JOURNAL OF CLINICAL MICROBIOLOGY, May 1988, p. 896–900
0095-1137/88/050896-05$02.00/0

Vol. 26, No. 5

Prevalence and Persistence of *Neisseria cinerea* and Other *Neisseria* spp. in Adults

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Received 26 May 1987/Accepted 26 January 1988

***Neisseria cinerea* is a commensal *Neisseria* sp. which was first described in 1906 but was subsequently misclassified as a subtype of *Branhamella catarrhalis*. *N. cinerea* resembles *Neisseria gonorrhoeae* in both cultural and biochemical characteristics and, thus, may also have been misidentified as *N. gonorrhoeae*. Of 202 patients whose oropharynges were colonized by *Neisseria* spp., *N. cinerea* was isolated in 57 (28.2%) patients, including 25 (30.1%) of 83 women, 22 (23.9%) of 92 heterosexual men, and 10 (37.0%) of 27 homosexual men in Seattle, Wash., in 1983 to 1984. *N. cinerea* was isolated from the urethra of only one (1.1%) patient. The oropharynges of many individuals were colonized persistently by strains of *N. cinerea* and other *Neisseria* spp.**

Neisseria cinerea was first described in 1906 (23), but strains of this species were subsequently misidentified as *Branhamella* (*Neisseria*) *catarrhalis* (14, 15, 26) because nitrate reduction was not used as a differential test for the classification of *Neisseria* spp. until 1961 (3). Although isolates resembling *N. cinerea* were referred to as *Neisseria pseudocatarrhalis* in 1934 (F. M. Huntoon, Abstr. Annu. Meet. Am. Soc. Bacteriol., 1934, M50, p. 108), this species was not recognized again until 1962 (8) and was not described in the United States until 1984 (18). We have been interested in *N. cinerea* because although strains are colistin sensitive (18), they have recently been isolated on selective media for pathogenic *Neisseria* spp. and resemble *Neisseria gonorrhoeae* in both cultural and biochemical characteristics (13, 18). Strains of *N. cinerea* may be misidentified as *N. gonorrhoeae* when many tests available for the rapid identification of gonococci are used (10–13, 18–20), with resulting serious social and medicolegal consequences (13).

We undertook a study in two parts to determine the prevalence of *N. cinerea* in the oropharynx and genital sites and to determine the ability of *N. cinerea* to persist in the oropharynx with other *Neisseria* spp.

MATERIALS AND METHODS

Strains. In the first part of the study, we determined the prevalence of *N. cinerea* in 209 unselected patients attending the Seattle-King County Sexually Transmitted Diseases Clinic at Harborview Medical Center between October 1982 and February 1983. Cervical and pharyngeal specimens from women and urethral and pharyngeal specimens from men were inoculated on a new medium, LBVT.SNR, which was selective for commensal *Neisseria* spp. (described below). Oropharyngeal specimens from the first 109 patients were plated only on LBVT.SNR medium, but oropharyngeal specimens from the next 100 patients were also plated on modified Thayer-Martin medium to ensure maximum recovery of *Neisseria meningitidis*, which was recovered at a lower frequency than expected from homosexual men among the first 109 patients. All urogenital specimens were also inoculated on Thayer-Martin medium.

In the second phase of the study, oropharyngeal specimens were collected weekly, for 15 weeks, from 16 volunteers in our laboratories to determine whether *N. cinerea* persisted in the oropharynx or whether it colonized the oropharynx transiently and thus would be isolated rarely. All specimens were inoculated directly on LBVT.SNR medium and modified Thayer-Martin medium. All volunteers gave verbal informed consent before specimen collection.

LBVT.SNR medium for the selective isolation of commensal *Neisseria* spp. The medium base, LB medium, contained 1% Bacto-Tryptone (Difco Laboratories), 0.5% yeast extract (Difco), 0.5% sodium chloride, and 1.5% Bacto-Agar (Difco). The ingredients were dissolved, 5.0 ml of neutral red indicator (0.3% [wt/vol]) per liter was added, and the medium was sterilized by autoclaving for 15 min at 121°C. After cooling to 56°C, sucrose was added to the base medium to a final concentration of 1% (wt/vol), and vancomycin and trimethoprim were each added to a final concentration of 3.0 μg/ml. The complete medium, designated LBVT.SNR, was used to isolate strains of commensal *Neisseria* spp. and *B. catarrhalis* and to test for polysaccharide production from sucrose. Known strains of commensal *Neisseria* spp. and *B. catarrhalis* were used to evaluate the selective medium. Strains of most species were stock cultures in the *Neisseria* Reference Laboratory and included strains of *Neisseria perflava* and *Neisseria sicca* provided by Ulrich Berger, Hygiene Institute, University of Heidelberg, Heidelberg, Federal Republic of Germany.

