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Short Communication

Molecular characterisation of the clonal emergence of high-level ciprofloxacin-monoresistant *Haemophilus influenzae* in the Region of Southern Denmark

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ABSTRACT

Haemophilus influenzae is an important human pathogen usually susceptible to quinolones. Here we report the emergence of high-level ciprofloxacin-monoresistant *H. influenzae* in the Region of Southern Denmark. Four isolates were collected for phenotypic and molecular characterisation using whole-genome sequencing (WGS). During an 18-month period, the occurrence of high-level ciprofloxacin-monoresistant *H. influenzae* in patients aged 1–77 years from sputum, ear and eye samples was detected. An epidemiological link between the patients could not be identified. The isolates were non-encapsulated, biotype III and were demonstrated by WGS to be clonal belonging to a single clade with an unknown multilocus sequence type (double-locus variant of ST196). The antibiogram demonstrated that they were all monoresistant to ciprofloxacin with a minimum inhibitory concentration (MIC) >32 mg/L. In silico resistome analysis revealed identical, both previously characterised and novel, putative resistance-related mutations in *gyrA* (S84L and D88 N), *parC* (K20R, S84I, D356A or T356A, and M481I) and *parE* (E151 K, I159A, D420 N and S599A) in all isolates. The isolates were otherwise negative for any resistance genes. This is the first description of the clonal emergence of high-level monoresistant *H. influenzae* due to amino acid substitutions in *gyrA*, *parC* and *parE*.

1. Introduction

Haemophilus influenzae is a human pathogen colonising the upper respiratory tract and is associated with diseases ranging from acute otitis media, sinusitis and conjunctivitis to deadly infections such as epiglottitis, respiratory tract infections, septicaemia and meningitis. Resistance to β-lactam antibiotics is increasingly acknowledged, in contrast to quinolones such as ciprofloxacin that is still considered highly active against *H. influenzae* [1–3]. In particular, high-level ciprofloxacin resistance in *H. influenzae* is extremely rare and is considered an exceptional phenotype by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4]. In December 2013, two ciprofloxacin-resistant *H. influenzae* isolates were identified at Odense University Hospital (Odense, Denmark) from two patients. Further investigations showed both to be high-level ciprofloxacin-resistant [minimum inhibitory concentration (MIC) > 32 mg/L] with no other resistance traits. As a result, a retrospective investigation was performed evaluating the incidence of high-level ciprofloxacin-monoresistant *H. influenzae*, and isolates were prospectively collected for characterisation.

Here we report the appearance of high-level ciprofloxacin-monoresistant *H. influenzae* in the Region of Southern Denmark. Four isolates were available for molecular characterisation and were demonstrated to be highly clonal based on genomic comparisons.

2. Materials and methods

2.1. Setting and strains

Between November 2011 and May 2015, the Laboratory Information Management System (LIMS) of the Clinical Microbiology at Odense University Hospital was searched for patients

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recorded as having infection or colonisation with a ciprofloxacin-monoresistant *H. influenzae*. Clinical and demographic data were extracted and recorded. Species identification on available isolates was done by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics, Bremen, Germany) and was confirmed by analysis of the 16S rRNA gene from whole-genome sequencing (WGS) data. The capsular type and biotype was determined by standard methods [5].

2.2. Susceptibility testing

Antibiotic susceptibility was determined on Mueller–Hinton agar with 5% horse blood + NAD incubated in ambient air supplemented with 5% CO_2 at 35 °C using Neo-Sensitabs (Rosco, Taastrup, Denmark) for disk diffusion, and determination of MICs was done using gradient Etest strips (bioMérieux, Craponne, France). Susceptibility testing and interpretation were done according to EUCAST (http://www.eucast.org).

2.3. Whole-genome sequencing

Four ciprofloxacin-monoresistant *H. influenzae* isolates as well as 69 invasive *H. influenzae* collected in 2015 from all Danish microbiology laboratories were sequenced by paired-end Illumina sequencing. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and fragment libraries were constructed using a Nextera XT Kit (Illumina, Little Chesterford, UK) followed by 251-bp paired-end sequencing (MiSeq™; Illumina) according to the manufacturer's instructions. The paired-end Illumina data were de novo assembled using CLCbio's Genomics Workbench v.7.5 (QIAGEN) reporting only contigs >500 bp using standard settings.

