International Journal of Antimicrobial Agents 62 (2023) 106991

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Detection of $bla_{\text{CTX-M-15}}$ in an integrative and conjugative element in four extensively drug-resistant *Haemophilus parainfluenzae* strains causing urethritis

L. Saiz-Escobedo[a], I. Cadenas-Jiménez[a,b], R. Olmos[a], A. Carrera-Salinas[a], D. Berbel[a,b], J. Càmara[a,b], F. Tubau[a,b], M.A. Domínguez[a,c,d], C. Ardanuy[a,b,d], A. González-Díaz[a,b,*], S. Marti[a,b,e,*]

[a] *Microbiology Department, Hospital Universitari de Bellvitge, IDIBELL-UB, Barcelona, Spain*
[b] *Research Network for Respiratory Diseases (CIBERES), ISCIII, Madrid, Spain*
[c] *Research Network for Infectious Diseases (CIBERINFEC), ISCIII, Madrid, Spain*
[d] *Department of Pathology and Experimental Therapeutics, School of Medicine, University of Barcelona, Barcelona, Spain*
[e] *Department of Medicine, School of Medicine, University of Barcelona, Barcelona, Spain*

ARTICLE INFO

Article history:
Received 24 July 2023
Accepted 21 September 2023

Editor: Dr. Po-Ren Hsueh

Keywords:
H. parainfluenzae
$bla_{\text{CTX-M-15}}$
Urethritis
XDR

ABSTRACT

Haemophilus parainfluenzae is a commensal organism with rising numbers of multidrug-resistant (MDR) strains. This pathogen is of increasing clinical relevance in urogenital infection. The aim of this work was to identify and characterise the molecular mechanisms of resistance associated with four cephalosporin-resistant *H. parainfluenzae* strains collected from patients with urethritis. Antimicrobial resistance was determined by microdilution following European Committee on Antimicrobial Susceptibility Testing criteria. Strains were then analysed by whole-genome sequencing to determine clonal relationship and the molecular basis of antimicrobial resistance. Finally, a phylogenetic analysis was performed on all urogenital MDR strains of *H. parainfluenzae* previously isolated in our hospital. All strains were resistant to β-lactams, macrolides, tetracycline, fluoroquinolones, chloramphenicol, cotrimoxazole, and aminoglycosides. The resistance profile was compatible with the presence of an extended-spectrum β-lactamase (ESBL). Whole-genome sequencing detected $bla_{\text{CTX-M-15}}$ that conferred high minimum inhibitory concentrations to cephalosporins in two novel integrative and conjugative elements (ICE*HpaHUB6* and ICE*HpaHUB7*) that also harboured a $bla_{\text{TEM-1}}$ β-lactamase. This study shows a novel $bla_{\text{CTX-M-15}}$ ESBL carried in an integrative conjugative element in four extensively drug-resistant *H. parainfluenzae* strains. This resistance determinant could be transmitted to other sexually transmitted pathogens and this is a cause for concern.

1. Introduction

Haemophilus parainfluenzae is an opportunistic Gram-negative pathogen normally found as a commensal in the human respiratory and urogenital tracts. Infections caused by *H. parainfluenzae* have recently gained more attention because this pathogen has been increasingly identified as a causative agent in urogenital infections over the last decade [1]. In particular, *H. parainfluenzae* has been isolated in numerous episodes of urethritis, with transmission presumed to be through unprotected sex [2].

The role of *H. parainfluenzae* in sexually-transmitted infections (STIs) [3], as well as the emergence and spread of multidrug-resistant (MDR) strains, highlights its clinical relevance. The first extensively drug-resistant (XDR) strain was reported in Switzerland in 2013 [4], and the number of MDR and XDR strains has been increasing ever since. Antibiotic resistance due to point mutations or the acquisition of transferable resistance genes has been shown with β-lactams, macrolides, tetracycline, fluoroquinolones, chloramphenicol, and cotrimoxazole [2,5]. Resistance is often associated with the presence of integrative and conjugative elements (ICEs) [6]. Several of these elements have been described in resistant *H. parainfluenzae*, including ICE*HpaHUB3* [7]. The transposons Tn*3*, Tn*10*, and Tn*7076* are also contained in this ICE. Tn*3* harbours the $bla_{\text{TEM-1}}$ gene, which encodes TEM-1 β-lactamase, the

