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ORIGINAL RESEARCH

# Alternations in DNA gyrase genes in low-level fluoroquinolone-resistant *Moraxella catarrhalis* strains isolated in Poland

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**Purpose:** The purpose of this study was to investigate the molecular mechanisms of fluoroquinolone resistance in *Moraxella catarrhalis* clinical strains isolated in Lublin, Poland.

**Materials and methods:** A total of 150 non-duplicate clinical strains of *M. catarrhalis* were obtained from individuals with signs of upper respiratory tract infection. Bacterial identification was corroborated on the basis of phenotypic and biochemical characteristics as well as with the use of molecular tests. The antimicrobial susceptibility of *M. catarrhalis* isolates was determined using the disk diffusion method and Etest. Mutations in the gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) genes were determined by polymerase chain reaction and sequencing.

**Results:** It was observed that 16.7% of the studied isolates were drug resistant. Resistance to tetracycline was detected for 12% of the strains. Resistance to nalidixic acid, moxifloxacin, and levofloxacin was exhibited by 2.7% of the strains; 1.3% of the strains were resistant to trimethoprim/sulfamethoxazole and 0.7% to erythromycin. Minimum inhibitory concentration values of the four strains demonstrating fluoroquinolone resistance were: 6–12 mg/L for nalidixic acid, 1–1.5 mg/L for levofloxacin, 1 mg/L for moxifloxacin, and 0.25–0.5 mg/L for ciprofloxacin. The research resulted in the detection of mutations in 4 strains, in gyrase *gyrA* and *gyrB* genes. In *gyrA* gene, there occurred mutation G412C as well as four silent transition mutations. Within *gyrB* gene, there occurred mutation, substitution A1481G, as well as two identical silent mutations.

**Conclusion:** Our findings reveal that resistance to fluoroquinolones in *M. catarrhalis* is connected with amino acid substitutions in *gyrA* and *gyrB* genes. To our knowledge, this work is the first description of fluoroquinolone-resistant clinical strains of *M. catarrhalis* with described mutations in *gyrA* and *gyrB* genes isolated in Poland and in Europe.

**Keywords:** *Moraxella catarrhalis*, drug resistance, quinolones, respiratory tract infections

## Introduction

*Moraxella catarrhalis* is an aerobic Gram-negative diplococcus that is frequently isolated from the human respiratory tract. For many years, *M. catarrhalis* has been considered a harmless commensal microorganism, but recent research proves the important pathogenic character of the bacterium. It is mainly responsible for the infections of upper and lower respiratory tract including acute otitis media in children and exacerbation of chronic obstructive pulmonary disease in adults. Less frequently, it may lead to severe infections of different locations which are heavily dependent on the host's condition.[1,2] Despite the widely described inherent resistance of the bacteria to vancomycin, clindamycin, and trimethoprim and their extremely common resistance to penicillins, resulting from the production of specific β-lactamases, most clinical

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isolates are susceptible to most of the antibiotics commonly used in the treatment of respiratory infections. Due to that, infections caused by *M. catarrhalis* are usually treated with a combination of penicillin and β-lactamase inhibitors. Moreover, most of macrolides, tetracyclines, quinolones, and trimethoprim/sulfamethoxazole are usually highly effective against these bacteria with good therapeutic effects.[3–5]

Fluoroquinolones have a broad spectrum of action and high therapeutic safety. Due to their good penetration into respiratory tract and laryngeal tissues, they are readily used in the treatment of infections of these areas. Newer generations of drugs in this class (eg, levofloxacin and moxifloxacin) are effective against Gram-positive, Gram-negative, aerobic, and anaerobic bacteria. They are also proven to be effective against some atypical bacteria. Fluoroquinolones inhibit DNA synthesis by targeting two essential topoisomerases in bacterial cell. The first one, DNA gyrase, an enzyme found exclusively in bacteria, uses the energy of ATP hydrolysis to introduce negative supercoils into DNA, which is essential for chromosome condensation, replication, and promoting transcription initiation. The second enzyme, topoisomerase IV, resolves interlinked daughter chromosomes after DNA replication and also relaxes positive supercoils of DNA strands. Both enzymes are composed of two pairs of subunits. The A subunit of DNA gyrase is encoded by the *gyrA* gene, whereas the B subunit is encoded by *gyrB* gene. The A and B topoisomerase IV subunits are encoded, respectively, by *parE* and *parC* genes. The resistance of bacteria to fluoroquinolones can result from three mechanisms. In Gram-negative bacteria, the most common of these is the presence of mutations (amino acid substitutions in quinolone resistance-determining region [QRDR]) within the genes encoding the A and B subunits of gyrase and topoisomerase IV. In addition, fluoroquinolones can be actively pumped out of the cell via efflux mechanism, and some species of bacteria also produce Qnr proteins that form stable complexes with gyrase and topoisomerase IV inhibiting the activity of these enzymes as a result.[6]

