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RESEARCH ARTICLE

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Genotyping of commensal *Neisseria* spp strains by pulsed-field gel electrophoresis and 16S rRNA gene sequencing

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Background: We investigated the diversity of the primary sequences of the 16S rRNA genes among 46 commensal *Neisseria* strains and evaluated the use of this approach as a molecular typing tool in comparison with PFGE analysis.

Methods: Identification to the genus was done using conventional methods and API NH (bio-Mérieux®). Identification to species level was based on 16S rRNA gene sequencing. PFGE analysis was done using *Spe*I.

Results: Fourteen, two, three and fourteen 16S rRNA sequence types were found among twenty *Neisseria flavescens*, two *Neisseria sicca*, five *Neisseria macacae* and nineteen *Neisseria mucosa* clinical isolates. Forty-three different PFGE patterns were found among the tested strains.

Conclusion: We demonstrated a high diversity among 16S rRNA genes which was reflected by PFGE analysis.

KEYWORDS

16S rRNA gene, commensal *Neisseria*, genotyping, pulsed-field gel electrophoresis

1 | INTRODUCTION

Phenotypic and genetic variation in bacteria can take bewilderingly complex forms even within a single genus. One of the most intriguing examples of this is the genus *Neisseria* which consists of Gram-negative bacteria that colonize the mucous membranes of mammals.[1] *Neisseria* spp colonization of the respiratory tract, a phenomenon commonly referred to as carriage, represents a successful commensal relationship between the host and the bacterium, with the host experiencing no detectable pathology. Commensal bacteria comprise a large part of the microbial world, playing important roles in human development, health and disease.[2] However, little is known about the genomic content of commensals or how related they are to their pathogenic counterparts. Complex relatedness among *Neisseria* species makes it challenging to study their genotypic characterization. Especially their rRNA genes which are often organized as part of a multigene family. Members of the genus *Neisseria* contain four rRNA operons.[3]

Genotyping methods may be compared on the basis of a range of criteria, including sensitivity, availability, reproducibility, rapidity, ease of use, and cost. One of the most important characteristics is discriminatory power.[4]

One aim of our study was to establish if sequence differences exist in 16S rRNA gene sequences of commensal *Neisseria* clinical isolates, because if intraspecific variation exists in these genes it may be possible to use the method for subtyping. Also, during the course of this study, we compared the abilities of 16S rRNA sequencing-based typing and pulsed-field gel electrophoresis (PFGE) typing to differentiate clinical isolates of commensal *Neisseria*.

2 | MATERIAL AND METHODS

We analyzed 55 *Neisseria* spp clinical isolates collected between 2003 and 2008, from various sources, from patients monitored in the Bone Marrow Transplant Center of Tunisia.

Neisseria perflava 8022, *N. mucosa* 8023, *N. sicca* 8024, *Neisseria cinerea* 8025, and *Neisseria lactamica* 9366 were used as quality-control reference strains (R. Leclercq, CHU Caen).[5]

2.1 | Bacterial DNA preparation

A bacterial pellet, obtained from centrifuging 2 mL of an overnight-grown culture from a single colony, was washed twice in TE buffer

(10 mmol/L Tris–Cl pH8, 1 mmol/L EDTA). Pelleted cells were suspended in 500 μL TEG (50 mmol/L Tris Hcl, 10 mmol/L EDTA, 100 mmol/L glucose) supplemented with 3 mg/mL lysozyme and 25 mg/L RNase. After an incubation period of 30 minutes at 37°C, the cells were lysed by the addition of 20 mg/mL proteinase K and 10% sodium dodecyl sulphate solution, and incubation for 30 minutes at 55°C. The solution was extracted twice with chloroform/isoamyl alcohol (24:1 v/v) and with phenol/chloroforme/isoamyl alcohol (25:24:1) by volume. Chromosomal DNA in the aqueous solution was pelleted with two volumes of ethanol and incubated for one night at −20°C. After brief centrifugation, the bacterial pellet was resuspended in 100 μL of sterile distilled water and stored at −20°C until use.

