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Sulphonamide resistant commensal *Neisseria* with alterations in the dihydropteroate synthase can be isolated from carriers not exposed to sulphonamides

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Abstract

Background: Development of sulphonamide resistance in *Neisseria meningitidis* has been suggested to involve horizontal DNA-transfer from a commensal *Neisseria* species. In this study, we isolated commensal *Neisseria* from throat specimens and examined the isolates with respect to sulphonamide resistance.

Results: Three resistant clones were identified and the resistance phenotype could be explained by amino acid variations in their dihydropteroate synthase, the target molecule for sulphonamides. Some of these variations occurred in positions corresponding to previously detected variations in resistant *N. meningitidis*.

Conclusions: Sulphonamide resistant commensal *Neisseria* were isolated from an environment not exposed to sulphonamides, suggesting that resistant *Neisseria* has become a natural part of the commensal throat flora.

Background

Sulphonamides inhibit the enzyme dihydropteroate synthase (DHPS) by acting as competitive inhibitors and thereby blocking the biosynthesis of folic acid in bacteria. We have earlier defined sulphonamide resistance in *Neisseria meningitidis* as amino acid variations in the chromosomal *folP*-gene, encoding DHPS [1]. The DHPS of the resistant strain 3976 had a Leu in position 31 and a Cys in position 194, instead of the Phe and Gly residues found in susceptible strains. These two alterations conferred sulphonamide resistance, but they also had a negative influence on substrate binding of the DHPS enzyme [2]. This disadvantage was compensated by an additional alteration in position 84, which improved the substrate binding without affecting the resistance. Such compensatory mutations can lead to irreversible resistance because the bacterium has little to gain in reverting to the susceptible state. This phenomenon can therefore explain the presence of sulphonamide resistant strains of *N. meningitidis* in an environment not exposed to sulphonamides.

Horizontal gene transfer via natural transformation has been suggested as the main mechanism of genetic diversity among *Neisseria* [3]. Genetic transfer between different species has also been reported to contribute to the spread of resistance to antimicrobials, for example penicillin [4] and sulphonamides [5]. The hypothesis put forward [2] was that the sulphonamide resistance of *N. meningitidis* originated from a commensal *Neisseria* and was transferred to *N. meningitidis* via natural transformation. If this