* Corresponding authors.
E-mail addresses: agonzalezd@bellvitgehospital.cat (A. González-Díaz), smartinm@bellvitgehospital.cat (S. Marti).

https://doi.org/10.1016/j.ijantimicag.2023.106991

main cause of β-lactam resistance in this species [8]. Tn*10* includes the *tet*(B)/(C)/(D)/(R) genes, and may also contain *catA2*, conferring resistance to tetracycline and chloramphenicol, respectively. Finally, Tn*7076* carries different aminoglycoside (*aph*(6)-Id, *aph*(3")-Ib, *aph*(3")-Ia, *ant*(2")-Ia) and cotrimoxazole (*sul2*) resistance genes, contributing to the XDR phenotypes [7].

The increasing prevalence of urogenital infections caused by *H. parainfluenzae* and the spread of MDR strains are a cause for concern. To date, β-lactam resistance has only been attributed to the presence of TEM-1 β-lactamase, which confers resistance to penicillins, and point modifications in the PBP3, which could cause low-level resistance to cephalosporins. The aim of this study was to describe a CTX-M-15 extended-spectrum β-lactamase (ESBL) in four XDR urogenital strains of *H. parainfluenzae* and fully characterise the molecular resistance determinants.

2. Methods

2.1. Study design and strain selection

Hospital Universitari de Bellvitge is a tertiary care hospital located in southern Barcelona. Microbiological samples from the hospital and primary care centres are routinely processed for diagnosis. In 2022, four *H. parainfluenzae* strains with significant clinical relevance presented a cefotaxime minimum inhibitory concentration (MIC) of >2 mg/L. The clinical charts of patients were reviewed to evaluate relevant clinical variables, including age, sex, symptoms, underlying disease, high-risk sexual behaviours, sexual orientation, HIV infections, presence of other microorganisms, and antibiotic therapy. The four strains were further analysed by whole-genome sequencing for in-depth characterisation.

2.2. Characterisation and antibiotic susceptibility

Strains were routinely grown in chocolate agar plates (BioMérieux) and incubated at 37°C in a 5% CO_2 atmosphere. Bacterial identification was performed by MALDI-TOF (Bruker). Antibiotic susceptibility was studied using a disk diffusion method. The double-disk synergy test (DDST) was utilised for ESBL, using amoxicillin and clavulanic acid and the β-lactams, cefotaxime, ceftazidime, aztreonam, and cefuroxime. MICs were determined by microdilution using STRHAE2 Sensititre commercial panels (Thermo Fisher Scientific). For certain β-lactams (cefotaxime and ceftriaxone), fluoroquinolones (ciprofloxacin and levofloxacin), and aminoglycosides (gentamycin, tobramycin, and amikacin), MICs were determined by Etest (bioMérieux). All procedures for susceptibility studies were performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria for *H. influenzae* (www.eucast.org/clinical_breakpoints).

2.3. Whole-genome sequencing

DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen) and quantified with Qubit 4 (Thermo Fisher Scientific). Short-read sequencing was conducted using Illumina Nextera XT to prepare genome libraries, with subsequent sequencing on a MiSeq platform (2 × 300 bp). For long-read sequencing, the Native Barcoding Expansion Kit (EXP-NBD196) and the Ligation Sequencing Kit (SQK-LSK109) were used for library preparation, with sequencing on FLOMIN106D flow cells (R9.4.1) (Oxford Nanopore Technologies). Reads were assembled using the Unicycler pipeline (github.com/rrwick/Unicycler). Short- and long-reads were deposited at the European Nucleotide Archive (PRJEB63685), with the final assemblies deposited at the NCBI.

2.4. Phylogenetic analysis

The phylogenetic relationship of the four *H. parainfluenzae* strains was established using the urogenital MDR isolates from a previous study in our hospital (2013–2017) as the epidemiological background [7]. Whole-genome alignment was constructed in Snippy (github.com/tseemann/snippy) using HUB-HP16991, the first strain isolated in this study, as the reference. A phylogenetic tree was constructed using Gubbins [9], the tree representation was done using FigTree (tree.bio.ed.ac.uk/software/figtree/), and metadata were incorporated with Inkscape.

