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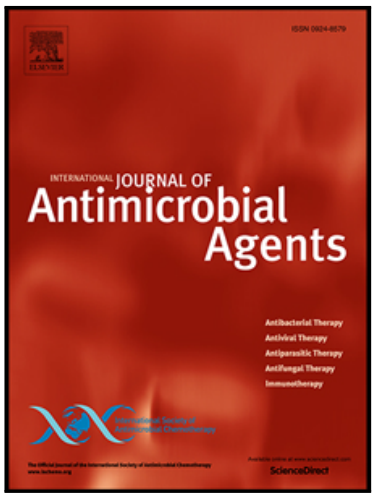

Emergence of Haemophilus parainfluenzae resistant to third-generation cephalosporins in Italy: potential role of PBP3 and PBP5 substitutions in high-level resistance

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Letter to the Editor

Emergence of *Haemophilus parainfluenzae* resistant to third-generation cephalosporins in Italy: potential role of PBP3 and PBP5 substitutions in high-level resistance

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Sir,

The potential role of *Haemophilus parainfluenzae* as a sexually transmitted genitourinary pathogen is well recognized, even though indistinctive clinical symptoms make difficult the differentiation of urethritis due to *Haemophilus* spp. from other pathogens [1]. Of note, *H. parainfluenzae* represents an example of how a community-acquired pathogen could become a serious concern for antimicrobial treatment due to emergence of resistance traits. In particular, resistance to β-lactams emerged from the diffusion of plasmid-mediated β-lactamases (TEM-1, ROB-1). Later, isolates resistant to third-generation cephalosporins (3-GCs) have been sporadically reported worldwide, mostly showing alterations in the transpeptidase domain of penicillin-binding protein 3 (PBP3), with variable phenotypic resistance profiles depending on the specific amino acid (AA) substitution patterns [2]. Resistance to carbapenems is considered exceptional [2-4], and so far, not reported in *H. parainfluenzae*.

With regard to Italy, though surveillance data are scarce and not updated, resistance to 3-GCs has never been reported. Here, we describe the first Italian 3-GC-resistant *H. parainfluenzae* isolated from a genitourinary infection and the related molecular investigation using whole genome sequencing (WGS).

In 2018, a 45-years-old Italian male with purulent urethral discharge was examined for sexually transmitted diseases. Anamnestic consultation revealed that he had unprotected sex with a woman of Serbian origin. A 7-day treatment with ceftibuten (400 mg, once daily) was initially prescribed as empirical therapy. At the end of this treatment, due to the persistence of clinical symptoms (urethral pain, dysuria, purulent urethral discharge), a microbiological culture from urethral swab was performed at the Microbiology Laboratory of A. Manzoni Hospital (Lecco, Italy).

Bacterial identification and antimicrobial susceptibility testing were performed using MALDI-TOF MS (VITEK MS, bioMérieux, Marcy l'Etoile, France) and broth microdilution method (YITHMN panel, Thermo Fisher Scientific, Waltham, MA, USA), respectively. Furthermore, Etest (bioMérieux) and CLSI standard broth microdilution methods were also performed for antibiotics and/or MICs not included in the YITHMN panel. Production of β-lactamases was investigated by nitrocefin-based test (Thermo Fisher Scientific). MICs were interpreted according to the EUCAST (v. 9.0) breakpoints recommended for *H. influenzae* (www.eucast.org).

As a result, an *H. parainfluenzae* (LC/1315.18) negative for β-lactamase production but resistant to penicillin, ampicillin, amoxicillin/clavulanate, cefotaxime, ceftriaxone was identified. The isolate also showed a non-susceptible profile for doxycycline and a borderline MIC for tetracycline. Fortunately, LC/1315.18 remained susceptible to carbapenems (though meropenem showed a borderline MIC of 2 mg/L), trimethoprim/sulfamethoxazole and ciprofloxacin (Table 1). Thus, ciprofloxacin (500 mg, bid) was administered for 10 days. The new antimicrobial treatment was successful in eradicating the infection, as demonstrated by culture follow-up analysis.

Since the antibiotic resistance profile of LC/1315.18 was exceptional, WGS was conducted with both NovaSeq (Illumina Inc.) and MinION (Oxford Nanopore) platforms as previously done [5]. Assemblies of the Illumina whole-genome shotgun and Nanopore hybrid assemblies were done with SPAdes (v.3.12.0) and Canu (v1.7), respectively [6-7]. All assemblies were deposited to GenBank under BioProjects PRJNA515889 (Nanopore hybrid) and PRJNA575148 (Illumina SPAdes). AA sequences of PBP3 and PBP5 were compared to their wild type counterparts of *H. parainfluenzae* T3T1 strain (GenBank accession no. NC_015964) by implementing the protein database Protein BLAST (blastp) of NCBI. The phylogenetic tree was constructed using the tool for microbial core genome alignment and variant detection Parsnp (v1.2; http://github.com/marbl/harvest) [8]. In short, a core-genome alignment was generated with the genomes of 26 *H. parainfluenzae (*WGS available in the GenBank nucleotide database) and the Illumina SPAdes assembly of LC/1315.18. Finally, the

FigTree software (v1.4.4; http://tree.bio.ed.ac.uk/software/figtree) was implemented to visualize the Parsnp core-genome SNV phylogenetic tree.

