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Molecular characterization of a clinical *Haemophilus parainfluenzae* isolate with cefotaxime resistance and decreased susceptibility to fluoroquinolones.

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Abstract.

We report an *H. parainfluenzae* clinical isolate resistant to cefotaxime and with decreased susceptibility to ciprofloxacin recovered from a patient with cystic fibrosis. The isolate had elevated MICs of ampicillin (256 mg/L), amoxicillin-clavulanate (8 mg/L), cefuroxime (8 mg/L) and cefotaxime (4 mg/L), and showed a ß-lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) phenotype. A bla_{TEM-1} plus five amino acid substitutions in the PBP3 were found: Ser385Thr, Val511Ala, Ile519Val, Asn526Lys and Asp551Leu. MIC of ciprofloxacin was 0.5 mg/L, and substitutions in *gyr*A (Ser84Tyr) and *par*C (Ser84Phe) genes were detected.

Keywords.

Haemophilus parainfluenzae; Cefotaxime; Ciprofloxacin; PBP3

Manuscript.

Haemophilus species are considered to be normal inhabitants of the human upper respiratory and urogenital tracts. *H. parainfluenzae* causes opportunistic human infections, mainly associated with respiratory and genitourinary tracts, although severe infections like meningitis, sepsis, septic arthritis, pericarditis and endocarditis were also described.[1-4] In our country the prevalence of resistance to ampicillin in *H. influenzae* clinical isolates is 22% and mainly associated to ß-lactamase production (http://antimicrobianos.com.ar/2015/?cat=16).

Decreased susceptibility or resistance to β-lactams in *H. parainfluenzae* can be mediated by the production of TEM β-lactamase, alterations in the penicillin binding protein 3 (PBP3), or the combination of both mechanisms[4-6]. There are key modifications in PBP3, that were found to reduce the susceptibility against β-lactams, like the amino acid substitutions of Asn for Lys at position 526 or Arg for His at position 517 in the *fts*I gene of PBP3[5-7]. A number of other PBP3 substitutions, in addition to those at positions 526 or 517, that are thought to contribute to decreased susceptibility to β-lactams, are close to the SSN (Ser-385) or the KTG (Val-511 and Ala-530) motifs that result in elevated MIC to cefotaxime (0.5-1.5 mg/L range).[4,5] Decreased susceptibility (0.12-1 mg/L) or resistance (≥ 2 mg/L) to fluoroquinolones was also reported in *Haemophilus* species being the main mechanism of resistance the acquisition of mutations in the *gyr*A gene with or without mutations in the *par*C gene[8-10]. Fluoroquinolone-resistant *H. parainfluenzae* isolates were recently reported in Spain and Switzerland.[3,4] Here, we report an *H. parainfluenzae* clinical isolate resistant to cefotaxime and with decreased susceptibility to ciprofloxacin.

H. parainfluenzae M11065 was recovered from the sputum of a fibrocystic two-year old patient in a general hospital from Buenos Aires City, Argentina. The isolate showed an unusual phenotype of resistance to both cefotaxime and ciprofloxacin, therefore it was submitted to the National Reference Laboratory for further characterization. Minimal inhibitory concentration (MIC) was determined by agar dilution using HTM medium, 10^4 CFU/spot, and incubated at 35ºC during 20-24hs in 5% CO_2. MIC was interpreted

according to CLSI M100-S25 guidelines[11]. MIC to ciprofloxacin was evaluated with and without 12.5 mg/L of reserpine.

The *ftsI* gene (PBP3) was amplified and sequenced using conditions described by Tristram et al.[6], but with a degenerate forward primer (FtsI-Hp-F1) which was designed in this work (Table 1). Amplification and sequencing of *gyr*A, *par*C and bla_{TEM} genes were performed using primers and anneling temperature described in Table 1. PCR reactions were performed using standard conditions[12], the elongation step was adjusted to 30 seconds for *gyr*A and *par*C amplification, and to 60 seconds for *fts*I and bla_{TEM} genes. Sequences of *fts*I, *gyr*A and *par*C amplification fragments were compared with sequences of *H. parainfluenzae* T3T1 isolate (Accession NC_015964).

The isolate had elevated MICs of ampicillin (256 mg/L), amoxicillin-clavulanate (8 mg/L), cefuroxime (8 mg/L) and cefotaxime (4 mg/L). ß-lactamase production was detected by the nitrocefin assay. The isolate showed a ß-lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) phenotype according to the susceptibility to ß-lactams as defined for *H. influenzae*[13]. PCR and sequencing revealed the presence of bla_{TEM-1} with a Pdel promoter region which explained the high level of resistance to ampicillin but not to amoxicillin-clavulanate and cephalosporins[14]. Then, five amino acid substitutions were detected in the PBP3: Ser385Thr, Val511Ala, Ile519Val, Asn526Lys and Asp551Leu. To our knowledge, this is the first description of Asp551Leu and Ile519Val modifications in a clinical isolate. The combination of substitutions at positions 385, 511 and 526 were previously described in three *H. parainfluenzae* isolates showing MIC values to cefotaxime between 0.5-1.5 mg/L (Table 2).[4,5] *H. parainfluenzae* M11065 presented a third amino acid substitution in position 519 of the KTG motif, in addition to 511 and 526, that could have contributed to the increased cefotaxime MIC value, even though it needs to be confirmed by transformation assays. In this genus, a cefotaxime MIC of 4 mg/L mediated by amino acid substitutions in PBP3, was only described in *H. influenzae*[7].