2.5. Antibiotic resistance and ICEs

To detect acquired resistance genes, the ResFinder Database [10] was used to screen the final assemblies. Point mutations in genes involved in antibiotic resistance and mobile genetic elements were detected using Geneious R9 (Biomatters) with *H. parainfluenzae* T3T1 (NC_015964) as the reference. ICE structures was characterised using the ICE*Hpa8f* (AM884335) sequence for reference.

3. Results

3.1. Clinical strains and patient background

The four *H. parainfluenzae* isolates were obtained from young men who had sex with men: three isolates were from urethral exudates (HUB-HP16991, HUB-HP17345 and HUB-HP17554) and one was from a genital ulcer (HUB-HP17357). All patients had a history of unprotected sex with multiple partners and had previously been diagnosed with other STIs. Three of the men had received antibiotic treatment in the previous 6 months (no records were available for the fourth patient). *H. parainfluenzae* was the only pathogen identified in the samples from two patients. The other two patients also had *Chlamydia trachomatis* (HUB-HP17357) or *Trichomonas vaginalis* (HUB-HP17554) detected by polymerase chain reaction (PCR). The episodes of urethritis were treated with a combination of intramuscular ceftriaxone and oral azithromycin or doxycycline.

3.2. Phylogenetics of urogenital isolates

Phylogenetic analysis of MDR *H. parainfluenzae* strains was conducted for context. Figure 1A shows the phylogenetic structure, with two major clades, one of which (marked in blue) accounted for most of the genomes (n = 31). Figure 1B shows a sub-tree constructed using the same methodology to account for the low resolution of the dominant clade. Although HUB-HP16991 and HUB-HP17554 clustered together, the core genome contained 5720 single nucleotide polymorphisms (SNPs). HUB-HP17345 and HUB-HP17357 were phylogenetically closer to 1086 SNPs. Finally, HUB-HP16991 and HUB-HP17345 were the most distant genomes and presented 11 755 different core SNPs.

3.3. Antimicrobial resistance

The four strains under study were resistant to penicillins and cephalosporins but were susceptible to amoxicillin/clavulanic acid and carbapenems (Table 1). The strains had high MICs for cephalosporins and were DDST-positive (Supplementary Figure S1). All strains were resistant to macrolides, fluoroquinolones, chloramphenicol, cotrimoxazole, and tetracycline. The strains from HUB-HP16991 and HUB-HP17554 had higher MICs to aminoglycosides (amikacin, 12 mg/L; gentamicin, >256 mg/L; tobramycin, 96 mg/L) than the strains from HUB-HP17345 and HUB-HP17357 (amikacin, 6 mg/L; gentamicin, 1 mg/L; tobramycin, 1 mg/L).