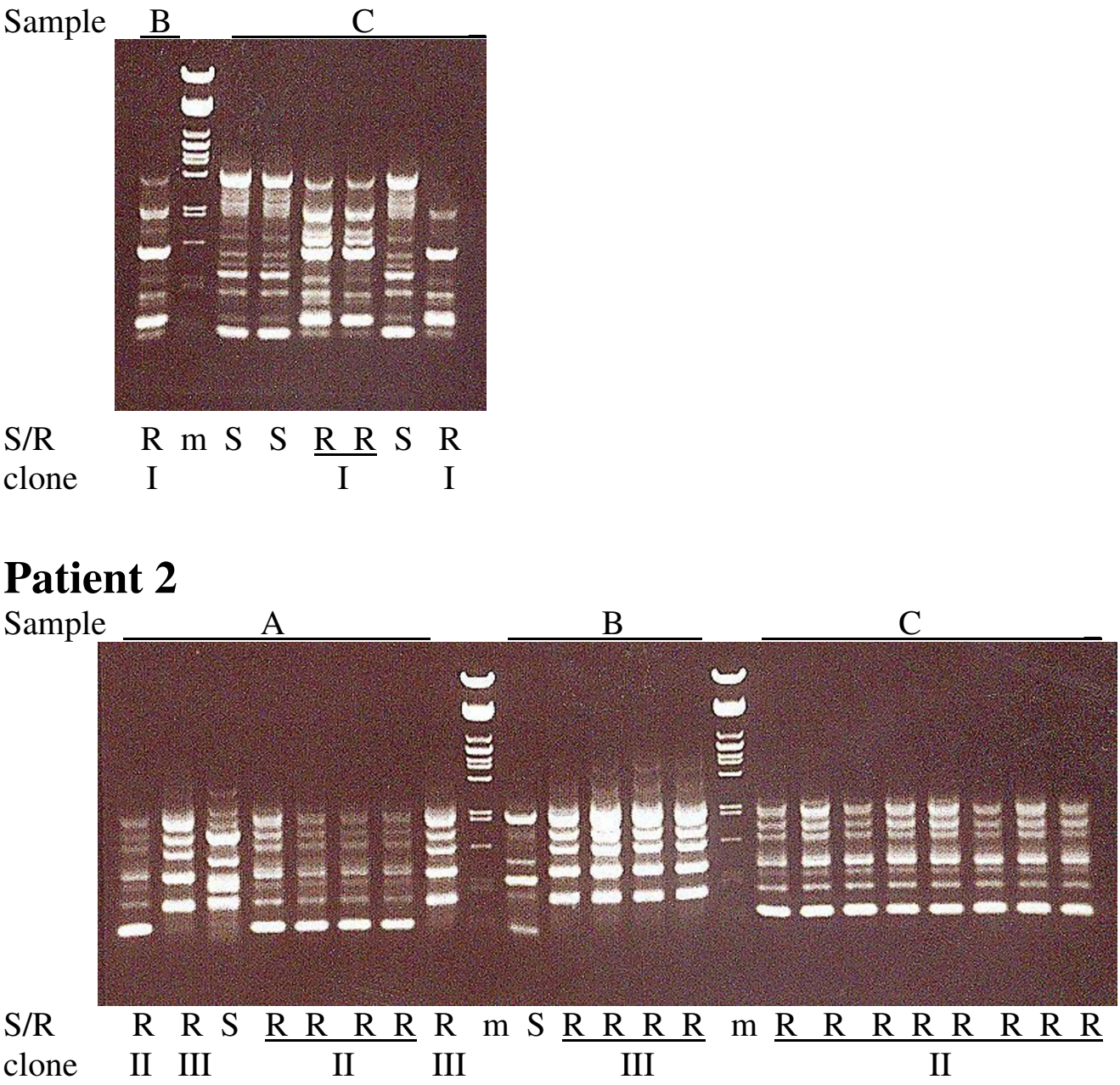

Figure 1
AP-PCR patterns of *Neisseria* isolates The isolates were obtained from samples collected from the same patients at three separate occasions: sample A at time zero, sample B two weeks later and sample C one year later. Sulphonamide sensitive isolates are marked with S. Resistant isolates are marked with R and the clone to which they belong. m=molecular size markers

hypothesis is correct, we should expect to find sulphonamide resistant commensal *Neisseria* among the throat flora of people.

In the present study, we have examined bacterial isolates belonging to commensal *Neisseria* species with reference to sulphonamide susceptibility. The strains were isolated from throat swabs collected from outpatients in Sweden.

The use of sulphonamides in Sweden is restricted to hospital care and the consumption is only 0.15 DDD (defined daily doses) per 1000 inhabitants and day (data from the National Board of Health and Welfare, Sweden). Thus, it could be concluded that the patients involved in this study had not been exposed to sulphonamides.

Results

The bacterial strains used in this work were isolated from throat swabs collected from 17 patients (of which 3 were untreated controls) enrolled in a treatment program for *Helicobacter pylori* infection. Each patient had donated specimens at three occasions during a one-year period. The patients was not allowed to use sulphonamides for three weeks preceding the study or during the study period.

The primary cultures of all throat swabs were examined for the presence of bacteria belonging to *Neisseria*, resulting in a collection of 165 individual *Neisseria* isolates. All isolates were subjected to species determination tests based on biochemical properties, revealing that all isolates belonged to the species subgroup *Neisseria subflava*/*Neisseria sicca*/*Neisseria mucosa*. The isolates were then tested for resistance to sulphonamides, resulting in the identification of 23 highly sulphonamide resistant isolates with MIC >256 μg per ml. Four of the sulphonamide resistant isolates originated from one patient whereas the other 19 were from one of the untreated control patients.

The phylogenetic relationships between the isolates were analysed with a molecular fingerprinting method based on arbitrarily primed PCR. All four resistant isolates from the first patient gave almost identical AP-PCR patterns (Figure 1a) and were therefore assumed to belong to one single clone, designated clone I. The sulphonamide resistant isolates from the second patient could be divided into two dissimilar clones (clone II and III) according to AP-PCR patterns (Figure 1b). All but one of the throat samples contained resistant and susceptible isolates with completely distinguishable AP-PCR patterns (Figure 1).

The chromosomal *folP*-genes from six sulphonamide resistant isolates and four susceptible isolates were sequenced. The sequence identity was 94–100% at the nucleotide level, indicating that all isolates belonged to one or a few closely related *Neisseria* species. The deduced amino acid sequences from six isolates (one representative from each resistant clone plus three susceptible isolates) are aligned in Figure 2. The sulphonamide resistant isolates had variations that previously were identified as necessary for sulphonamide resistance in *N. meningitidis*: clone I and III carried a Leu at position 31 and clone II carried a Cys at position 194. Clone II also had a Met at the highly conserved position 66, where all other isolates had Thr, and a one-amino-acid insertion at position 76.

