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Juan A. Sáez Nieto
Carmen Marcos
Ana Vindel

Servicio de Bacteriología. Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

Multicolonization of human nasopharynx due to *Neisseria* spp.

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Correspondence to:
Juan A. Sáez Nieto.
Servicio de Bacteriología. Centro Nacional de Microbiología. Instituto de Salud Carlos III.
28220 Majadahonda, (Madrid). España.
Tel.: +34-915097972. Fax: +34-915097966.
E-mail: jasaez@isciii.es

Summary The colonization due to *Neisseria* spp. in the nasopharynx of forty healthy adults was studied by using a selective medium that allows the differentiation of *Neisseria* species and inhibits the rest of pharyngeal microbiota. The medium detected a variety of colonial morphology types and some metabolic characteristics of the isolates. We demonstrated the multicolonization by several *Neisseria* spp. in the same individual, and we isolated several strains of the same species, after analysis by pulsed-field gel electrophoresis (PFGE) patterns obtained from the different colonial types previously identified as the same species. The forty adults studied were colonized by 112 forms of *Neisseria* spp., and twelve colonization patterns were obtained: one species (45%), two (45%), three (7.5%) and four (2.5%). *N. perflava-N. sicca*, either alone or in combination with other species was the most frequent isolate (92.5%). The analysis of PFGE patterns obtained from different colonial types revealed the multicolonization by several strains of the same species in some individuals. This fact was found in *N. perflava-N. sicca* (50%) and *N. mucosa* (2.5%).

Key words *Neisseria* spp. · Pulsed-field gel electrophoresis (PFGE) · Nasopharynx colonization · Nasopharyngeal microbiota · Colony morphology

Introduction

The commensal *Neisseria*, differently from pathogenic strains, rarely grows on media selective for meningococci. Only *N. lactamica* [11] and *N. polysaccharea* [5], and occasionally other species, allow to carry out prevalence studies, mainly in children [3, 8, 13].

To determine nasopharyngeal infection rates by other *Neisseria* spp. a colistin-free medium is needed, but this medium inhibits the growth of other bacterial species and does not allow to differentiate groups of *Neisseria* [1, 17]. Some studies have been performed by using media selective for commensal *Neisseria* which also differentiated asaccharolitic species [2] or several different groups of species [10].

We used a selective media previously described [10], which allows to differentiate several morphological colonial types and some metabolic characteristics of *Neisseria* isolates. We also used pulsed-field gel electrophoresis (PFGE) of total bacterial DNA to determine the possible multicolonization of carriers by several isolates of the same species.

Finally we studied the prevalence of isolates with moderate resistance to penicillin, because the commensal *Neisseria* spp. can play a major role in the recent appearance of moderate resistance to penicillin in *N. meningitidis* [6, 12, 14].

Materials and methods

Strains, pharyngeal cultures and identification We studied 112 isolates from forty randomly chosen individuals among the personnel of the Departments of Bacteriology and Parasitology of our center. Nasopharyngeal specimens were inoculated on LBVT.SNR medium, previously described by Knapp and Hook [10]. This medium contained 1% Bacto-Tryptone (Difco), 0.5% yeast extract, 0.5% sodium chloride, and 1.5% Bacto-Agar (Difco). Neutral Red (0.3% wt/vol) at a concentration of 5 ml/l was added. After the sterilization, the medium was cooled to 56°C for addition of 1% of sucrose; vancomycin and trimethoprim were each added to a final concentration of 3.0 µg/ml. The complete medium was used to isolate strains of *Neisseria* spp. and *Moraxella catarrhalis* and to test for polysaccharide production from saccharose. Specimens were also plated on Thayer Martin medium to ensure the recovery of *N. meningitidis* and *N. lactamica*.The plates were incubated at 37°C in 5% CO_2 atmosphere during 48 h. One colony of each morphologic type was subcultured in the same medium.