2.2 | Bacterial identification

The identification of genus was based on conventional methods: Gram strain, oxidase test and API NH (bio-Mérieux®) characterization. The identification of species was based on 16S rRNA gene sequencing. For all strains, the 16S rRNA gene was amplified by PCR and sequenced using the primers up1-F: AGAGTTTGATCCTGGCTCAG and up1-R: GTTACCTTGTTACGACTT.[6] The PCR thermal program consisted of initial denaturation at 95°C for 5 minutes, 30 cycles of amplification (denaturation at 95°C for 60 seconds, annealing at 54°C for 60 seconds and elongation at 72°C for 90 seconds) and final elongation at 72°C for 10 minutes.[7] The products (1406 bp) were separated with an Applied Biosystems 3730xl DNA analyzer (96 capillary type). The resultant DNA sequences were assembled using the CLC WORKBENCH of computer programs. The nucleotide sequence of the 16S rRNA gene fragments were aligned and compared using the GenBank and EzGenome (http://ezgenome.ezbiocloud.net) databases. In our study, the criteria established for identification of the bacterial isolates were: ≥95% to ≥99% sequence similarity prototype strain sequence in the database defined a bacterial genus; ≥99% sequence similarity to the prototype strain sequence defined a bacterial species. Only strains showing concordant results with the two databases were included in this study.

2.3 | 16S rRNA type determination

The nucleotide sequence of the 16S rRNA gene fragments were aligned and compared using the ClustalW program. A number was assigned to each different 16S rRNA gene sequence; a single base change was considered a new 16S rRNA type. When discrepancies in the alignment were obtained, the 16S rRNA gene amplification and sequencing of the entire gene were repeated.

2.4 | PFGE

PFGE was done as described previously by Mechergui et al.[8] Our 46 isolates were digested with *Spe*I, and the resulting fragments were separated by electrophoresis in CHEFF DR II (Biorad) in 1% MP agarose gel (Boehringer Mannheim) made with 0.5× TBE (Tris Borate 45 mmol/L/EDTA 1 mmol/L pH 8). The pulse was ramped from 0.5 to 30 seconds over 20 hours.

Lambda ladder DNA (New England BioLabs, Beverly, USA) was used as molecular weight PFGE marker. Banding patterns were inspected manually, and the criteria of Tenover et al.[9] were used to determine strain relatedness.

3 | RESULTS

The nucleotide sequence of 16S rRNA gene fragment (1406 bp) from all our strains were compared to the previously identified sequences hosted on Genbank and EzGenome databases. We obtained the same identification result for 46 strains. These strains, based on the BLAST results, showed 99%-100% sequence similarity to their nearest database entries.

They were assigned to four diverse species represented by *N. flavescens* (44%), *N. mucosa* (41%), *N. macacae* (11%) and *N. sicca* (4%).

Three (16S rrn type I-1 to 16S rrn type I-3), two (16S rrn type II-1 and 16S rrn type II-2), fourteen (16S rrn type III-1 to 16S rrn type III-14), and fourteen (16S rrn type IV-1 to 16S rrn type IV-14) 16S rRNA sequence types were, respectively, found among 5 *N. macacae*, 2 *N. sicca*, 19 *N. mucosa* and 20 *N. flavescens* isolates.

PFGE analysis detected 46 unique genome patterns among the tested strains (Figure S1) (Table 1).

4 | DISCUSSION

Since no reference "gold standard" is commonly used for speciation of *Neisseria*, we used 16S rRNA gene as an identification tool because the use of another identification method could lead us into error. Indeed, we could study the presumptive intraspecific diversity of 16S rRNA gene among strains belonging actually to different species when using 16S rRNA sequence–based identification.