Despite the generally high antibiotic sensitivity of *M. catarrhalis*, sporadic reports show a slow gradual growth of the number of drug-resistant strains. Fluoroquinolone resistance in *M. catarhalis* is still a very rare phenomenon; however, it has been reported by several countries. These include, among others, the USA,[7] Europe,[8] India,[9,10] Taiwan,[11] Thailand,[10] and Japan.[12] To date, the exact mechanism of fluoroquinolone resistance in *M. catarrhalis* has not been fully clarified, and there exist only a few reports dealing with this subject.[12,13] Thus, the purpose of this study was to investigate the molecular mechanisms of fluoroquinolone resistance in *M. catarrhalis* clinical strains isolated in Lublin, Poland.

# Materials and methods

## Bacterial strains and culture conditions

The bacterial strains were obtained from the upper respiratory tract specimens (nasal or pharyngeal swabs) collected from individuals with signs of infection of this area. The only inclusion criteria for patients were primary care doctor's recommendation for microbiological testing and positive cultures for *M. catarrhalis* in the respiratory sample. One hundred fifty non-duplicate clinical strains of *M. catarrhalis* were used in this study. Bacterial cultures were grown routinely for 24 h at 37°C on Columbia agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in an atmosphere comprising 95% air and 5% $CO_2$.

## Identification

The fact that the obtained isolates belonged to *M. catarrhalis* species was corroborated on the basis of phenotypic and biochemical characteristics as well as with the use of molecular tests. Bacterial colonies on blood agar with phenotype characteristics for *M. catarrhalis*, that is, round, convex, shiny, opaque, about 2 mm in diameter, light gray, of equal thickness, not hemolytic, and which can be pushed along the surface of the agar (hockey disk test), were transferred to a fresh medium to obtain pure cultures. Colonies of Gram-negative, catalase- and oxidase-positive diplococci were additionally identified using API® NH identification strips (bioMerieux, Marcy l'Etoile, France).

For a definitive identification of the examined strains, polymerase chain reaction (PCR) for marker gene *copB* was performed. DNA extraction was performed using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). The nucleotide primer sequences to amplify this gene were previously described by Verhaegh et al[14] and are given in Table 1. A standard PCR protocol was used. It comprised 1 cycle of initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 s, annealing at a temperature of 55°C for 1 min, and extension at a temperature of 72°C for 1 min, each for 35 cycles. Thermocycling was conducted with the use of LabCycler Gradient thermocycler (SensoQuest, Gottingen, Germany). PCR products were visualized by electrophoresis on 2% agarose gels (Basica LE, ABO, Gdańsk, Poland) and stained with ethidium bromide (ICN Biomedicals, Aurora, OH, USA). *M. catarrhalis* ATCC 25238 was used as a positive control.

**Table 1** PCR primers for *copB* and quinolone resistance-determining region (QRDR) amplification in *Moraxella catarrhalis*

| Name | Sequence (5′→ 3′) | Temperature (°C) | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *copB* forward | GGCGTGCGTGTTGACCGTTTTG | 58.6 | 564 | 14 |
| *copB* reverse | GTTTGGCAGGCGATAGGCGACAT | 58.8 | | |
| *gyrA* forward | TGCGTGATGGACTTAAGCCT | 51.8 | 370 | 13 |
| *gyrA* reverse | GGCAACACGCTTGGCATTTT | 51.8 | | |
| *gyrB* forward | GTGGTTGAGACCGCCATGCA | 55.9 | 460 | |
| *gyrB* reverse | CATCCGCATCGGTCATGATG | 53.8 | | |
| *parC* forward | GCATCGTCTATGCCATGAGT | 51.8 | 390 | |
| *parC* reverse | CGGCGATGCCTGTCGTGCCA | 60.0 | | |
| *parE* forward | GTCAGTCAGCGGGTGGATCA | 55.9 | 340 | |
| *parE* reverse | CCTGCATCAATACGATACAA | 47.7 | | |

## Antimicrobial susceptibility testing

Cefinase disks (MAST ID Graso Biotech, Poland) were used to investigate β-lactamase production. Antimicrobial susceptibility testing was conducted using the Kirby–Bauer disk diffusion method on MH-F agar (bioMerieux) in compliance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and interpreted in accordance with the "breakpoint tables for interpretation of minimum inhibitory concentrations (MICs) and zone diameters" specified by EUCAST for each year.[15] The study involved the use of antibiotic disks in appropriate concentrations from Becton, Dickinson and Company. Furthermore, fluoroquinolone susceptibility of the four fluoroquinolone-resistant *M. catarrhalis* strains was examined on MH-F agar using an Etest (bioMerieux) to estimate the MIC value. *M. catarrhalis* ATCC 25238 strain was used as a control to ascertain the accuracy of the performed identification.

