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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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To Jean-Marc Rolain, September 2, 2020
Editor in Chief
International Journal of Antimicrobial Agents

Enclosed please find the revision (R3) of the manuscript entitled "*Emergence of Haemophilus parainfluenzae resistant to third-generation cephalosporins in Italy: potential role of PBP3 and PBP5 substitutions in high-level resistance"*, which I am re-submitting as a *Letter* to *International Journal of Antimicrobial Agents*.

The text has been revised, as required by the Section Editor. I am providing the files with the highlighted text, a clean copy and the point-by-pint reply to Reviewers' comments.

The revised manuscript has been seen and approved by all the authors. The material is original, unpublished, and has not been simultaneously submitted to another medical journal.

We hope that the information provided with this manuscript will be interesting to the audience of *International Journal of Antimicrobial Agents*, and that it will meet with the Editor's approval. We look forward to hearing from you at your earlier convenience.

Yours sincerely,

Luigi Principe

Please, address the correspondence concerning this manuscript to:
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POINT BY POINT REPLY TO REVIEWERS' COMMENTS

Section Editor:

SPAdes, Canu, and ParSNP have been published in peer-reviewed journal and their authors should get credit for using the product of their research. As previously requested, please insert regular citations to published articles for all these bioinformatics tools, even if the number of citations then exceeds the usual limit for letters.

Reply: References have been added (#6, #7, #8), as required.

HIGHLIGHTS

- The spread of resistance traits in *H. parainfluenzae* is a matter of concern

- Here we describe the first case of resistance to 3-GCs in *Haemophilus* spp. in Italy

- Several AA substitutions in PBP3 and PBP5 were detected in 3-GCs-resistant isolate

- AA substitutions in PBP5 were not so far reported in 3-GCs-resistant *Haemophilus* spp.

- Empirical therapy without microbiological investigations could be harmful to patients

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

Luigi Principe[1*], Odette J. Bernasconi[2], Valentina Viaggi[1], Edgar I. Campos-Madueno[2], Andrea Endimiani[2], Francesco Luzzaro[1]

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Sir,

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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).

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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.

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Table 1

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*.

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