All isolates of Gram-negative, oxidase-positive diplococci were identified by their patterns of acid production with sugars, nitrate reduction and other biochemical tests included in the

Table 1 Frequency of isolation of *Neisseria* spp. and *Moraxella catarrhalis* from the nasopharynges of 40 adults

Species	No. colonized	No. strains[a]	No. strains[b]
N. perflava/sicca	37 (92.5)[c]	78	64
N. mucosa	10 (25.0)	14	11
N. flava	8 (20.0)	8	8
N. cinerea	4 (10.0)	4	4
N. lactamica	2 (5.0)	2	2
N. flavescens	2 (5.0)	2	2
N. subflava	1 (2.5)	1	1
M. catarrhalis	1 (2.5)	1	1
N. meningitidis	2 (5.0)	2	2
Totals	40	112	95

[a] Strains isolated according with colonial types and biochemical identification. [b] Strains characterized by means PFGE. [c] Percentage

Rosco System (Rosco Diagnostica, Denmark) (ONPG, beta-lactamase, tributyrin, gamma-glutamyl aminopeptidase) according to the instructions of the manufacturer. Some strains with atypical patterns or with doubtful test results were confirmed in CTA medium and MHBT medium for sugars fermentation and other conventional tests employed in our Neisserial Reference Laboratory [5, 12].

Susceptibility to penicillin was studied by using the agar dilution method, which detects the minimal inhibitory concentration of penicillin [14].

Pulsed-field gel electrophoresis Genomic DNA was prepared in agarose plugs as has been previously described [4]. For restriction endonuclease digestion of DNA, small slices of the agarose plugs were placed into a mixture of 125 µl of reaction buffer and 15 units of *Spe*I, and incubated at 37°C overnight. The slices were placed in wells of a 1% MP agarose gel (Boehringer Mannheim) made with 0.5 × TBE (Tris Borate 45 mM/EDTA 1 mM pH 8) and the wells were sealed with the same agarose. Gels were electrophoresed in CHEFF DR II (Biorad) at 12°C and 200 V. The pulse was ramped from 0.5 to 30 s over 20 h. DNA fragments were visualized under short wave UV after staining with ethidium bromide and then photographed.

Results

Isolation of *Neisseria* spp. in the nasopharynx Gram-negative, oxidase-positive cocci were isolated from the nasopharynx of the 40 (100%) persons studied. The frequency of isolation of *Neisseria* spp. and *Moraxella catarrhalis* is shown in Table 1. In the first step, 112 neisserial strains were isolated according to the different colonial morphologic types. We considered for this classification: color, surface, opacity, form and size after 48 h of incubation. *N. perflava/sicca* was the most frequent species (92.5%) in carriers, and only two persons had *N. meningitidis* in their nasopharynges. We found concurrent colonization of the nasopharynges of carriers by several species (Fig. 1) in 22 persons (55%), with 12 colonization patterns (P1-P12).