Isolation of commensal *Neisseria* spp. and *B. catarrhalis* on LBVT.SNR medium. Specimens were plated directly onto LVBT.SNR medium, placed in a candle extinction jar, and incubated at 37°C. After 44 to 48 h of incubation, at least one colony representative of each morphologic type was subcultured onto supplemented GC-base medium (GCK; 24). To ensure maximum recovery of isolates of *N. cinerea* and to detect concurrent colonization by *N. cinerea*, *N. meningitidis*, and *Neisseria lactamica*, several translucent, pink colonies characteristic of these species were isolated. A small

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TABLE 1. Colonial morphologic characteristics of commensal *Neisseria* spp. and *B. catarrhalis* grown on LBVT.SNR medium selective at 37°C for 48 h in a CO_2-enriched atmosphere

Species	Diam (mm)	Color	Elevation	Surface	Opacity	Color when stained with Lugol's iodine	Final color[a] of medium
N. mucosa, *N. sicca*, *N. perflava*[b]	1–2	Yellow-pink	Convex	Glistening	Opaque	Blue-black	Pink
N. flava[c], *N. subflava*[d]	1–2	Orange	Flat	Matt	Opaque	No reaction	Orange
N. flavescens	1	Orange	Convex	Glistening	Opaque	Blue-black	No change
N. lactamica, *N. meningitidis*, *N. cinerea*	1–2	Pink	Convex	Glistening	Translucent	No reaction	No change
B. catarrhalis	1–2	Pink	Convex	Matt	Opaque	No reaction	No change

[a] Final color of medium inoculated with a pure culture of each species.
[b] *N. perflava*, *N. subflava* biovar *perflava*.
[c] *N. flava*, *N. subflava* biovar *flava*.
[d] *N. subflava*, *N. subflava* biovar *subflava*.

section of the culture plate was then flooded with Lugol's iodine to detect polysaccharide-negative colonies of *B. catarrhalis* that had a colonial morphologic appearance similar to that of the polysaccharide-producing *Neisseria* spp. on this medium. In this way, it was possible to detect strains of the polysaccharide-positive *N. polysaccharea* (21) that also produce pink, translucent colonies on this medium. All isolates of gram-negative, oxidase-positive diplococci were identified by their pattern of acid production from glucose, maltose, sucrose, fructose, and lactose (17), reduction of nitrate (17), and production of polysaccharide from sucrose on LBVT.SNR medium (18). Colistin disk susceptibility tests were performed on strains resembling *N. cinerea* (18). Sucrose-positive, nitrate-negative isolates, *Neisseria subflava* biovar *perflava* (*Neisseria perflava*) and *N. sicca*, were recorded as *N. perflava-N. sicca* because these species could not be differentiated biochemically.

Statistical analysis. Statistical analyses were performed by using a chi-square test.

RESULTS

Growth of commensal *Neisseria* spp. and *B. catarrhalis* on LBVT.SNR medium. Five strains each of *N. lactamica*, *N. cinerea*, *N. sicca*, *Neisseria mucosa*, *N. subflava* biovars *subflava* (*N. subflava*), *flava* (*Neisseria flava*), and *perflava* (*N. perflava*), and *B. catarrhalis* and one strain each of *Neisseria flavescens* and *Neisseria polysaccharea* repeatedly grew well on LBVT.SNR medium both alone and in combination with each other. Some strains of *N. meningitidis* did not grow as well on LBVT.SNR medium as on GCK medium. LBVT.SNR medium did not support growth of *N. gonorrhoeae* strains. The colonial morphologic characteristics of *Neisseria* spp. and *B. catarrhalis* after growth on LBVT.SNR medium at 37°C for 48 h in a CO_2-enriched atmosphere are summarized in Table 1. All strains produced colonies 1 to 2 mm in diameter. Although many, but not all, *N. sicca* colonies produce wrinkled, adherent colonies, few colonies of this type were found in the isolates studied, and we presumed that most of the *N. perflava-N. sicca* isolates were *N. perflava*. We isolated a few strains of *N. mucosa* that produced wrinkled, adherent colonies similar to those described for *N. sicca*.