WGS analysis showed several point mutations in PBP3 and PBP5 sequences, thus suggesting the potential role of derived AA substitutions in determining high-level resistance to 3-GCs. Of note, PBP3 showed 98% AA identity (Supplemental Fig. 1) with respect to the wild type *H. parainfluenzae* strain T3T1 (GenBank accession no. FQ312002) (i.e., Gly11Ala; Val34Met; Val356Leu; Ser385Thr; Ile442Phe; Val511Ala; Ile519Val; Asn526Lys; Asp551Leu; Thr574Ala; Phe602Leu). Instead, PBP5 displayed only 91% AA identity (Supplemental Fig. 2) due to 37 AA substitutions scattered through the entire protein sequence. Since no β-lactamases were detected, resistance to β-lactams was reasonably attributable to the substitutions in PBP3 and/or PBP5.

Comparison with other genomes deposited in GenBank showed that LC/1315.18 had a unique profile, with no clonal relationship with other previously reported *H. parainfluenzae* strains (Supplemental Fig. 3).

We described the first 3-GC-resistant *H. parainfluenzae* strain from Italy. More importantly, the isolate showed very high MIC values for 3-GCs (the one of cefotaxime was notable: >32 mg/L) and a borderline MIC for meropenem (data not previously described in *H. parainfluenzae*). Our molecular analysis showed several AA substitutions in PBP3 and PBP5 (most of them not so far described) that are probably responsible for this phenotype. However, we could only evaluate the cumulative effect of these substitutions providing this particular resistance phenotype. As a limitation, we did not explore *in vitro* the effect of individual polymorphisms on the resistance to 3GC by cloning or site mutagenesis experiments, so not assessing which substitution could have a major role for this resistant phenotype.

The spread of resistance traits in *H. parainfluenzae* is a matter of concern. To this regard, our findings highlight that empirical prescription of antibiotics in the community setting without using microbiological data could be harmful to patients. Moreover, continuous education and awareness of general practitioners toward resistance to antibiotics, including rare phenotypes, could be of utmost importance in promoting antibiotic and diagnostic stewardship, as well in the community setting.

Declarations

Funding: None.

Competing Interests: None declared.

Ethical Approval: Not required.

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LEGEND TO THE FIGURES

Supplemental Figure 1. NCBI protein BLAST results of PBP3 (i.e., query *H. parainfluenzae* LC/1315.18, subject *H. parainfluenzae* T3T1).

Supplemental Figure 2. NCBI protein BLAST results of PBP5 (i.e., query *H. parainfluenzae* LC/1315.18, subject *H. parainfluenzae* T3T1).

Supplemental Figure 3. Phylogenetic tree based on single nucleotide variants (SNV) of 26 *H. parainfluenzae* WGS available in the NCBI database and LC/1315.18 strain.

Tree construction was performed using *Parsnp* (http://github.com/marbl/harvest) with HiSeq paired-end reads assembled with the genome assembly algorithm SPAdes. The parameter *–c* was used for the curated genome directory, and *-C* for the maximal cluster D value (reference coverage) was set to 5000. Remaining options were let as default. Final graphical visualization of the phylogenetic relationships was created with FigTree software (http://tree.bio.ed.ac.uk/software/figtree). Tip labels correspond to *H. parainfluenzae* strains. NA, not available

Table 1: Antibiotic susceptibility profile of *Haemophilus parainfluenzae* (LC/1315.18)

Antibiotic	MIC values, mg/L (S, I, R)	
	Etest	Broth microdiluition
Ampicillin		4 (R)
Amoxicillin/clavulanate	24 (R)	>2/2 (R)
Ceftriaxone	2 (R)	2 (R)
Cefotaxime	> 32 (R)	>2 (R)
Ciprofloxacin		0.016 (S)
Doxycycline		2 (I)
Levofloxacin		0.06 (S)
Meropenem	2 (S)	2 (S)
Penicillin		>1 (IE)
Tetracycline		1 (S)
Trimethoprim/sulfamethoxazole		≤0.12/2.37 (S)
Ertapenem		0.125 (S)
Imipenem	0.75 (S)	

Abbreviations: MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible; IE, insufficient evidence. Since no specific criteria are currently available by EUCAST, results were interpreted according to those recommended for *H. influenzae*.