## PCR and DNA sequencing of fluoroquinolone-resistant genes

In order to detect mutations in the QRDR regions, the following gene fragments of fluoroquinolone-resistant strains were analyzed: nt 172–447 region of *gyrA*, nt 1135–1527 region of *gyrB* , nt 187–507 region of *parC*, and nt 1321–1590 region of *parE*. These gene fragments underwent PCR reaction. Then, the obtained products of these reactions underwent sequencing. DNA was extracted with the use of a commercial kit (QIAamp DNA Mini Kit) in compliance with producer's guidelines. Primer sequences and the expected quantities of the reaction products are presented in Table 1. The PCR reaction protocol was identical to the one performed for *copB* gene, except for the fact that 30 cycles were used in the reaction process. The products of PCR reaction were sequenced with the use of the Sanger method (Genomed, Warsaw, Poland). The obtained results underwent a comparative analysis involving a gene sequence of wild-type *M. catarrhalis* strain BBH18 (GenBank Accession No CP002005) with the use of BLAST software (software available online: www.ncbi.nlm.nih.gov). Simultaneous comparison of multiple sequences was conducted using MEGA 6 software (Molecular Evolutionary Genetics Analysis software version 6.0.).

## Ethics approval and informed consent

The research protocol was approved by the Ethics Committee of the Medical University of Lublin (number KE-0254/155/2015). The study was conducted in the Department of Medical Microbiology of the Medical University of Lublin (Poland) in the years 2013–2015. All patients gave their written informed consent to the use of the isolated strains for the purpose of this study. STATISTICA 13.0 software (StatSoft, Cracow, Poland) was used to set up and maintain a database.

# Results

## Characteristics of the studied group

A total of 150 non-duplicate *M. catarrhalis* isolates were collected from 62 males and 88 females. One hundred twenty-seven (84.7%) isolates were obtained from nasal swabs and 23 (15.3%) from pharyngeal swabs. The average age of individuals who provided materials for isolates was 6.23±10.52 years, and the age range of the patients was from 3 months to 68 years. Most of the *M. catarrhalis* isolates were recovered from children younger than 4 years of age (141; 94%).

## Antimicrobial susceptibility

About 98.7% (n=148) of the studied strains produced β-lactamases (positive cefinase test), whereas 1.3% (n=2) did not produce these enzymes. The study assessed the

sensitivity of *M. catarrhalis* isolates to the following groups of antimicrobial substances: penicillins (amoxicillin/clavulanate 20/10 µg/disk), cephalosporins (cefuroxime 30 µg/disk), fluoroquinolones (nalidixic acid 30 µg/disk, ciprofloxacin 5 µg/disk, moxifloxacin 5 µg/disk, levofloxacin 5 µg/disk), macrolides (erythromycin 15 µg/disk), tetracyclines (tetracycline 30 µg/disk), and trimethoprim/sulfamethoxazole (trimethoprim/sulfamethoxazole 1.25–23.75 µg/disk). The studied isolates were classified based on breakpoint values of growth inhibition zones determined by EUCAST into two categories: resistant or susceptible, and in case of tetracycline also into intermediate category. Twenty-five (16.7%) of the studied isolates were drug resistant. Almost all drug-resistant strains were isolated from children under the age of 4 (93.9%) years and from materials obtained from nasal swabs (84.8%). In general, 8 drug-resistant strains were isolated in 2013, 6 in 2014, and 11 in 2015. Resistance to tetracycline was demonstrated by 18 (12%) of the strains, whereas a decreased sensitivity to this substance was reported for 22 (14.7%) of the strains. Among fluoroquinolone drugs, resistance to nalidixic acid, moxifloxacin, and levofloxacin was demonstrated by 4 (2.7%) strains. In case of the rest of the antimicrobial substances, 2 (1.3%) strains were resistant to trimethoprim/sulfamethoxazole and 1 (0.7%) was resistant to erythromycin. Resistance to amoxicillin/clavulanate, ciprofloxacin, and cefuroxime was not detected (Table 2). MIC values of the 4 strains demonstrating fluoroquinolone resistance are presented in Figure 1. These were: 6–12 mg/L for nalidixic acid, 1–1.5 mg/L for levofloxacin, 1 mg/L for moxifloxacin, and 0.25–0.5 mg/L for ciprofloxacin (Figure 1).