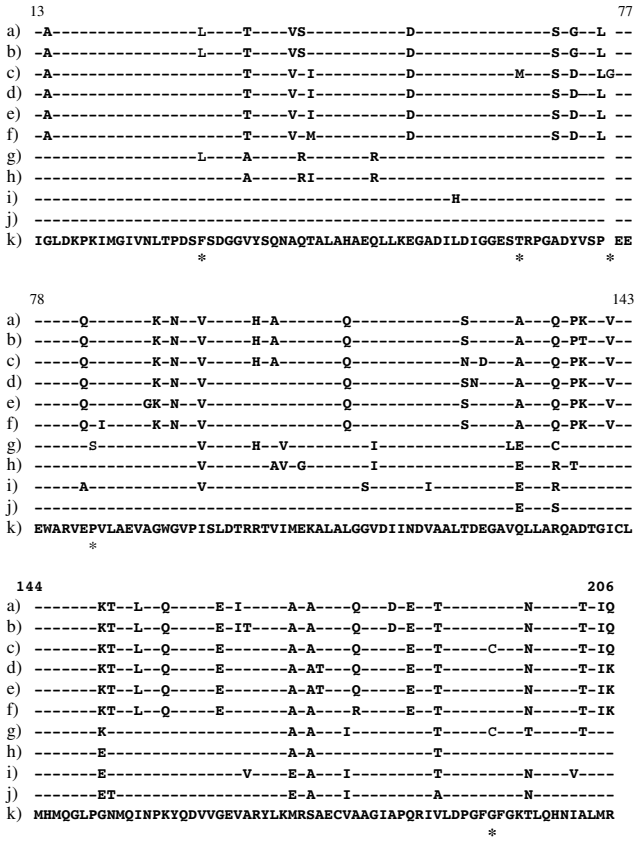

Figure 2
Amino acid sequences of DHPS from *Neisseria* Alignment of deduced amino acid sequences from internal fragments of *folP*-genes from *Neisseria*: **a**) isolate 10B:1 (Su^R clone I); **b**) isolate 102A:2 (Su^R clone III); **c**) isolate 102A:1 (Su^R clone II); **d**) isolate 102A:3 (Su^S); **e**) isolate 10C:1 (Su^S); **f**) isolate 102B:3 (Su^S); **g**) *N. meningitidis* 3976 (Su^R)[1]; **h**) *N. meningitidis* BT054 (Su^S)[5]; **i**) *N. mucosa*; **j**) *N. subflava*; **k**) *N. sicca*. Amino acid residues discussed in the text are bolded and marked with * below the sequences. Accession numbers in the EMBL database are AJ457071, AJ457074, AJ457073, AJ457075, AJ457072, AJ457076, X87405, X68067, AJ457077, AJ457079 and AJ457078, respectively.

The *folP* sequences from the isolates were compared to corresponding sequences from ATCC strains of the commensal species *Neisseria mucosa, Neisseria sicca*, and *Neisseria subflava* (see Figure 2). Two clinical *Neisseria meningitidis* isolates were also included in the comparison. The sequence variation between the *Neisseria* species mentioned above is 10% or less at amino acid level. However, the sequence divergence between the isolates from this study and the other *Neisseria* species was twice as high, 20%, at the amino acid level. This high sequence divergence suggested that the isolates were only distantly related to the other species.

Experiments were conducted to reveal if the sulphonamide resistance phenotype could be transferred from the isolates to the pathogenic *Neisseria meningitidis* by natural transformation. The recipient strain was efficiently transformed with DNA from sulphonamide resistant *Neisseria meningitidis* strains, but no transformation with DNA from the isolates was detected. One reason for this could be the substantial difference in DNA sequence between donor and recipient, which probably led to transformation frequencies below the detection limit. Efficient transformation of *Neisseria* requires the presence of an uptake sequence in the donor DNA [6] and it is possible that the isolates studied here lacked this uptake sequence in the proximity of the *folP*-gene.

Discussion

Sulphonamide resistant commensal *Neisseria* were detected in throat swabs from two patients not exposed to sulphonamides. The resistant strains displayed sequence variations in the *folP*-gene that could explain the resistance phenotype. In addition to some well-documented alterations previously reported as important for sulphonamide resistance in *Neisseria meningitidis* [1], a new variant for the resistance phenotype was suggested: a Met at position 66 in combination with a Gly-insertion between positions 75 and 76. Position 66 has not previously been discussed in relation to sulphonamide resistance, but the residue in the *Plasmodium falciparum* DHPS corresponding to position 67 in *Neisseria* is often found altered in sulphonamide resistant strains of this parasite [7] and residue 68 in *Neisseria meningitidis* strain MO035 is important for high level sulphonamide resistance [8]. Thus, alterations at position 66 are likely to affect the susceptibility to sulphonamides. Likewise, insertions of one or two amino-acids in different positions in the DHPS have previously been reported to confer sulphonamide resistance in *Neisseria meningitidis* [1] and *Streptococcus pneumoniae* [9]. Thus, the resistance phenotype of the isolates in this study could be explained by amino acid variations in their DHPS enzymes.

