Emergence of Extensively Drug-Resistant (XDR) 

*Haemophilus parainfluenzae* in Switzerland

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**Running title:** XDR *H. parainfluenzae*

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ABSTRACT

Two homosexual men were colonized in the urethra with *Haemophilus parainfluenzae* non-susceptible to ampicillin (MIC 8 μg/ml), amoxicillin-clavulanate (MIC 4 μg/ml), cefotaxime (MIC 1.5 μg/ml), cefepime (MIC 3 μg/ml), meropenem (MIC 0.5 μg/ml), cefuroxime, azithromycin, ciprofloxacin, tetracycline and chloramphenicol (all MICs ≥32 μg/ml). Rep-PCR showed that the strains were indistinguishable. The isolates had amino acid substitutions in PBP3, L4, GyrA, ParC, and possessed Mef(A), Tet(M) and CatS resistance mechanisms. This is the first report of extensively drug-resistant (XDR) *H. parainfluenzae.*
*Haemophilus influenzae* isolates producing TEM-1/ROB-1 β-lactamases and/or with altered PBP3 (β-lactamase negative ampicillin-resistant, BLNAR) are nowadays increasingly reported. This species can also be multidrug-resistant (MDR) due to expression of co-associated mechanisms involving quinolones, macrolides, trimethoprim-sulfamethoxazole, tetracyclines and chloramphenicol (1-3). In contrast, little attention has been paid to *Haemophilus parainfluenzae*. Only a few BLNAR and/or TEM-producing isolates have been described (4, 5) and those with resistance to quinolones are rarely reported (6, 7). To our knowledge, MDR *H. parainfluenzae* isolates (e.g., simultaneously resistant to β-lactams, quinolones and macrolides) have never been found.

In April 2012, a homosexual man was treated with penicillin intramuscularly for primary syphilis. Four months later, he presented with urethritis. A pan-susceptible *Neisseria gonorrhoeae* (numerous colonies) of sequencing type (ST) 7616 (Por: 4578; TpbB: 29; [www.ng-mast.net](http://www.ng-mast.net)) and an *H. parainfluenzae* (few colonies) were isolated from the urethral swab implementing blood and chocolate agar plates. The infection was treated empirically with ciprofloxacin and led to complete resolution of symptoms. Retrospectively, the *H. parainfluenzae* (AE-2096513) was considered as colonizer but its very unusual MDR phenotype observed using the standardised disk diffusion method drew our attention ([http://www.eucast.org/mic_distributions/](http://www.eucast.org/mic_distributions/)). Species identification was routinely achieved by indole test (negative) and direct analysis of colonies by MALDI-TOF mass spectrometry (Bruker Daltonik). It was also confirmed by sequencing of the 16S rRNA (MicroSeq® 500; Applied Biosystem). Production of β-lactamases was initially tested using the cefinase paper disc (BBL) and further evaluated assessing the hydrolytic activity against nitrocefin [100 µM] for 1 hour. Although it is not a standardized methodology, the Etest (bioMérieux) was used to obtain the MICs for antibiotics. Mueller-Hinton
agar plus 5% defibrinated horse blood and 20 mg/L β-NAD plates (Oxoid) were used and results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (8).