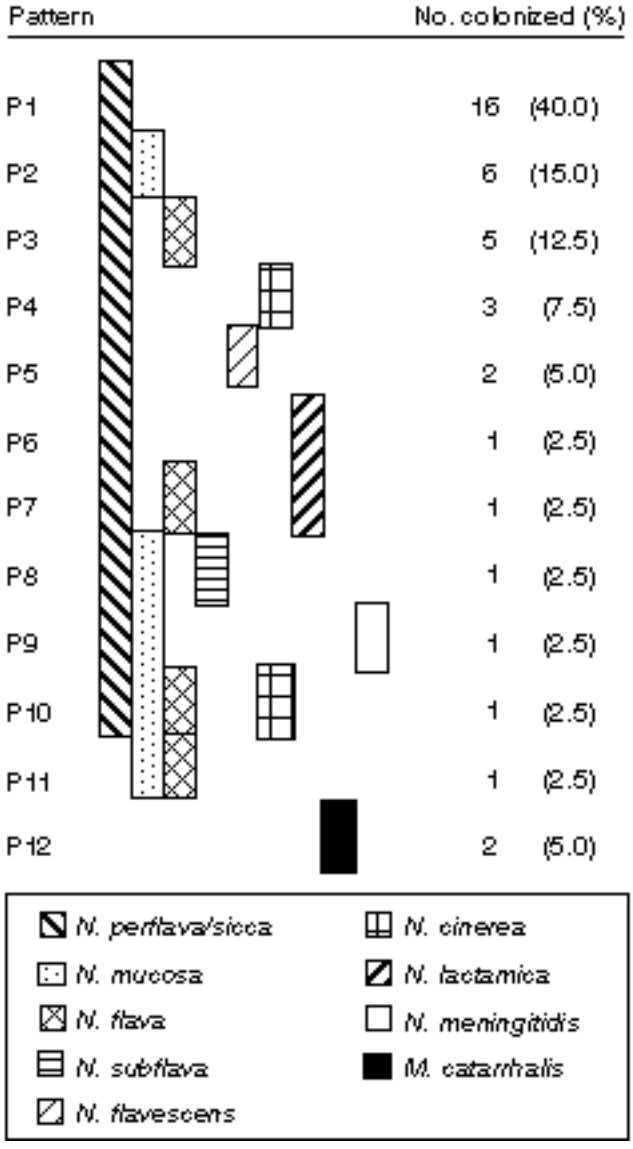

Fig. 1 Frequency and colonization pattern of nasopharingeal *Neisseria* spp. from 40 adults. Boxes represent the different species isolated from the number of carriers indicated

Of the individuals, 40% were colonized by strains of *N. perflava/sicca* alone (P1), and others were colonized by a variety of species (two, three or four). In most cases, *N. perflava/sicca* was one of the species isolated.

Pulsed-field gel electrophoresis We studied all the strains of the same species isolated in one individual (according to the different colonial types found) to determine multicolonization due to strains of the same species in several individuals. This fact was observed in the case of *N. perflava/sicca* and *N. mucosa* isolates. So, we studied the PFGE patterns of 85 strains (78 *N. perflava/sicca* and 7 *N. mucosa*). Table 1 shows also the identity of the different strains of the same species in the carriers. After application of PFGE, several strains (with distinct colonial types) had identical patterns, 95 being the definite number of strains obtained from the 40 carriers (Table 1).

Table 2 shows several representative examples; in the carrier AG, five distinct colonial types of *N. perflava/sicca* were isolated. PFGE analysis showed that this carrier had only 2 different strains of *N. perflava/sicca*. In the carrier MS, the three initial *N. mucosa* strains isolated turned out to have the same PFGE pattern.

Figure 2 shows that 20 colonization patterns were obtained after the application of PFGE. The results showed that the multicolonization by the same species (22/40; 55%) is very frequent (in both *N. perflava/sicca* and in *N. mucosa* was detected in a carrier). Figure 3 shows some representative PFGE patterns. In all strains studied, identical PFGE correlated with the same minimal inhibitory concentration of penicillin (Table 2).

***Neisseria* spp susceptibility to penicillin** Only 8 neisserial strains were sensitive to penicillin (MIC < 0.06 µg/ml) (3 *N. perflava/sicca*, 2 *N. mucosa*, 1 *N. flava*, 1 *N. subflava* and 1 *Moraxella catarrhalis*), 13 strains were resistant (MIC > 2 µg/ml), six of them being beta-lactamase producers (4 *N. perflava/sicca*, 1 *N. mucosa*, 1 *N. flava*). The other strains had MICs ranging from 0.12 to 1 µg/ml.

Discussion

Commensal *Neisseria* are usual components of the nasopharyngeal microbiota [11], although they can be occassionally isolated from other sites either as contaminants or as casual agents of several infections such as endocarditis, bacteriemia and meningitis [7, 9, 11, 16]. Besides, their coexistence in the nasopharynx with a major pathogen (*N. meningitidis*), as well as the recent demonstration of the transmission of moderate resistance to penicillin in meningococci [6, 14], explain both the prevalence and distribution of these commensal species and their penicillin sensitivity or resistance rates.

Currently, only prevalence studies for *N. lactamica* are reliable because this species can grow well in selective media for meningococi [3, 8, 13]. Most prevalence studies of *Neisseria* spp. were performed with nonselective media [17] and poor methods for species identification. Recently Knapp and Hook [10] studied the prevalence of *Neisseria* spp. by using a selective medium that inhibits the rest of the pharyngeal microbiota. We studied the prevalence of *Neisseria* spp. in nasopharynx from 40 adults with that selective medium.

Neisseria spp. was isolated from 100% of the individuals studied, and *N. perflava/sicca* was isolated from 92.5%. Twelve

Table 2 Identification of multiple species and strains variants of *Neisseria* spp.