Strains of different *Neisseria* spp. or multiple strains of *N. perflava-N. sicca* were isolated from the oropharynges of individuals. The colonial morphology of isolates and production of polysaccharide were useful for initial differentiation between strains and species on the isolation medium. The final color of the culture medium was not useful for differentiating between species because the color of the indicator reflected the proportion of sucrose-positive and *N. flava* colonies present. If *N. flava* was present, the final color of the medium was orange. If *N. flava* was not present, the final color of the medium was pink because of acid production from sucrose by the *N. perflava-N. sicca* and *N. mucosa* strains.

Isolation of commensal *Neisseria* spp. from clinical specimens. Gram-negative, oxidase-positive diplococci were isolated from the oropharynges of 202 (96.6%) of 209 persons, including 83 women, 92 heterosexual men, and 27 homosexual men. Of these patients, only one woman had pharyngeal gonorrhea. The frequency of isolation of *Neisseria* spp. and *B. catarrhalis* from the 202 patients is shown in Table 2. Colonies representative of different colonial morphologic types of *N. perflava-N. sicca* isolated from individuals

TABLE 2. Frequency of isolation of *Neisseria* spp. and *B. catarrhalis* from the oropharynges of 202 adults attending the Seattle-King County Sexually Transmitted Diseases Clinic, Harborview Medical Center, 1982 to 1983

Species	No. (%) of individuals colonized		
	Men		Women (n = 83)[a]
	Heterosexual (n = 92)[a]	Homosexual (n = 27)[a]	
N. perflava[b]-*N. sicca*[c]	89 (97)	26 (96)	79 (95)
N. mucosa	21 (23)	6 (22)	23 (28)
N. flava[d]	26 (28)	9 (33)	18 (22)
N. cinerea	25 (27)	10 (37)	22 (27)
N. meningitidis	4 (7)	4 (29)	0
N. lactamica	1 (1)	0	1 (1)
B. catarrhalis	4 (4)	1 (4)	4 (5)

[a] These denominators are for all species, with the exception of *N. meningitidis*. The denominators for *N. meningitidis* are 54, 14, and 30, respectively.
[b] *N. perflava*, *N. subflava* biovar *perflava*.
[c] *N. sicca* strains cannot be distinguished from *N. subflava* biovar *perflava* on the basis of biochemical tests; on the basis of colonial morphology, very few isolates were *N. sicca*.
[d] *N. flava*, *N. subflava* biovar *flava*.

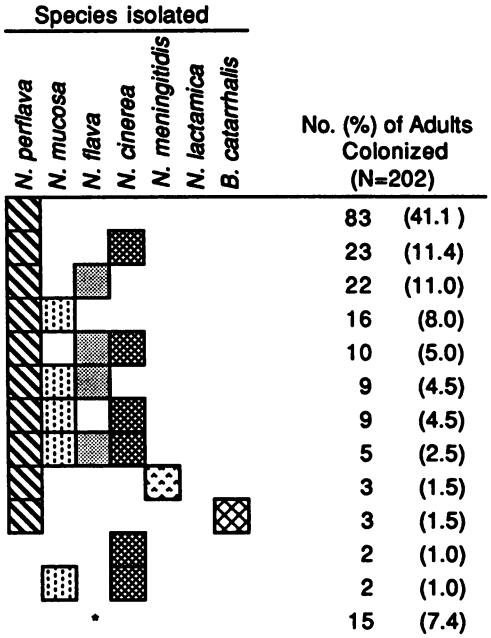

FIG. 1. Frequency and colonization patterns of the oropharyngeal neisserial flora from 202 adults in Seattle, Washington, from 1982 to 1983, by *Neisseria* spp. and *Branhamella catarrhalis*. Shaded boxes represent the isolation of the corresponding species from the number of patients indicated. The patterns of colonization are arranged in order of decreasing frequency of isolation. Some patterns of colonization, indicated by the asterisk, were each detected in only one patient and included the following isolates: 11 *N. perflava*, 9 *N. mucosa*, 7 *N. flava*, 6 *N. cinerea*, 5 *N. meningitidis*, 2 *N. lactamica*, and 6 *B. catarrhalis*.

remained morphologically distinct when subcultured onto GCK medium and were considered to be different strains. More than one colony form of *N. perflava-N. sicca* was isolated from 155 (76.7%) of 202 individuals. *Kingella denitrificans* was not isolated.