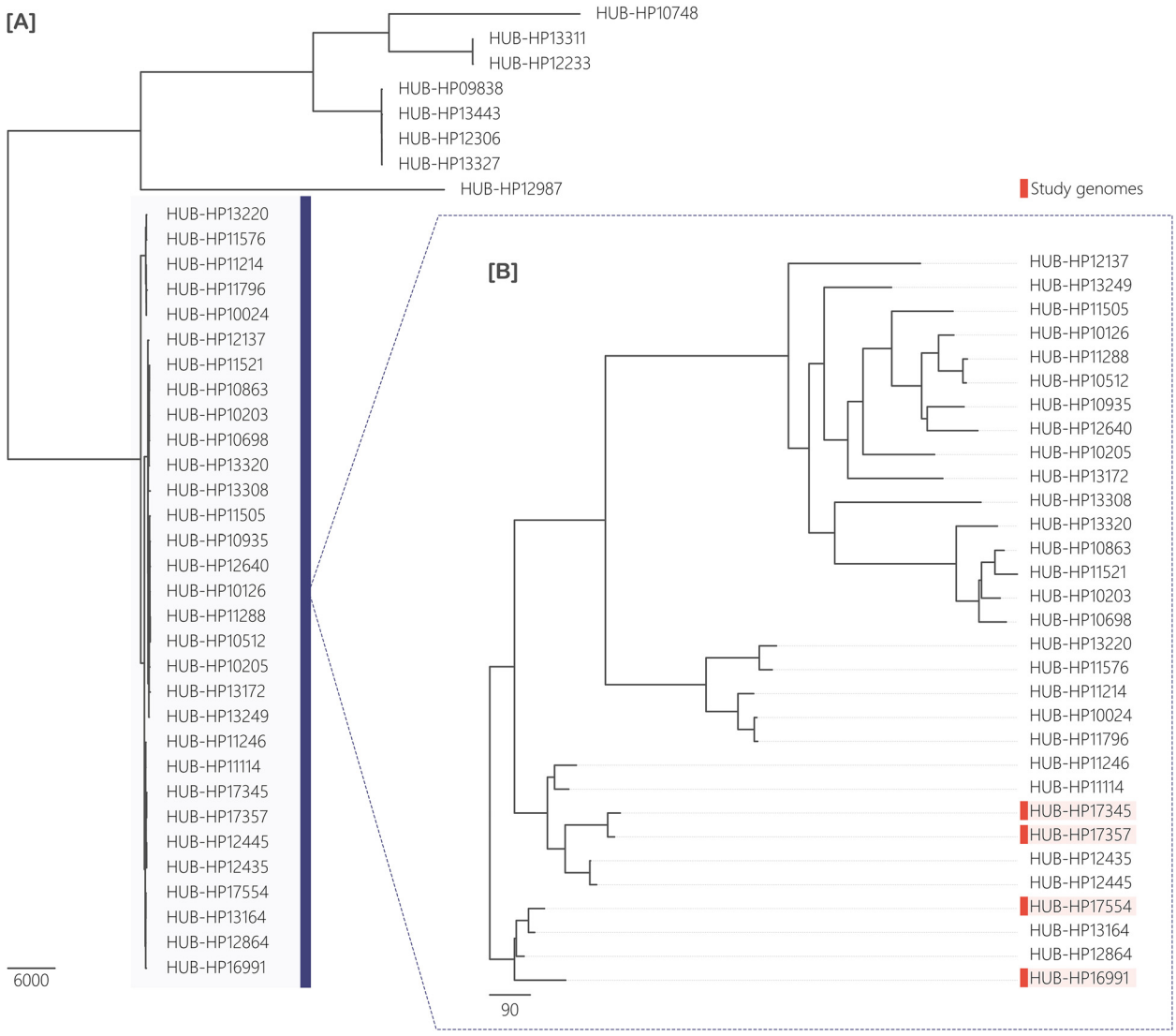

Figure 1. Phylogenetic tree of the urogenital *H. parainfluenzae* strains isolated in our hospital. [A] Phylogenetic tree including 36 urogenital *H. parainfluenzae* genomes from a previous study (7) and the 4 studied genomes carrying $bla_{CTX\text{-}M\text{-}15}$. The major clade is marked in blue. [B] Phylogenetic subtree of the major clade (blue), showing the four genomes containing $bla_{CTX\text{-}M\text{-}15}$ marked in orange.

β-lactam resistance was attributed to the presence of β-lactamases (TEM-1 and CTX-M-15). All strains also had resistance-associated modifications in PBP3 (i.e., V329I, S385T, I442F, V511A, N526K, and V526I). Macrolide and tetracycline resistance was caused by the insertion of a *tet*(M)-MEGA element formed by the *mef*(E), *msr*(D), and *tet*(M) genes, present in a partial Tn*2009* transposon lacking the *int-Tn* and *xis-Tn* genes. The HUB-HP16991 strain also had the tetracycline resistance genes, *tet*(B), *tet*(C), and *tet*(D) inserted in the genome. Fluoroquinolone resistance was explained by amino acid substitutions in GyrA (i.e., S84F and D88Y) and ParC (i.e., S84F/Y). All strains had both modifications in GyrA, but only HUB-HP17345 and HUB-HP17357 had all described substitutions in ParC. HUB-HP17554 had lower MICs for ciprofloxacin and levofloxacin and did not have the S84F/Y mutation in ParC. Resistance to chloramphenicol was attributed to the acquisition of *catS*, and aminoglycoside resistance (HUB-HP16991 and HUB-HP17554) was linked to *aac*(6')-*Ie-aph*(2'')-*Ia*. Finally, resistance to cotrimoxazole was conferred by mutations in *folA* (i.e., I95L and S135N), *folP* (i.e., P64S), and the *folA* promoter (i.e., -10 motif [G>A]).