Carrier	Strain	Specie[a]	Colonial type[b]	Size (mm)	MIC[c]	PFGE pattern	Final identification
J	J1	*N. mucosa*	PEGS	4	0.12		*N. mucosa*
	J2	*N. perflava/sicca*	YEGS	4	0.5	12	*N. perflava/sicca*
	J3	*N. perflava/sicca*	YUMR	2	0.5	13	(3 distinct strains)
	J4	*N. perflava/sicca*	PUMR	1	0.5	1	
M	M1	*N. perflava/sicca*	BEMS	2	0.06		*N. perflava/sicca*
	M2	*N. perflava/sicca*	YEGS	1	0.06	6	(2 distinct strains)
	M3	*N. perflava/sicca*	PEGS	2	> 4	5	
	M4	*N. perflava/sicca*	PUMS	2	0.06	6	
MS	MS1	*N. mucosa*	YEGS	3	1	10	*N. mucosa*
	MS2	*N. mucosa*	PEGS	2	1	10	(1 strain)
	MS3	*N. mucosa*	OEGS	2	1	10	
	MS4	*N. perflava/sicca*	OEGS	4	0.12		*N. perflava/sicca*
AG	AG1	*N. perflava/sicca*	YUMR	2	1	8	*N. perflava/sicca*
	AG2	*N. perflava/sicca*	YEGS	2	0.25	14	(2 distinct strains)
	AG3	*N. perflava/sicca*	BEMS	3	1	8	
	AG4	*N. perflava/sicca*	PEGS	1	1	8	
	AG5	*N. perflava/sicca*	PUGR	3	1	8	

[a] Biochemical test identification. [b] P, pink; O, orange; Y, yellow; B, brown; U, undulate; E, entire; M, matt; G, glistennig; R, rough; S, smooth. [c] Minimal inhibitory concentration of penicillin (µg/ml)