**Table 2** Drug susceptibility of *Moraxella catarrhalis* isolates

| Antibiotic | Number of resistant strains |
|---|---|
| Tetracycline | 12% (n=18) |
| Nalidixic acid | 2.7% (n=4) |
| Moxifloxacin | 2.7% (n=4) |
| Levofloxacin | 2.7% (n=4) |
| Ciprofloxacin | 0 |
| Erythromycin | 0.7% (n=1) |
| Trimethoprim/sulfamethoxazole | 1.3% (n=2) |
| Cefuroxime | 0 |
| Amoxicillin/clavulanate | 0 |

## Sequencing

The work adopts a chronological numbering of strains with regard to the order of their isolation. The research resulted in the detection of mutations in 4 strains, in gyrase *gyrA* and *gyrB* genes (Figure 2). Mutations did not occur in topoisomerase *parC* and *parE* genes. Mutations in *gyrA* gene occurred in the genetic material of 1 strain (no 52). A single missense mutation G412C resulting in the substitution of Gln138 (**C**AG) for Glu138 (**G**AG) was observed in case of this strain. The analyzed gene fragment of strain 52 also featured four silent transition mutations: C234T,

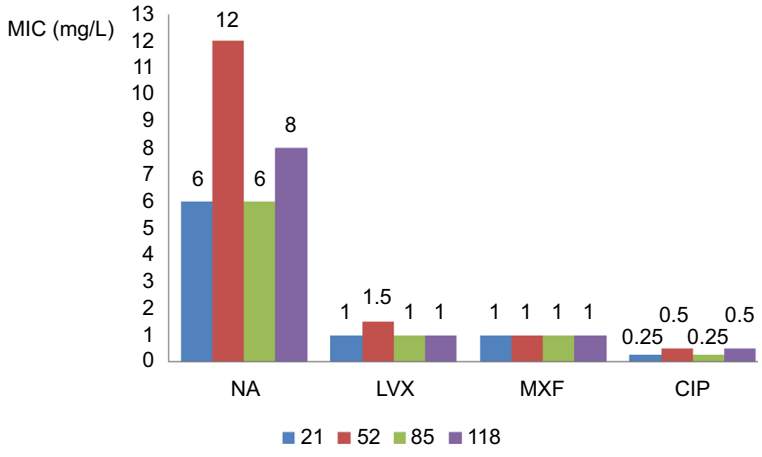


**Figure 1** MIC values of examined fluoroquinolones.
**Note:** 21, 52, 85, and 118 are strain numbers.
**Abbreviations:** NA, nalidixic acid; LVX, levofloxacin; MXF, moxifloxacin; CIP, ciprofloxacin; MIC, minimum inhibitory concentration.

| Strain number | *gyrA* | | | *gyrB* | | |
|---|---|---|---|---|---|---|
| | 138 Gln | | | 494 Gly | | |
| *M. catarrhalis* BBH18 CP002005 | C | A | G | G | G | C |
| 21 | • | • | • | • | **A** | • |
| 52 | **G** | • | • | • | • | • |
| 85 | • | • | • | • | **A** | • |
| 118 | • | • | • | • | **A** | • |

**Figure 2** Nonsynonymous mutations in bacterial DNA gyrase genes, *gyrA* and *gyrB*, in fluoroquinolone-resistant *Moraxella catarrhalis* strains.
**Notes:** The dots indicate the absence of a mutation in the tested sequence. The bold texts indicate the place where the mutation occurred in the tested sequence. ***indicates missense mutation (which results in a protein in which one amino acid is substituted for another).

C243T, T306C, and C336T. Within *gyrB* gene of strains 21, 85, and 118, there occurred an identical missense mutation, substitution A1481G, which resulted in the substitution of Gly494 (G**G**C) for Asp494 (G**A**C), as well as two identical silent mutations in case of each strain: transition A1446G and transversion A1404T.

## Discussion

Antimicrobial agents from the fluoroquinolone family are widely and willingly applied in the therapy of multiple infections. However, the increased prevalence of fluoroquinolone-resistant strains of various bacterial species has recently become a serious risk in several places around the world.[12,16–18] In this work, most of the drug-resistant clinical isolates of *M. catarrhalis* were obtained from children. Fluoroquinolone antibiotics are not commonly used in this age group owing to the risk of side effects. At the same time, this age group is characterized by the highest rate of *M. catarrhalis* colonization. It might suggest that fluoroquinolone-resistant strains isolated from children can be strains acquired from adults.