Genomic extraction was performed using the QIAamp® DNA Mini kit (QIAGEN). Two microarray platforms (AMR-ve 0.5m and AMR+ve-3; Alere technologies) were implemented to detect numerous antibiotic resistance genes (9). Some of them may be potentially present in *Haemophilus* spp. such as (targeted antibiotics): *bla*TEM (β-lactams); *qnr*A/B/S, *qep*A, *aac*6-Ib-cr (quinolones); *erm*(A)/(B)/(C)/(D)/(F), *mef*A (macrolides); *tet*(A)/(B)/(C)/(D)/(E)/(G)/(K)/(M) (tetracyclines), *dfr*A/D/G/K, *sul*1/2/3 (trimethoprim-sulfamethoxazole); and *cat*A/B/D/P/Q/S/III, *fex*A, *flo*R, *cml*A1 (chloramphenicol) (2, 3). Previously reported, or designed primers, were also used to amplify and sequence further resistance traits (targeted antibiotics): PBP3 transpeptidase domain of the *fts*I (5), *bla*TEM [primers TEM-F1: 5’-CGTGTCGCCCTTATTCCC-3’; TEM-B1: 5’-AGGCACCTATCTCAGCGATC-3’], *bla*TEM promoter region [primers proTEM-F1: 5’-AATTCTTGAAGACGAAAGGG-3’; proTEM-R2: 5’-CGCTGTTGAGATCCAGTTCG-3’] and *bla*ROB (β-lactams) (10); quinolone resistance determining region of *gyr*A and *par*C (quinolones) (6); *rpl*ID and *rpl*IV (L4 and L22 proteins, respectively) of the 50S ribosomal subunit (11) and *mef*A (macrolides) (12); *tet*M (tetracyclines) (13); *cat*D, *cat*P and *cat*S [primers catDPS-F, catDPS-R (9); catHP-F: 5’-GAGATGATGCAGCCTTTG-3’; catHP-R 5’-AGTCCGACAACTGGAAG-3’] (chloramphenicol). Presence of mutations into the copies of the 23S rRNA (resistance to macrolides) were assessed amplifying the genes from base 1902 to 2956 (Escherichia coli numbering) [primers 23S-3 (11); 23S-R: 5’-CGCCAGATTCCCTTTA-3’], cloning them with TOPO® XL PCR kit (Invitrogen), and sequencing five randomly selected colonies. Results for all of the above genes were compared to the deposited genome of *H.*
parainfluenzae T3T1 (NC_015964) or searching for homologies in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Repetitive extragenic palindromic PCR (rep-PCR) was performed as previously done and interpreted using the Agilent bio-analyzer (14).

As shown in Table 1, the H. parainfluenzae was resistant to β-lactams, macrolides, quinolones, tetracyclines and chloramphenicol, leaving only trimethoprim-sulfamethoxazole and rifampicin in the susceptible range. The MIC of meropenem (0.5 μg/ml) was interpreted as susceptible, though this value is defined as intermediate when the isolate causes meningitis (8). Thus, the H. parainfluenzae can be defined as an extensively drug-resistant (XDR) isolate (1).

Molecular analysis indicated that AE-2096513 was resistant to β-lactams and amoxicillin-clavulanate because of seven amino acid substitutions in the PBP3 (Table 1). A similar pattern of substitutions have been recently described in one BLNAR H. parainfluenzae found in Spain (4). Remarkably, AE-2096513 carried the \textit{bla}_{TEM-1} but the β-lactamase was not expressed based on both nitrocefin tests implemented. This observation was partially supported by the finding that the \textit{bla}_{TEM-1} possessed a P3 promoter, which is recognized as the weakest in driving gene expression among those previously described (15).

High level resistance to macrolides was attributed to the presence of the efflux-mediated resistance mechanism Mef(A) and to the Ala69Ser in the L4 protein, a substitution previously associated to macrolides resistance in H. influenzae (11). The L22 protein did not contain substitutions. Two different copies of the 23S rRNA were cloned and sequenced (GenBank: KC559885 and KC559886), but previously reported mutations conferring macrolide resistance were not recorded (11, 16).

AE-2096513 was also highly-resistant to quinolones due to the classic GyrA (Ser84Phe and Asp88Tyr) and ParC (Ser84Phe) amino acid substitutions (6). Finally, tetracycline and
chloramphenicol resulted resistant due to production of the ribosomal protective protein Tet(M) and the CatS acetyltransferase enzyme, respectively (Table 1) (13).

Surprisingly, five months after the identification of the above strain, a further XDR *H. parainfluenzae* (few colonies) was isolated from a urethral swab of another homosexual man.

Again, it was together with a *N. gonorrhoeae* (numerous colonies) of ST8371 (por: 5033 - tbpB: 137) resulting resistant to penicillin, tetracycline and ciprofloxacin, but susceptible to azithromycin and ceftriaxone. This second case was empirically treated with ciprofloxacin and doxycycline without clinical improvement and then with ceftriaxone plus azithromycin with resolution of the infection. Interestingly, the strain (AE-2137638) possessed exactly the same phenotypic and molecular characteristics of AE-2096513 (Table 1). More importantly, the rep-PCR analysis showed that the two *H. parainfluenzae* were indistinguishable (Figure 1). This was also supported by identical DNA sequences of the *gyrA* and *parC* housekeeping genes.

This is the first report of XDR *H. parainfluenzae* isolates. The strains possess a pattern of multiple resistance traits that has never been described in a single isolate. To our knowledge, this is also the first time that *mef*(A), *tet*(M), *cat*S and the Ala69Ser substitution in L4 are found in *H. parainfluenzae*.