Disk diffusion assay to nalidixic acid and ciprofloxacin resulted in no-inhibition zone and a 22 mm inhibition zone, respectively. MIC of ciprofloxacin was 0.5 mg/L with or without

reserpine discarding a possible contribution of efflux pumps to this phenotype. As a result, we detected substitutions in *gyr*A (Ser84Tyr) and *par*C (Ser84Phe) genes. In *H. parainfluenzae* clinical isolates, Ser84Phe amino acid substitution in both *gyr*A and *par*C genes have been the most commonly reported, while the Ser84Tyr mutation in *gyr*A gene were not previously described in this species (Table 2).[3,4, 10] Both Ser and Tyr are hydrophilic amino acids, however Tyr has an additional bulky hydrophobic group which could affect the interaction between the DNA gyrase with quinolones. Additionally, we evaluated the presence of plasmid-mediated quinolone-resistance genes by PCR obtaining negative results for *qnr*A, *qnr*B, *qnr*S, *qep*A and *aac-6´-Ib-cr* genes[15]. The nalidixic acid disk has been previously proposed as a useful screening method to detect decreased susceptibility or resistance to fluoroquinolones in *H. influenzae*[9]. In this work, we have observed a good performance of the nalidixic acid disk for *H. parainfluenzae* isolates as well.

ß-lactams are first line treatment drugs for Haemophili infections, therefore the emergence of resistance to cefotaxime is of clinical concern. A recent study documented the intra and interspecies recombination of FstI in *H. influenzae* and *Haemophilus haemolyticus in vitro* resulting in mosaic structure of the gene[16]. The capacity of *Haemophilus* species to acquire resistance, potentially not only to ß-lactams, via mosaic genes, contextualizes the relevance of this report. These facts highlight the importance of antimicrobial surveillance systems to monitor the emergence or increase of resistance in Haemophili clinical isolates causing infectious diseases.

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Transparency declarations

None to declare.

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Table 1. Primers details, annealing temperatures and product size for specific PCR.

Primer	Gene	Sequence (5´-3´)	Annealing (°C)	Product size (bp)
FtsI-Hp-F1	*ftsI*	GAYGGTGCWCGYGTTGTTCG	50	1038
FtsI-Hp-R		GCTAGAGAATACCGGGGCAG	50	
gyrA-Hp-F	*gyr*A	TTCYTACCTTGACTACGCSA	52	441
gyrA-Hp-R		AGTGCTGGAATACGAGTTGG	52	
parC-Hp-F	*par*C	CATGGATCGTGCRTTGCCTT	52	471
parC-Hp-R		GTGTGGTGGAATATCMGTRG	52	
TEM-Fb	bla_{TEM}	GTATTGCCCGCTCCACGGT	50	1117
TEM-Rb		GAGTAAACTTGGTCTGACAGTTACCA	50	

Table 2. Summary of amino acid substitutions described in the *fts*I, *gyr*A and *par*C genes for *H. parainfluenzae* clinical isolates.

A.

Isolate	β-lactamase	Ser385	Ile442	Val511	Ile519	Asn526	Cefotaxime MIC (mg/L)	Additional substitutions	Reference
M11065	TEM-1	Thr	-	Ala	Val	Lys	4	Asp551Leu	This study
AE-2096513	TEM-1[c]	Thr	Phe	Ala	-	Lys	1.5	Lys276Asn; Ala307Asn; Val329Ile;	6
III-like[a]	None	Thr	Phe	Ala	-	Lys	0.5	Val562Ile	5
SF2/SF3[b]	TEM-15[d]	Thr	-	-	-	His	16/8	Ala343Val;	4

B.

Isolate	gyrA		parC		Ciprofloxacin MIC (mg/L)	Additional substitutions	Reference
	Ser84	Asp88	Ser84	Tyr88			
M11065	Tyr	-	Phe	-	0.5	None	This study
617	Phe	Tyr	Phe	-	32	parC: Ser138Thr; Met198Leu	3
AE-2096513	Phe	Tyr	Phe	-	>32	None	4
07-020	Phe	Tyr	Phe	-	4	None	14
07-028	Phe	Tyr	Phe	-	12	None	14

Footnote. *H. parainfluenzae* clinical isolates with: decreased susceptibility, MIC 0.5-2 mg/L, or resistance, MIC ≥ 4 mg/L, to cefotaxime (panel A); or decreased susceptibility, MIC 0.12-1 mg/L, or resistance, MIC ≥ 2 mg/L, to ciprofloxacin (panel B). a, include two isolates with the same gBLNAR group III-like genotype defined in reference 7; b, isolates SF2 and SF3 were indistinguishable by rep-PCR; c, TEM-1 not expressed; d, extended-spectrum TEM β-lactamase.

Highlights

- *H. parainfluenzae* recovered from a patient with cystic fibrosis.
- Resistant to cefotaxime and decreased susceptibility to ciprofloxacin.
- A bla_{TEM-1} plus five amino acid substitutions in the PBP3 were found.
- Substitutions in *gyr*A and *par*C genes were detected.