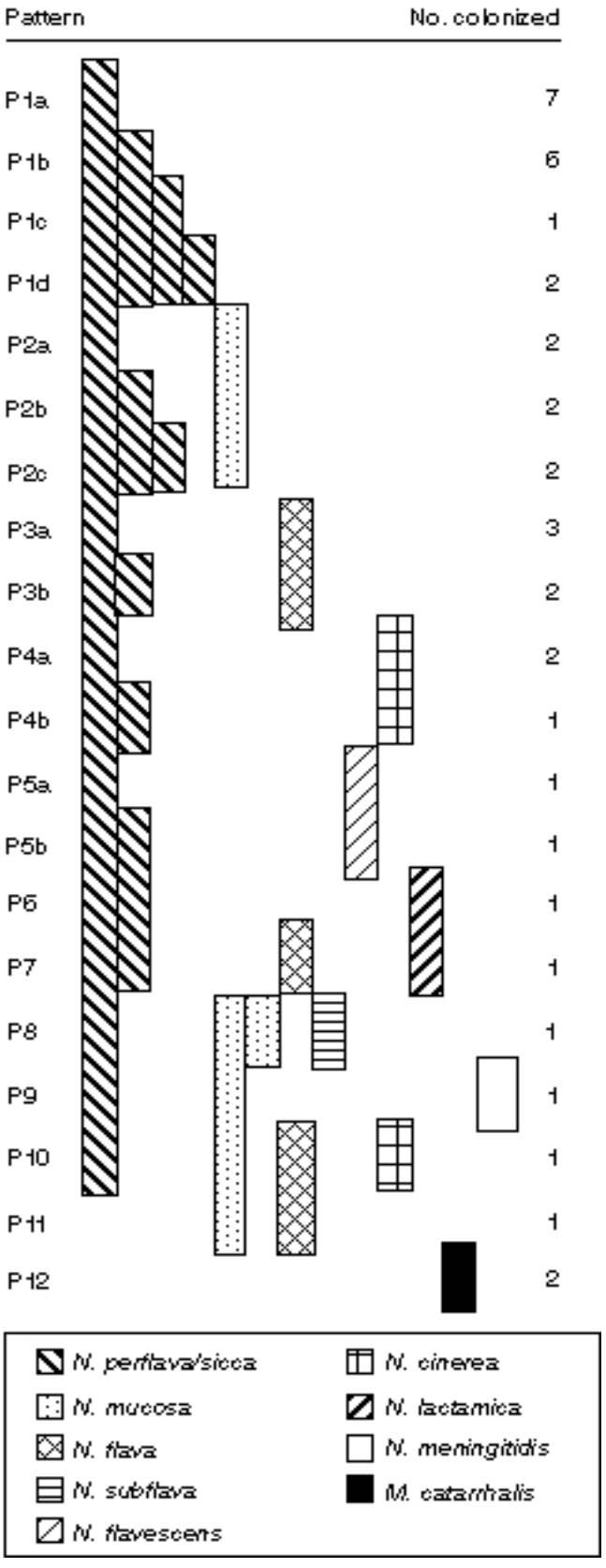

Fig. 2 Frequency of multicolonization of strains of the same species after the differentiation with the PGFE method

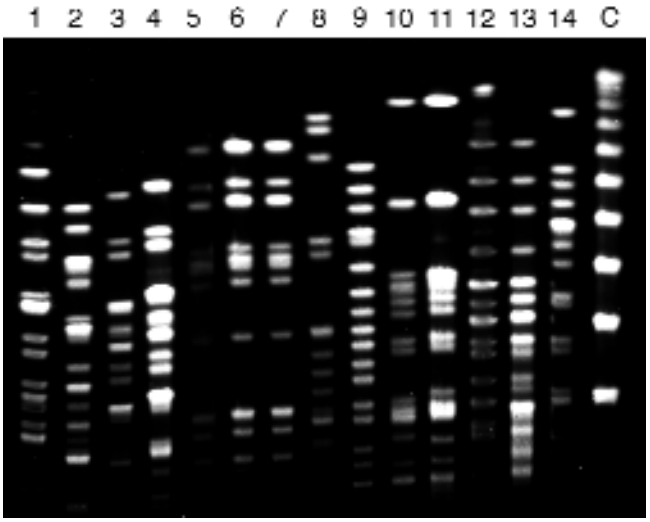

Fig. 3 Representative *SpeI* PFGE patterns of strains of *N. perflava/sicca* and *N. mucosa*. Lanes 1-2 *N. mucosa* (IS1, IS4 strains); lanes 3-4 *N. mucosa* (R1, R2); lanes 5-7 *N. mucosa* (MS1, MS2, MS3); lanes 8-9 *N. perflava/sicca* (A1, A2); lanes 10-11 *N. perflava/sicca* (E1, E2); lanes 12-14 *N. perflava/sicca* (IJ1, IJ2, IJ3); lane C, DNA size standard

patterns of colonization were found, and in ten of them *N. perflava/sicca* was isolated either alone or with several other species. The selective medium shows a great variety of morphological types of colonies. By using PFGE we could determine whether a given carrier was colonized by several isolates of the same species.

The application of this method revealed a higher complexity of the neisserial microbiota studied. In fact, we found the concurrent colonization of several strains of the same species in an individual (Fig. 2). The complexity observed according to the morphological type of colonies was confirmed when we used PFGE. There were 20 definite patterns of colonization (Fig. 2), some of which with four different strains of the same or different species. The high prevalence of saccharolitic species found is similar to that found by Knapp and Hook and others [1, 10]. However, we found that the prevalence of *N. cinerea* was very low, and also that, in the case of two persons carriying *N. meningitidis* in their nasopharynges, only this species was isolated. Both strains were moderately resistant to penicillin.

A high percentage of strains moderately resistant to penicillin (MIC 0.12–1 µg/ml) (77.9%) was found, and 8% of strains were penicillin resistant (with 6 strains beta-lactamase producers). This is very relevant; in fact, the transmission of resistance from commensal species to meningococcus has been demonstrated and could explain to some extent the current emergence of meningococcal strains moderately resistant to penicillin in Spain and other countries [15].

Selective media are very useful for studies of prevalence of normal microbiota because they allow a first step to differentiate strains. Besides, the concurrent utilization of a molecular marker allows to differentiate strains within a given

species. This strategy could be used for other groups of species such as "viridans" streptococci, and other commensal inhabitants. It can also provide a better insight of the complexity of human "normal" microbiota.

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