3.4. Characterisation of $bla_{CTX\text{-}M\text{-}15}$ and novel ICEs

An ICE*Hpa8f-like* was detected in all strains, carrying not only a $bla_{TEM\text{-}1}$ β-lactamase but also a $bla_{CTX\text{-}M\text{-}15}$ ESBL, and identified for the first time in *H. parainfluenzae* strains (Figure 2A). This $bla_{CTX\text{-}M\text{-}15}$ had been integrated with its own *tnpA* gene (also known as ISE*cp1*) in transposon Tn*3* that carries the $bla_{TEM\text{-}1}$ gene, its own transposase (*tnpA*), and a regulator (*tnpR*). This Tn*3-like* structure has already been described in *Escherichia coli* and *Klebsiella pneumoniae* plasmids [11,12]. The CTX-M-15-containing Tn*3* transposon was inserted in an ICE*Hpa8f*-like mobile element, resulting in the formation of a new ICE variant named ICE*HpaHUB6*. This was found in three of the strains (HUB-HP17554, HUB-HP17345, and HUB-HP17357) and was inserted in the same position of the genome, between tRNA(Leu)-tRNA(Lys)-tRNA(Gly) and the gene encoding cytochrome c peroxidase (Figure 2B).

The HUB-HP16991 strain also harboured an ICE*Hpa8f*-like element with the same Tn3-$bla_{CTX\text{-}M\text{-}15}$ structure, but this also included tetracycline resistance genes. An integrative mobilis-

Table 1
Clinical data and antimicrobial resistance for *H. parainfluenzae* isolates from patients with acute urethritis.

	HUB-HP16991	HUB-HP17345	HUB-HP17357	HUB-HP17554
Age/Sex	46/Male	27/Male	35/Male	40/Male
MSM	Yes	Yes	Yes	Yes
Risk exposures				
Unprotected sex	Yes	Yes	Yes	Yes
Multiple partners	Yes	Yes	No data	No data
PrEP/HIV	Yes/No	No/No	Yes/No	No/Yes
Clinical symptoms				
Abdominal pain	No	No	Yes	Yes
Diarrhoea and anal bleeding	No	No	No	Yes
Dysuria	No	Yes	Yes	Yes
Skin lesions	No	No	Yes	No
Urogenital discharge	Yes	Yes	Yes	Yes
Previous STI				
Chlamydia	Yes	No	Yes	No data
Gonorrhoea	Yes	Yes	Yes	No data
Syphilis	Yes	No	Yes	Yes
Trichomoniasis	No	No	No	Yes
Previous treatment (<6 months)				
Ceftriaxone IM and Azithromycin OR	Yes	No	No data	No
Ceftriaxone IM and Doxycycline OR	No	No	No data	Yes
Ciprofloxacin OR	No	Yes	No data	No
Antimicrobial therapy (episode)				
Ceftriaxone IM and Azithromycin OR	Yes	No	No	No
Ceftriaxone IM and Doxycycline OR	No	Yes	No	Yes
Ceftriaxone IM, Doxycycline OR and Penicillin G IM	No	No	Yes	No
Antimicrobial resistance (MIC)[a]				
Ampicillin	>4 (R)	>4 (R)	>4 (R)	>4 (R)
Amoxicillin/clavulanic acid	2/1 (S)	1/0.5 (S)	2/1 (S)	2/1 (S)
Cefotaxime[b]	32 (R)	>32(R)	>32 (R)	>32 (R)
Cefuroxime	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Ceftriaxone [b]	32 (R)	>32 (R)	>32 (R)	>32 (R)
Imipenem	0.25 (S)	≤0.12 (S)	≤0.12 (S)	≤0.12(S)
Azithromycin	>4 (R)	>4 (R)	>4 (R)	>4 (R)
Ciprofloxacin [b]	>32 (R)	16 (R)	>32 (R)	0.5 (R)
Levofloxacin [b]	>32 (R)	>32(R)	>32 (R)	0.75 (R)
Chloramphenicol	>8 (R)	8 (R)	>8 (R)	>8 (R)
Cotrimoxazole	>2/38 (R)	>2/38 (R)	2/38 (R)	>2/38 (R)
Tetracycline	>4 (R)	>4 (R)	>4 (R)	>4 (R)