2.4. Molecular characterisation

Multilocus sequence typing (MLST) was performed from the assembled contigs using the MLST service (https://cge.cbs.dtu.dk/services/MLST/) with the MLST configuration for *H. influenzae*. The Illumina sequence data of the four *H. influenzae* isolates were aligned against the chromosome of the R2846 *H. influenzae* reference genome (GenBank accession no. **CP002276**) using the Burrows–Wheeler Aligner. Similarly, the chromosome of *H. influenzae* strains Rd KW20, F3047 and KR494 (GenBank accession nos. **NC_000907**, **NC_014922** and **CP005967**, respectively) were included in the analysis. Identification of single nucleotide polymorphisms (SNP) variants was performed using the GATK UnifiedGenotyper with filtering using the Northern Arizona SNP Pipeline (NASP) (http://tgennorth.github.io/NASP/) to remove positions with <10× coverage and <90% unambiguous variant calls, or within duplicated regions of the reference using NUCmer (http://mummer.sourceforge.net/). Phylogenetic reconstruction on the identified SNPs was performed using the FastTree v.2.1.5 implementation in Geneious v.9.02 [6].

Demonstration of the quinolone resistance-determining region (QRDR) genes (*gyrA*, *gyrB*, *parC* and *parE*), plasmid-mediated quinolone resistance genes [*qnrA1*, *qnrB6*, *qnrD*, *qnrS* and *aac(6′)-Ib*-cr] and β-lactam resistance due to mutations in *ftsI* was done by extracting the gene in question and comparing by BLAST against *H. influenzae* Rd KW20 as well as 69 (phenotypic) ciprofloxacin-susceptible *H. influenzae* blood culture isolates submitted to Statens Serum Institut (Copenhagen, Denmark) in 2015 as part of the National Surveillance Programme on Invasive *H. influenzae*. ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (80% ID threshold and 60% minimum length settings) was used to search for (non-chromosomal mutation) resistance genes.

3. Results and discussion

During the investigated period, six clinical samples with growth of ciprofloxacin-monoresistant *H. influenzae* were detected in the LIMS of the Department of Clinical Microbiology at Odense University Hospital, of which four isolates were available for molecular characterisation. The characteristics of the four patients are shown in Table 1. Patients were aged 1–77 years and the origin of samples were from sputum (two patients) and one each from the eye and ear. The residence of the patients were either located around the greater area of Odense on Funen or from Esbjerg at the Southwestern part of Jutland. No information on prior antibiotic use was available and an epidemiological link between the patients could not be identified. The four clinical isolates were collected for extended investigation by molecular characterisation using WGS. All four strains were identified as *H. influenzae* by MALDI-TOF/MS and verified by sequence analysis of the 16S rRNA gene. The four ciprofloxacin-resistant strains were all non-capsular and belonged to biotype III with an unknown MLST type (double-locus variant of ST196 with the allelic profile 14:8:18:11:161:138:3). All isolates were monoresistant to ciprofloxacin with an MIC >32 mg/L.

Molecular characterisation of the strains including four diverse references revealed a core genome of ca. 1.4 Mb (76%) and with only three SNPs differentiating them. Analysis including the 69 invasive isolates from Denmark identified a total of ca. 68 000 identified SNPs in an ca. 1.2 Mb core genome and showed the four monoresistant isolates to form a distinct cluster (Fig. 1).

Genome-based resistome analyses revealed no β-lactamase genes (*tem* and *rob*), no mutations in the *ftsI* gene mediating β-lactamase-negative ampicillin resistance, and the isolates were negative for resistance genes towards aminoglycosides, sulphonamides, trimethoprim, chloramphenicol, fosfomycin and tetracycline. However, both previously characterised and novel putative resistance-related mutations conferring resistance towards quinolones were detected.

Ciprofloxacin-resistant *H. influenzae* was first described in 1993 [7] and although still rare is seen increasingly, mostly in chronic obstructive pulmonary disease (COPD) patients [1–3]. Resistance to quinolones among *H. influenzae* strains occurs primary via alterations in the genes encoding DNA gyrase (encoded by *gyrA* and *gyrB*) or topoisomerase IV (encoded by *parC* and *parE*), the so-called QRDR [8]. Less frequent are acquisition of resistance genes of the *qnr* type and the transferable plasmid-mediated acetyl transferase gene *aac(6′)-Ib*-cr [8].

Table 1
Characteristics of four patients with ciprofloxacin-monoresistant *Haemophilus influenzae*.

Isolation date	Age (years)	Sex	Ward speciality	Specimen	Clinical condition
3 December 2013	66	Male	Pulmonary	Sputum	Pneumonia
4 December 2013	1	Female	Paediatric	Eye swab	Eye infection
5 January 2014	1	Female	General practice	Ear swab	Ear infection
6 April 2014	77	Male	Gastroenterology	Sputum	COPD

COPD, chronic obstructive pulmonary disease.