It is established that heterogeneity exists even within the well-conserved 16S rRNA gene. This is due to the frequently recombinant genotype of members of *Neisseria* genus.[10] In fact, 16S rRNA gene of commensal *Neisseria* is affected by recombination events leading to genetic diversity.[11] Also, when segments of the 16S rRNA gene are transferred from one species to another, this is likely to happen in just one of the copies of the ribosomal operons first.[12] This would lead to strains with two different types of 16S rRNA genes.

Previous reports of transformation in *Neisseriae* have suggested that different regions of the genome may have different recombination rates. In *N. meningitidis*, analysis of the 16S sequence data show that the 16S rRNA gene may recombine as frequently as the housekeeping genes.[13] Lateral transfer and recombination do not lead to rapid changes in rRNA genes. One of the main reasons would be that the tertiary structure of the ribosomal RNAs is essential for the association with components of the translation apparatus, such as the ribosomal proteins. During evolution, gradual changes may have been introduced in the rRNA genes and ribosomal protein genes, leading to the slow emergence of new ribosomal complexes. This hypothesis is

supported by the fact that mutations in stems of the stem-loop structures in the 16S rRNA gene are often associated with mutations in opposing parts of the rRNA strand, leading to restoration of the stem structure.[12]

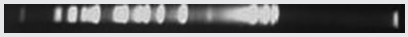
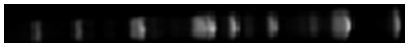
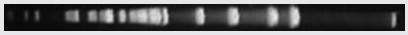
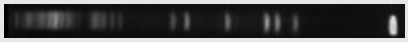
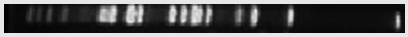
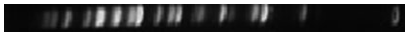
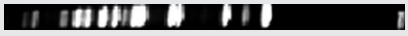
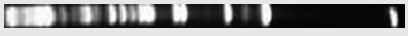
In the present paper, we found a large degree of diversity among 16S rRNA genes of commensal *Neisseria* strains, suggesting that 16S rRNA gene sequencing might be a useful molecular typing tool. In previous studies, interstrain variation in the 16S rRNA gene of *N. meningitidis* was used as a molecular typing tool.[13,14] In these studies, 16S typing was the most sensitive and specific typing tool to discriminate outbreak-related isolates from sporadic isolates. The performance was even better than that of multilocus sequence typing. Also, some authors conclude that molecular typing by 16S rRNA sequence determination is not only more rapid but also more accurate than traditional typing methods.[15] In fact, because of the strong conservation of regions in the rRNA genes and the presence of highly variable (noncoding) flanking regions, these genes are suitable targets for subtyping purposes.[3] Also, the rRNA genes are universally present in all organisms, can be easily obtained using PCR with universal primers, and are easy to sequence. However, other authors show some shortcomings associated with 16S rRNA use as a typing tool due to the fact that it is, relatively, a so well conserved gene that it results in a limited resolving power.[16] Indeed, the use of the 16S rRNA gene sequences for phylogeny studies has become extremely popular and has led to the reconstruction of the tree of life. In addition, the rRNA based analysis, is now a standard approach for studying microbial

TABLE 1 PFGE and 16S rRNA gene types of the commensal *Neisseria* clinical isolates