N. cinerea was isolated from the oropharynges of 25 (30.1%) of 83 women, 22 (23.9%) of 92 heterosexual men, and 10 (37.0%) of 27 homosexual men. Although differences were not significant, *N. cinerea* was isolated more frequently from homosexual men than from either heterosexual men ($P = 0.32$) or women ($P = 0.29$). *N. meningitidis* was the only other *Neisseria* sp. isolated more frequently from homosexual men. There was no difference in the frequency of isolation of other *Neisseria* spp. according to gender or sexual preference. *N. cinerea* was isolated from the urethra of only one heterosexual man, and no other *Neisseria* spp. were isolated from genital specimens.

The frequency of concurrent colonization of the oropharynges of patients by *N. cinerea* and other *Neisseria* spp. or *B. catarrhalis* is shown in Fig. 1. *N. cinerea* was isolated with most other species on at least one occasion. None of the colistin-sensitive species were isolated on Thayer-Martin medium in this study.

Two general patterns of neisserial oropharyngeal flora were observed. A total of 41% of individuals were colonized by strains of *N. perflava-N. sicca* alone. Some individuals were colonized heavily by as many as three or four different strains of *N. perflava-N. sicca*, and rarely by other species. Other individuals were colonized by a variety of species, each of which was isolated in low numbers. In general, detection of *N. cinerea* in patients heavily colonized with sucrose-positive *Neisseria* spp. was more difficult than in those who were not. There were no differences between homosexual men, heterosexual men, and women with respect to the patterns of colonization by other *Neisseria* spp.

Persistence of *N. cinerea* in adults. In the second phase of these studies, cultures were obtained from 16 volunteers weekly to determine whether *N. cinerea* persisted in the oropharynx; *Neisseria* spp. were isolated from 15 (93.8%) of 16 volunteers. As observed in the first phase of the study, volunteers were either heavily colonized by *N. perflava-N. sicca* or were sparsely colonized by a variety of species and the pattern of colonization was generally consistent for each person throughout the study period. *N. cinerea* was isolated from 10 (62.5%) of 16 volunteers during the study but was not isolated in each week. *N. cinerea* was isolated in at least 3 weeks from six (37.5%) individuals who were consistently colonized sparsely by a variety of species.

DISCUSSION

In this study, *N. cinerea* was a common inhabitant of the oropharynges of adults but was rarely isolated from genital sites. *N. cinerea* was isolated more frequently from homosexual men than from heterosexual men or women, but the difference was not significant. Strains of *N. cinerea* were not present in large numbers in any specimen and concomitantly colonized the oropharynx with other *Neisseria* spp. Serially cultured samples from volunteers generally had consistent patterns of colonization by *Neisseria* spp. characterized either by heavy colonization with several strains of *N. perflava-N. sicca* or by sparse colonization with a larger number of species.

No prospective studies of the commensal neisserial flora of the oropharynx have been made since nitrate reduction was introduced as a differential test for the identification of *Neisseria* spp. (3), the redescription of *N. cinerea* (18) and *N. mucosa* (22), or the description of *N. lactamica* (16). We isolated *N. cinerea* more frequently than previous investigators did. In previous studies, specimens were inoculated on nonselective media such as blood agar or ascitic agar (9, 14, 15). Our own preliminary studies were made by using sheep blood agar (data not shown). Cultures were heavily contaminated by normal flora; it was difficult both to isolate *Neisseria* spp. that occurred in relatively small numbers in the oropharynx and to differentiate between *Neisseria* spp. on the basis of their colonial morphology on sheep blood agar.