*H. parainfluenzae* can be part of the nasopharyngeal flora and a possible cause of urethritis among homosexual men (17, 18). In our cases, contact between the two carriers was not explored to preserve the patients’ privacy. Therefore, though this *H. parainfluenzae* phenotype was previously neither observed nor reported, we can only speculate that the two men had direct or indirect (via a third or fourth person) sexual contact. However, we highlight that *H. parainfluenzae* can be responsible for serious infections such as meningitis, sepsis, septic arthritis and endocarditis (19-
22). Thus, larger studies should be planned to establish the epidemiologic relevance of these difficult to treat XDR organisms.
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REFERENCES


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Results of the rep-PCR analysis. L: ladder; 1: strain AE-2096513 (first patient, August 2012); 2: strain AE-2137638 (second patient, January 2013). The two XDR *H. parainfluenzae* isolates are indistinguishable.
Table 1. Antimicrobial phenotype and molecular mechanisms of resistance found in the two XDR H. *parainfluenzae*. Since the isolates are indistinguishable, the phenotypic and molecular background of resistance is the same.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC, µg/ml Interpretation</th>
<th>Molecular mechanism(s) of resistance</th>
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<tbody>
<tr>
<td>β-lactams</td>
<td></td>
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<tr>
<td>- Ampicillin</td>
<td>8, R</td>
<td>PBP3: Lys276Asn, Ala307Asn, Val329Ile, Ser385Thr, Ile442F, Val511 Ala, Asn526Lys (TEM-1 not expressed)</td>
</tr>
<tr>
<td>- Amoxicillin</td>
<td>6, R</td>
<td></td>
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<tr>
<td>- Amoxicillin-clavulanate</td>
<td>4, R</td>
<td></td>
</tr>
<tr>
<td>- Cefuroxime</td>
<td>32, R</td>
<td></td>
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<tr>
<td>- Ceftriaxone</td>
<td>0.25, R</td>
<td></td>
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<tr>
<td>- Cefotaxime</td>
<td>1.5, R</td>
<td></td>
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<tr>
<td>- Cefepime</td>
<td>3, R</td>
<td></td>
</tr>
<tr>
<td>- Meropenem</td>
<td>0.5, S (I)</td>
<td></td>
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<tr>
<td>Macrolides</td>
<td></td>
<td></td>
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<tr>
<td>- Erythromycin</td>
<td>&gt;256, R</td>
<td>Mef(A)</td>
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<tr>
<td>- Clarithromycin</td>
<td>&gt;256, R</td>
<td>L4: Ala69Ser b</td>
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<tr>
<td>- Azithromycin</td>
<td>&gt;256, R</td>
<td></td>
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<tr>
<td>Quinolones</td>
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<tr>
<td>- Ciprofloxacin</td>
<td>&gt;32, R</td>
<td>GyrA: Ser84Phe, Asp88Tyr</td>
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<tr>
<td>- Levofloxacin</td>
<td>&gt;32, R</td>
<td>ParC: Ser84Phe</td>
</tr>
<tr>
<td>- Tetracycline</td>
<td>32, R</td>
<td>Tet(M)</td>
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<tr>
<td>- Trimethoprim-sulfamethoxazole</td>
<td>0.25, S</td>
<td></td>
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<tr>
<td>- Rifampicin</td>
<td>0.75, S</td>
<td></td>
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<tr>
<td>Chloramphenicol</td>
<td>96, R</td>
<td>CatS</td>
</tr>
</tbody>
</table>

* Interpretation of MICs according to the EUCAST criteria (S, susceptible; I, intermediate; R, resistant) (8): ampicillin, cefuroxime, rifampicin, clarithromycin, levofloxacin, tetracycline (S ≤ 1 µg/ml); amoxicillin, amoxicillin-clavulanate, chloramphenicol (S ≤ 2 µg/ml); ceftriaxone, cefotaxime, azithromycin (S ≤ 0.12 µg/ml); cefepime (S ≤ 0.25 µg/ml); meropenem (S ≤ 2 µg/ml; S ≤ 0.25 µg/ml and R ≥ 2 µg/ml for meningitis); erythromycin, ciprofloxacin, trimethoprim-sulfamethoxazole (S ≤ 0.5 µg/ml)

b The Ala69Ser substitution was observed after comparison with H. influenzae Rd but not with H. parainfluenzae T3T1