MSM, men who have sex with men; PrEP, pre-exposure prophylaxis; HIV, human immunodeficiency virus; STI, sexually transmitted infection; IM, intramuscular; OR, oral.
[a] Interpretation of MICs according to the EUCAST criteria for *Haemophilus influenzae* (S, susceptible; R, resistant). Data are shown as a range of values for microdilution (mg/L).
[b] MIC for cefotaxime, ceftriaxone, ciprofloxacin, levofloxacin and aminoglycosides was determined by Etest (bioMérieux).

able element encoding *tet*(D), *tet*(C), and *tet*(B) surrounded by two transposases was inserted between the genes for replication and type 4 secretion in the ICE structure, resulting in a different ICE*HpaHUB7* that was, in turn, inserted adjacent to tRNA(Leu)-tRNA(Gly). A second copy of this integrative mobilisable element, which mobilises the tetracycline resistance genes, was also integrated into the chromosome.

4. Discussion

The role of *H. parainfluenzae* in STIs has been debated since the 1980s. This pathogen can be present as a normal coloniser of the urogenital tract and may coexist with other pathogens in urogenital STIs (e.g., *Neisseria gonorrhoeae* or *C. trachomatis)*, complicating efforts to determine its pathogenicity [13,14]. The current increase in the incidence of acute urethritis caused by *H. parainfluenzae* in patients who have had unprotected sex highlights its potential as a causative agent in STIs [1], and the emergence of multidrug resistance since 2013 [4] should raise concern. In this study, four XDR *H. parainfluenzae* strains were isolated from cases of acute urethritis and the molecular resistance mechanisms characterised. This resulted in the first description of $bla_{\text{CTX-M-15}}$ ESBL strains.

All four patients in the current study were men who had sex with men, had unprotected sex, and presented with the most common signs of infection (i.e., dysuria and urethral discharge) [15]. *H. parainfluenzae* was detected as the sole causative agent of infection in two patients, and there was co-infection with another STI-related pathogen in the other two patients. In a previous study analysing the emergence of MDR *H. parainfluenzae* urogenital strains in our hospital [2], this was the only pathogen isolated in 10 of 40 patients with symptoms of urethritis, indicating it is a relevant cause of STIs. *H. parainfluenzae* has also been considered the main cause of episodes of urethritis in other studies [16], but its pathogenic role has been difficult to determine. However, the increased prevalence of MDR *H. parainfluenzae* strains [2], and the frequent coexistence of these strains with other STI-related pathogens [1,2,4,16], underlines their clinical importance as potential reservoirs of resistance determinants.

All strains included in the current study were resistant to multiple antibiotics (XDR), with a $bla_{\text{CTX-M-15}}$ ESBL located in a mobile genetic element. This ESBL confers resistance to cephalosporins [17], including ceftriaxone, which is used to treat uncomplicated gonococcal infections [18]. This has clinical relevance in the management of urethritis because co-infection with the most common pathogen, *N. gonorrhoeae*, could transfer $bla_{\text{CTX-M-15}}$ from *H. parainfluenzae* and pose a therapeutic problem. In addition, all strains had other resistance elements inserted in their genomes, including the *tet*(M)-MEGA element, conferring resistance to macrolides and tetracyclines, which are first-line antibiotics in the treatment of non-gonococcal urethritis [19]. Again, co-infection with another