The sulphonamide resistant isolates in this study were found in mixed bacterial populations containing both susceptible and resistant *Neisseria*. The resistant isolates were also present in throat swabs collected with an interval of one year from the same persons. These observations indicated that the resistant bacteria were equally well adapted to natural conditions as susceptible bacteria. This can be explained by two theories: the resistant isolates had evolved from susceptible ancestors, including mutations compensating for any negative effects of the resistance, as seen in some *Neisseria meningitidis* strains [2]. Alternatively, the enzymes expressed by the resistant isolates could be naturally resistant variants of DHPS that were either endogenous or originated from another species. The possibility of the *folP*-genes originating from another species may also explain the discrepancy between the species determination results, which suggested that the isolates were members of the *N. sicca*/*N. subflava*/*N. mucosa* subgroup, and the *folP* sequences, which indicated a remote relation to these species: the sequence divergence between the isolates and the ATCC strains would then be the result of horizontal gene transfer and thus limited to this part of the genome.

Conclusions

When sulphonamides were heavily used as antibacterial drugs, genes encoding resistant variant of DHPS were most likely enriched in the population and transferred between different *Neisseria* species by natural transformation. The development of resistant *N. meningitidis* is assumed to be the result of such gene transfer, but the origin of the resistance genes remains unidentified. The resistant isolates in this study had little sequence similarity to the variants found in *N. meningitidis* and the resistance was not transferable to *N. meningitidis* by natural transformation. However, the presence of well-adapted resistant *Neisseria* in the small population examined in this study suggested that sulphonamide resistance has become a natural element among commensal *Neisseria*. Thus, resistance to sulphonamides seems to remain in the commensal throat flora despite the lack of selection pressure.

Methods

Bacterial strains and media

Throat specimens were plated on chocolate agar and incubated over night at 37°C in 5% CO_2. From primary cultures where *Neisseria* were present, 1–10 colonies containing Gram-negative, oxidase-positive diplococci were isolated and kept on ISA plates (Difco Laboratories, USA). Chocolate agar- and IM-plates (Iso-Sensitest Agar complemented with nutrients required for growth of Haemophilus influenzaea) were obtained from Huddinge hospital, Sweden.

Etest® (AB Biodisk, Sweden) was used as recommended by the manufacturer to determine sulphonamide resistance levels. Species determinations were performed with API HN tests (bioMèrieux, France).

Reference strains used in this study were *Neisseria sicca* ATCC9919, *Neisseria mucosa* ATCC19696, *Neisseria subflava* ATCC19243, *Neisseria cinerea* ATCC159/62 and *Neisseria lactamica* ATCC23970. *Neisseria meningitidis* strain 952 [5] were used as a recipient in transformations.

Molecular typing

Primer D8635 [10] was used in an arbitrarily primed PCR (AP-PCR) method. 25–50 μg total chromosomal DNA

was amplified using conditions previously described [11] and the resulting band patterns were visualised in ethidium-bromide-stained agarose gels.

Sequencing of folP

Total chromosomal DNA was purified using Wizard® Genomic DNA Purification Kit (Promega, UK). PCR primers designed for previous work on meningococci, NM6 [1] and NM7 (5'-TTGGCAGGCAGGACGGTTTG-3') were used to amplify 589 bp fragment of *folP*. The obtained PCR-products were cloned in pCR®2.1-TOPO vectors (Invitrogen™, Netherlands). The plasmids were purified using QIAprep Spin Miniprep (QIAGEN, Germany) and the inserts were sequenced on an ALFexpress™ DNA Sequencer using Thermo Sequenase Primer Cycle Sequencing and Cy5-labelled primers (Amersham Biosciences, Sweden).

Transformations

Neisseria meningitidis were resuspended to OD = 0.1 in a rich broth consisting of Brain-Heart-Infusion Blood-Agar Base (Difco Laboratories, USA) supplemented with 0.4% yeast extract, 10 mM $MgCl_2$, 2x Kellogg's supplement and 0.042% $NaHCO_3$ [12]. 2–5 μg of chromosomal DNA was added to 250 μl bacterial suspension and the mixture was incubated at 37°C in 5% CO_2: first for six hours without shaking and then, after addition of 1 ml of the enriched broth described above, over night with shaking. The transformation mixtures were incubated on IM plates containing 64 μg sulphametoxazol ml^{-1}. Chromosomal DNA from sulphonamide resistant *Neisseria meningitidis* (strains MO035 and BT227) was used as positive controls.

Authors' contributions

YQ conceived of the study, conducted all the experimental work, including the sequence analysis, and drafted the manuscript. GS participated in the design of the study and in interpretation of the results. Both authors read and approved the final manuscript.

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