Specie (n*)	Strain	Sample	16S type	GenBank acc. no	PFGE profile
N. sicca	1458b	Respiratory tract	II-2	KC178524	
(2)	2763	Throat swabs	II-1	KC178523	
N. macacae	3767c	Mouth	I-3	KC178518	
(5)	1364b	Respiratory tract	I-1	KC178521	
	4818	Throat swabs	I-2	KC178519	
	6250	Respiratory tract	I-1	KC178520	
	5556b	Respiratory tract	I-1	KC178522	
N. flavescens	3742c	Respiratory tract	IV-11	KC178492	
(20)	4413d	Respiratory tract	IV-3	KC178493	
	6148b	Throat swabs	IV-8	KC178494	
	598b	Respiratory tract	IV-1	KC178495	
	898b	Respiratory tract	IV-5	KC178496	
	1697a	Throat swabs	IV-2	KC178497	
	2478c	Respiratory tract	IV-10	KC178498	
	3129b	Respiratory tract	IV-1	KC178499	
	3984c	Throat swabs	IV-9	KC178500	
	5009a	Throat swabs	IV-6	KC178501	
	5870	Respiratory tract	IV-2	KC178502	
	3974a	Throat swabs	IV-2	KC178503	
	6862b	Throat swabs	IV-2	KC178504	
	1258a	Throat swabs	IV-13	KC178505	
	3349a	Throat swabs	IV-4	KC178506	
	129	Respiratory tract	IV-2	KC178507	
	5232b	Throat swabs	IV-2	KC178508	

(continued)

TABLE 1 (Continued)

Specie (n*)	Strain	Sample	16S type	GenBank acc. no	PFGE profile
	6597	Throat swabs	IV-14	KC178509	
	6819	Respiratory tract	IV-12	KC178510	
	8642	Throat swabs	IV-7	KC178511	
N. mucosa	1602a	Respiratory tract	III-8	KC178487	
(19)	3869c	Throat swabs	III-3	KC178472	
	3742	Respiratory tract	III-11	KC178470	
	5078d	Respiratory tract	III-5	KC178486	
	2268an	Blood culture	III-2	KC178475	
	1942b	Throat swabs	III-13	KC178490	
	269a	Throat swabs	III-6	KC178489	
	3619a	Respiratory tract	III-12	KC178483	
	5355b	Respiratory tract	III-5	KC178484	
	6950	Throat swabs	III-10	KC178485	
	968	Throat swabs	III-2	KC178471	
	4883	Throat swabs	III-7	KC178476	
	4940a	Throat swabs	III-9	KC178473	
	5219b	Throat swabs	III-1	KC178474	
	5556a	Respiratory tract	III-4	KC178479	
	5567a	Throat swabs	III-14	KC178491	
	5884	Respiratory tract	III-4	KC178478	
	269	Throat swabs	III-6	KC178482	
	8643	Throat swabs	III-2	KC178477	

*No. of isolates.

community dynamics at high resolution[17] and to explore microbial diversity.[18]

As revealed by 16S rRNA sequences, there was a wide genetic diversity among PFGE patterns of commensal *Neisseria* spp. However, commensal *Neisseria* isolates with the same 16S rRNA type seemed to be genomically different since they belong to different clones. These data demonstrate that PFGE is more discriminating than is 16S rRNA sequence analysis for differentiating commensal *Neisseria* isolates. Indeed, the high discriminatory power of PFGE makes it the "gold standard" for DNA fingerprinting techniques. Compared with other methods, it often has superior discriminatory power because of two reasons. First, PFGE typing involves the whole genome rather than a short target sequence, thus, strains are effectively compared on a broad basis. Second, there is no requirement for prior knowledge of sequence data; all strains can be typed. However, there are also many disadvantages in using PFGE as a genotyping tool. Intra- and inter-laboratory variability is a major problem, while bands on a gel can be difficult to interpret and data are not easily portable. In addition, the nature of the genetic variation being indexed is poorly understood; there is the question of whether bands of the same size in two isolates really represent the same pieces of DNA.

In the current study, no epidemiologic connection exists between our strains since they belong to the commensal endogenous flora of different patients. Indeed, it is established that the *Neisseria* species are believed to be non-clonal bacteria with a high degree of genetic transfer within and between different species.[19]

In conclusion, high genetic diversity was identified among non-clonal clinical isolates of commensal *Neisseria*, either by 16S rRNA gene sequencing or by PFGE, with better discriminatory power for the latter technique.