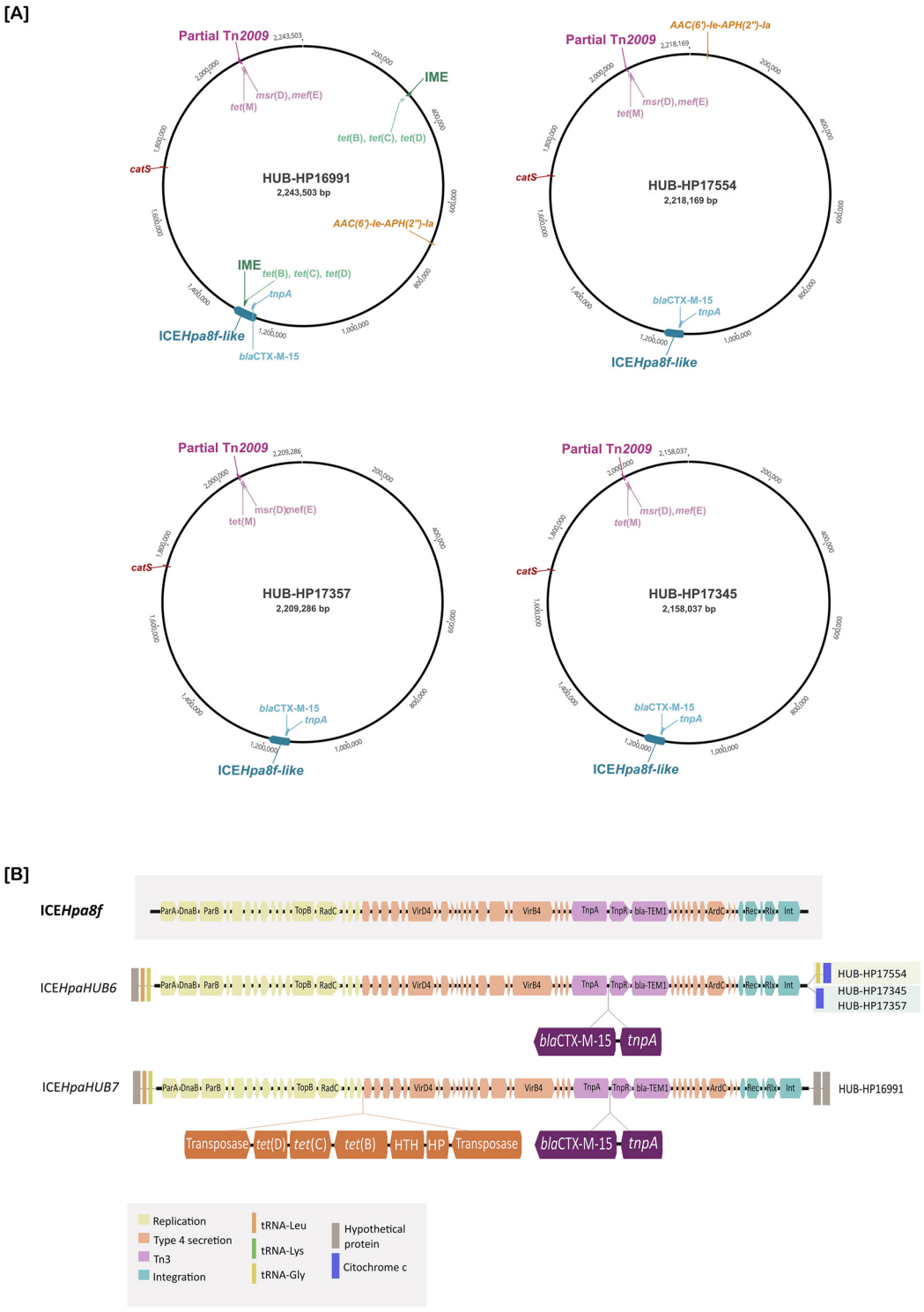

Figure 2. Acquired resistance genes and mobile elements for *H. parainfluenzae* cephalosporin-resistant strains. [A] $bla_{CTX\text{-}M\text{-}15}$ was inserted in an ICE structure (blue), the *tet*(M) gene and the MEGA element were inserted in a partial Tn*2009* (pink), and the *catS* (red) gene was observed in all the isolates. Additional tetracycline (green) and aminoglycoside (yellow) resistance was detected in HUB-HP16991 and HUB-HP17554. [B] ICE*HpaHUB6* and ICE*HpaHUB7* carried $bla_{CTX\text{-}M\text{-}15}$ and *tnpA* genes; the second ICE structure (ICE*HpaHUB7*) also carried the tetracycline resistance genes *tet*(B), *tet*(C) and *tet*(D).

