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## Characterization of a clinical isolate of *Haemophilus influenzae* with a high level of fluoroquinolone resistance

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Sir,

*Haemophilus influenzae* is one of the bacterial pathogens most likely to be involved in community-acquired respiratory infections in patients with chronic obstructive pulmonary disease. In this species, resistance to fluoroquinolones occurs at very low frequency although in such cases treatment failure has been reported.[1] In 2003, a fluoroquinolone-resistant *H. influenzae* isolate was recovered from the sputum of an 80-year-old male patient with a history of bronchiectasis and residual lesions from old tuberculosis but with no history of treatment with fluoroquinolones. Identification of this *H. influenzae* (strain number 79105) was carried out by Gram stain, tests for catalase, oxidase, X and V factor requirements (MAST), Api NH (bioMerieux, Madrid, Spain) and antisera to serotypes a-f (Difco, Sparks, MD, USA).

Susceptibility tests were performed by disc diffusion and Etest on HTM agar and Chocolate agar at 35°C and 5% $CO_2$ and by microdilution in HTM broth according to CLSI (formerly NCCLS) guidelines.[2] *H. influenzae* ATCC 49247 was used as a reference strain. Isolate 79105 was resistant to nalidixic acid, fluoroquinolones and co-trimoxazole and showed MICs of >256, 32, 32, 8 and 2 mg/L for nalidixic acid, ciprofloxacin, norfloxacin, levofloxacin

and moxifloxacin, respectively, and >4/76 mg/L for co-trimoxazole. Moreover, this strain was non-typeable and a non-β-lactamase producer like most of the previously described fluoroquinolone-resistant isolates.

Interestingly, this clinical strain showed dependence with nalidixic acid, ciprofloxacin and co-trimoxazole on HTM agar, growing only round the disc or Etest strip containing these antibiotics. In contrast this effect was not seen with organisms grown on Chocolate agar. Surprisingly, the dependence disappeared after several subcultures.

In our hospital the proportion of isolates of *H. influenzae* resistant to new quinolones is <1% and even lower rates have been reported in other studies.[3,4] At the present time only a few isolates of *H. influenzae* with a high level of fluoroquinolone resistance (MIC of ciprofloxacin >16 mg/L) have been described.[5] Since mutations in the DNA gyrase and topoisomerase IV genes are the most common mechanisms of fluoroquinolone resistance, the corresponding regions of the *gyrA* and *parC* genes of *H. influenzae* ATCC 49247 (as control) and of *H. influenzae* 79105 strains were sequenced (the primers used have been described previously).[1] The sequence of the quinolone-resistant isolate showed two substitutions in GyrA (S84Y and D88A) and two in ParC (S84N and E88A). This combination of mutations has not been described previously and only one *H. influenzae* isolate has previously shown four substitutions in these genes.[5] Additionally, the QRDR regions of the *gyrB* and *parE* genes were analysed with the set of previously described primers.[1] The sequence of the quinolone-resistant isolate showed the E559G substitution in GyrB and the L445F substitution in ParE. In summary, this strain contained six mutations in four different genes associated with quinolone resistance. In all cases, the sequence of the ATCC 49247 *H. influenzae* strain was identical to those obtained from the database.

To explain observed differences in susceptibility between ciprofloxacin and moxifloxacin, we tried to identify the presence of a possible efflux pump with a different affinity for those antimicrobials. Efflux of fluoroquinolones was determined indirectly by measuring the intracellular accumulation of norfloxacin by fluorometric assay.[6] A standardized cell suspension ($OD_{520}$ = 2.0) was preincubated with 10 mg/L norfloxacin, following which half of the cell suspension was exposed to 0.2 mM carbonyl cyanide chlorophenylhydrazone (CCCP). Cell-fluorescence was measured with a fluorescence spectrophotometer (Hitachi 2000; at 279/445 nm). The addition of CCCP to the cells did not increase the level of intracellular norfloxacin. Thus, there was no evidence that efflux pumps inhibited by CCCP contributed to the observed differences in susceptibility of the strain to ciprofloxacin, norfloxacin and moxifloxacin.

In conclusion, an *H. influenzae* isolate resistant to fluoroquinolones by means of a new combination of mutations in topoisomerase genes has been described. It is unknown whether other different efflux pumps or additional mechanisms are implicated in quinolone resistance and explain the different susceptibility to ciprofloxacin and moxifloxacin in this strain.

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## Transparency declarations

None to declare.

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