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REFERENCES

1. Knapp JS. Historical perspectives and identification of *Neisseria* and related species. *Clin Microbiol Rev*. 1988;1:415-431.
2. Marri PR, Paniscus M, Weyand NJ, et al. Genome sequencing reveals widespread virulence gene exchange among human *Neisseria* species. *PLoS ONE*. 2010;5:e11835.
3. Walcher M, Skvoretz R, Montgomery-Fullerton M, Jonas V, Brentano S. Description of an unusual *Neisseria meningitidis* isolate containing and expressing *Neisseria gonorrhoeae*-specific 16S rRNA gene sequences. *J Clin Microbiol*. 2013;51:3199-3206.
4. Wassenaar TM, Newell DG. Genotyping of *Campylobacter* spp. *Appl Environ Microbiol*. 2000;66:1-9.
5. Mechergui A, Touati A, Baaboura R, Achour W, Ben Hassen A. Phenotypic and molecular characterization of β-lactams resistance in commensal *Neisseria* strains isolated from neutropenic patients in Tunisia. *Ann Microbiol*. 2011;61:695-697.
6. Petata′n-Sagaho′n I, Anducho-Reyes MA, Silva-Rojas HV, et al. Isolation of bacteria with antifungal activity against the phytopathogenic fungi *Stenocarpella maydis* and *Stenocarpella macrospora*. *Int J Mol Sci*. 2011;12:5522-5537.
7. Mechergui A, Achour W, Ben Hassen A. Comparison of 16S rRNA sequencing with biochemical testing for species-level identification of clinical isolates of *Neisseria* spp. *World J Microbiol Biotechnol*. 2014;30:2181-2188.
8. Mechergui A, Achour W, Giorgini D, Baaboura R, Taha MK, Ben Hassen A. Molecular typing of *Neisseria perflava* clinical isolates. *APMIS*. 2012;121:843-847.
9. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial typing. *J Clin Microbiol*. 1995;33:2233-2239.
10. Linz B, Schenker M, Zhu P, Achtman M. Frequent interspecific genetic exchange between commensal *Neisseriae* and *Neisseria meningitidis*. *Mol Microbiol*. 2000;36:1049-1058.
11. Smith NH, Holmes EC, Donovan GM, Carpenter GA, Spratt BG. Networks and groups within the genus *Neisseria*: analysis of *argF*, *recA*, *rho*, and *16S rRNA* sequences from human *Neisseria* species. *Mol Biol Evol*. 1999;16:773-783.
12. Schouls LM, Schot CS, Jacobs JA. Horizontal transfer of segments of the 16S rRNA genes between species of the *Streptococcus anginosus* group. *J Bacteriol*. 2003;185:7241-7246.
13. Sacchi CT, Whitney AM, Reeves MW, Mayer LW, Popovic T. Sequence diversity of *Neisseria meningitidis* 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. *J Clin Microbiol*. 2002;40:4520-4527.
14. Popovic T, Sacchi CT, Reeves MW, et al. *Neisseria meningitidis* serogroup W135 isolates associated with the ET-37 complex. *Emerg Infect Dis*. 2000;6:428-429.
15. Springer B, Stockman L, Teschner K, Roberts GD, Bottger EC. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. *J Clin Microbiol*. 1996;34:296-303.
16. Achenbach LA, Carey AJ, Madigan MT. Photosynthesis and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Appl Environ Microbiol*. 2001;67:2922-2926.
17. Cai Y, Sun Y. ESPRIT-Tree: hierarchical clustering analysis of millions of 16S rRNA pyrosequences in quasilinear computational time. *Nucleic Acids Res*. 2011;39:e95.
18. Yadav V, Prakash S, Srivastava S, et al. Identification of *Comamonas* species using 16S rRNA gene sequence. *Bioinformation*. 2009;3:381-383.
19. Qvarnstrom Y, Swedberg G. Variations in gene organization and DNA uptake signal sequence in the folP region between commensal and pathogenic *Neisseria* species. *BMC Microbiol*. 2006;6:11.

SUPPORTING INFORMATION

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