STI-related pathogen, such as *C. trachomatis*, could lead to the pathogen acquiring this antimicrobial resistance element. Notably, the strains in the current study were susceptible to the combination of amoxicillin and clavulanic acid, which is common in ESBL-producing Enterobacteria (e.g., *E. coli*). This susceptibility, coupled with the high MIC for cefotaxime (>32 mg/L), points towards the presence of ESBL-producing strains, with MICs indicating susceptibility to third-generation cephalosporins. However, detecting ESBL could be a challenge and clinical microbiologists should be aware of *H. parainfluenzae* strains with deceased susceptibility to cephalosporins. Classical DDST could help identify this resistance mechanism.

The $bla_{CTX\text{-}M\text{-}15}$ ESBL was integrated into a novel ICE*Hpa8-like* [20] within a Tn*3-like* that also harboured a $bla_{TEM\text{-}1}$ β-lactamase.

This Tn*3-like* contained $bla_{CTX\text{-}M\text{-}15}$ with ISE*cp1* located between *tnpA* and *tnpR* genes. The insertion of ISEcp1-$bla_{CTX\text{-}M\text{-}15}$ in Tn*3* has been described in plasmids of *E. coli* (e.g., pEC_B24, pEC_L8, and pEC_L46) [12]. The Tn*3-like* in the current study shared 98.23% identity with those found in pEC_B24, only differing in the intergenic region between the *tnpR* gene and $bla_{TEM\text{-}1}$. As reported previously, the transposition of $bla_{CTX\text{-}M\text{-}15}$ with ISE*cp1* is common in *E. coli*, and the ISE*cp1*-$bla_{CTX\text{-}M\text{-}15}$ element usually inserts at the *tnpA* gene of Tn*3* [12]. To our knowledge, this is the first time that this transposition has been observed in *H. parainfluenzae*. Although plasmid acquisition is uncommon in *H. parainfluenzae*, this pathogen has the capacity to acquire mobile genetic elements and to introduce additional resistance genes into these elements [7], thereby enhancing the potential role of these pathogens as a reservoir for resistance genes.

5. Conclusions

This is the first study to show a $bla_{CTX\text{-}M\text{-}15}$ ESBL in *H. parainfluenzae*. The potential of *H. parainfluenzae* to accumulate resistance determinants, coupled with the possibility of transmitting these elements to other STI-associated pathogens, is a cause for concern. Given the threat posed to public health, this new resistance mechanism requires surveillance.

Acknowledgements: We thank the staff of the Microbiology Laboratory of Bellvitge University Hospital, who contributed daily to this project.

Declarations

Funding: This study was funded by the Fundación Española del Pulmón SEPAR (1116/2020 to S.M.); Fondo de Investigaciones Sanitarias (PI22/00257 to S.M.); Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES; CB06/06/0037) and Centro de Investigación Biomédica en Red de Enfermedades Infecciosas (CB21/13/0009), an initiative of the Instituto de Salud Carlos III (ISCIII). The European Regional Development Fund/European Social Fund (ERDF/ESF; "Investing in your future") also provided financial support, and CERCA Program/Generalitat de Catalunya provided institutional support. S.M. was supported by Miguel Servet contract (CP19/00096) (ISCIII). We declare that there is no conflict of interest regarding.

Competing Interests: None.

Ethical Approval: This study was in accordance with the Declaration of Helsinki from the World Medical Association. Written informed consent was not required as this was a retrospective, observational study with isolates obtained as part of routine microbiological tests, which was approved by the Clinical Research Ethics Committee of Bellvitge University Hospital (PR210/23). Patient confidentiality was always protected, and all personal data were anonymised following the current legal normative in Spain (LOPD 15/1999 and RD 1720/2007). This project followed Law 14/2007 on Biomedical Research for the management of biological samples in clinical research.

Sequence Information: European Nucleotide Archive (PRJEB63685).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106991.

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