Early studies of the neisserial flora of the oropharynx were also limited by the use of inappropriate serum-containing media to detect acid production from carbohydrates by the oxidative *Neisseria* spp. (14, 15, 25, 26). Because of their inability to obtain consistent patterns of acid production from carbohydrates from many strains, even with prolonged incubation, Wilson and Smith (25) suggested that the "fermentation" tests did not provide a reliable method for distinguishing between *Neisseria* species and that *N. catarrhalis*, *N. flavus*, *N. cinereus*, *N. mucosus*, and *N. siccus* be combined into a single species, "*Neisseria pharyngis*." Berger subsequently showed that *Neisseria* spp. were oxidative and thus produced less acid than fermentative organisms (1). By using a medium formulated to detect acid production from *Neisseria* spp., he found that they produced less acid from the monosaccharides glucose and fructose, than from the disaccharides maltose and sucrose (4) and that, because strains of *N. flava* and *N. subflava* also produced ammonia from peptone (9), they would neutralize

any acid produced in media that had been used previously. Berger (5) also suggested that, although *N. flava* and *N. subflava* appeared to be variants of the one species, they were distinctly different from *N. perflava* and *N. sicca*. Serologic studies also supported these findings (7).

Although *B. catarrhalis* subtypes, which almost certainly included *N. cinerea*, were isolated in early studies (14, 15, 26), the proportions of isolates belonging to each subtype were not determined. Thus, it is impossible to estimate the frequency of occurrence of *N. cinerea* from these studies.

Berger and Wulf (9) characterized the neisserial flora isolated on sheep blood agar from the oropharynges of 202 individuals. Strains of *N. perflava-N. sicca* were isolated from 88% of patients, and on the basis of serologic studies, it was estimated that 55% of these strains were *N. perflava* and 45% were *N. sicca*, although many *N. sicca* strains did not produce wrinkled colonies. The frequencies of isolation of *N. perflava* and *N. sicca* were almost certainly overestimated in these studies, however, because strains of *N. mucosa* were not differentiated. Strains of *N. flava* and *N. subflava* were isolated from 4 and 11% of individuals, respectively. Asaccharolytic isolates (*B. catarrhalis* and *N. cinerea*) accounted for only 3% of strains. It is unclear whether the isolates represented one isolate from each patient or multiple isolates from fewer patients. Because none of the six asaccharolytic isolates reacted with *B. catarrhalis*-specific or *N. flavescens*-specific antiserum (9), it is likely that these isolates were *N. cinerea*. No information is given about patterns of colonization of these patients by *N. cinerea* and other *Neisseria* spp.

Using a selective medium for *B. catarrhalis*, Berger (2) isolated "*N. catarrhalis*" from 15% of patients. Because this medium did not permit differentiation between the "saccharolytic" *Neisseria* spp., there is no information on the coexistence of *Neisseria* spp. in healthy adults. In a retrospective study of these isolates, Berger and Paepcke (8) found that strains of *N. cinerea* accounted for 93% of the asaccharolytic isolates obtained previously (2).

The use of a selective medium that also permitted differentiation between many commensal *Neisseria* spp. enhanced the detection and isolation of *N. cinerea* in our study and also permitted an immediate appreciation of the complexity of the neisserial flora of the oropharynx. The frequency of isolation of strains of *N. cinerea*, both in persons colonized heavily by strains of *N. perflava-N. sicca* and in those persons colonized more sparsely by a variety of species, may be a conservative estimate of the true prevalence of this species. Strains of *N. cinerea* generally occurred in small numbers in all cultures from which the species was isolated. Strains of *N. cinerea* may have been present in the persons heavily colonized by sucrose-positive species but may not have been observed because of overgrowth. In those persons sparsely colonized by several species, it is possible that strains of *N. cinerea* were present in such low numbers that they were not detected.

These data confirm the complexity of the neisserial flora of the oropharynx and demonstrate that *N. cinerea* is a frequent inhabitant of the oropharynx in adults. The exact frequency of isolation of *N. cinerea* is affected by the type of neisserial flora of the patient from whom the specimen is collected. In a recent survey, *N. cinerea* was isolated from the oropharynges of 27% of adults in DeKalb County, Ga. (data not shown). Thus, it does not appear that *N. cinerea* will be isolated only in limited geographic regions. Although strains of *N. cinerea* were isolated infrequently from genital specimens and were not isolated on gonococcus-selective medium during this study, the possibility that this species may be isolated must be considered when strains resembling the gonococcus are isolated from adults at low risk for gonorrhea. The laboratory identification of *N. gonorrhoeae* from patients at low risk for gonorrhea must be carefully substantiated by using tests appropriate for differentiating between *N. cinerea* and *N. gonorrhoeae*.

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