unlikely that differences in the glucose growth indices obtained with *N. cinerea* and *N. gonorrhoeae* are due solely to differences in the inocula used. The first two times that UMC 2768 was tested as a fresh clinical isolate, it yielded glucose growth indices of 650 and 687 (2). Fresh gonococcal isolates often yield glucose growth indices of >900. Neither the age of the culture nor the number of times the isolate was subcultured after removal from the freezer had a predictable effect on the glucose growth indices obtained in BACTEC kits. However, further studies are needed to determine if such variables may affect glucose growth indices obtained with *N. cinerea* and *N. gonorrhoeae*.

Contamination of the radiolabeled glucose with significant amounts of another ^{14}C-labeled substrate seems to be an unlikely explanation for the $^{14}CO_2$ produced by *N. cinerea*, because the tagged glucose used in this system was >98% pure. If the ^{14}C-labeled glucose was contaminated by another tagged carbon source, this must have occurred on a regular basis, because *N. cinerea* yielded positive readings with five different lots of ^{14}C-labeled glucose.

The pathway(s) used by *N. cinerea* for the metabolism of glucose is not known at this time. In studies by Holten (4), *N. cinerea* 165/61 did not produce CO_2 from [1-^{14}C]glucose. The difference between Holten's (4) findings and ours could be related to differences in the *N. cinerea* strains, the inocula, or the labeled glucose substrates used in the two studies. Holten (4) used [1-^{14}C]glucose, whereas the BACTEC kits that we used contain [U-^{14}C]glucose. Further studies are necessary to determine the mechanism by which *N. cinerea* utilizes glucose.

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