Previous works demonstrated that fluoroquinolone resistance in Gram-negative bacteria is most often conditioned by mutations in *gyrA* and/or *parC* genes, whereas mutations in *gyrB* and *parE* genes result in low-level resistance phenotype-wise. Additionally, there is an increase of fluoroquinolone resistance in case of many QRDR mutations.[6,19] Other mechanisms were described in the group of Gram-negative bacteria as well. These, however, were not connected with the changes in the QRDR region, which condition this type of drug resistance. Molecular basis of Gram-negative bacterial resistance to fluoroquinolones has already been described both for cocci and for bacilli such as, among others, *Neisseria gonorrhoeae*, *N. meningitidis*, *Escherichia coli*, *Salmonella enterica*, *Shigella* spp., *Campylobacter jejuni*, *Haemophilus influenzae*.[20–27] The present work describes 4 strains resistant to fluoroquinolones which possessed mutations in *gyrA* and *gyrB* genes within their genome. In these strains, the occurrence of mutations was connected to low-level resistance to levofloxacin and moxifloxacin as well as with resistance to nalidixic acid. All 4 isolates were sensitive to ciprofloxacin. The occurrence of mutations in *gyrA* gene resulted in a slightly higher levofloxacin and nalidixic acid MIC values in comparison to the strains with mutations only in *gyrB* gene. There exist only a few studies describing the resistance mechanisms of *M. catarrhalis* to fluoroquinolones in world literature, and all of them were conducted in Japan. The first work describing mutations in the QRDR region of *M. catarrhalis* was conducted by Yamada and Saito.[12] It involved the analysis of 5 *M. catarrhalis* strains exhibiting a decreased sensitivity to fluoroquinolones (ciprofloxacin

MIC of 0.5 mg/L and levofloxacin MIC of 1 mg/L). All 5 studied strains featured a *gyrA* gene mutation – C239T (Thr 80> Ile). The work corroborated that a Thr-to-Ile substitution at amino acid 80 (T80I) in *gyrA* gene had a considerable influence on the lowering of strains' sensitivity to fluoroquinolones, especially levofloxacin. During the study, there were no changes detected in *parC* gene. The same authors and associates described 6 novel mutations in their latest work. These mutations occurred in *gyrA* (Asp 84> Tyr, Thr594dup, and Ala722dup), *gyrB* (Glu 479> Lys and Asp 439> Asn), and *parE* (Gln 395> Arg), all of which are involved in *M. catarrhalis* resistance to fluoroquinolones.[27] Iwata et al,[13] in a work also conducted in Japan, analyzed a single clinical *M. catarrhalis* strain resistant to macrolides and fluoroquinolones (levofloxacin MIC of 4 mg/L). The researchers discovered the presence of mutation C239T (Thr 80> Ile) in *gyrA* gene and A1481G (Asp 494> Gly) in *gyrB* gene. There was also a single silent mutation in *parC* gene, whereas there were no mutations detected in *parE* gene. The mutation in *gyrB* gene that was described in their study was identical to the one observed in the research conducted by the authors of the following work, whereas the Glu 138> Gln mutation detected in *gyrA* gene has not been described so far. In conclusion, our findings reveal that the resistance to fluoroquinolones in *M. catarrhalis* is connected with amino acid substitutions in *gyrA* and *gyrB* genes. The acquisition of mutations in QRDR region of *M. catarrhalis* is most probably a direct cause of the occurrence of fluoroquinolone resistance in this species, and the mutations in *gyrA* gene condition a higher level of resistance. However, other mechanisms conditioning this type of resistance, that is, efflux mechanism or the production of Qnr proteins, were not researched in this study and they require further verification.

## Conclusion

To our knowledge, this work is the first description of fluoroquinolone-resistant clinical strains of *M. catarrhalis* with described mutations in *gyrA* and *gyrB* genes isolated in Poland and in Europe. The work was conducted within a small area. Thus, there exists a strong need to expand and continue the studies across the whole country. Due to the occurrence of fluoroquinolone resistance in *M. catarrhalis* as well as the resistance to other antibiotics that has been observed increasingly more often, drug susceptibility testing of this pathogen to antibiotics should be implemented as a routine procedure as a part of the diagnostic process. The possibility to conduct epidemiological surveillance of the occurrence of drug-resistant strains of this species in Poland and other countries would be worth taking into consideration as well. It is also vital to draw the attention to the increasing number of drug-resistant *M. catarrhalis* strains in Europe and worldwide and to monitor this alarming phenomenon.

## Disclosure

The authors report no conflicts of interest in this work.

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