Hafnia, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods

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Objectives: To determine the susceptibility to colistin of *Hafnia alvei* and *Hafnia paralvei*, and to compare methods for colistin resistance detection in the *Hafnia* genus.

Methods: A collection of 25 *Hafnia* isolates was studied. Species were identified by using 16S rRNA gene sequencing with subsequent phylogeny analysis. Susceptibility to colistin was determined using the broth microdilution (BMD) reference method, the Phoenix automated system, the Rapid Polymyxin NP test, the Etest system and the disc diffusion method.

Results: The collection consisted of 15 *H. alvei* and 10 *H. paralvei* isolates. Based on the 16S rRNA analysis, a close relationship of the *Hafnia* genus with naturally colistin-resistant enterobacterial genera (*Proteus*, *Morganella*, *Providencia* and *Serratia*) was identified. Susceptibility testing performed using the BMD method, the Phoenix automated system and the Rapid Polymyxin NP test revealed a high rate of colistin resistance (96%). Underestimation of colistin resistance using Etest strips (72%) and the disc diffusion method (0%) was observed.

Conclusions: The high rate of colistin resistance observed within the *Hafnia* genus and its close phylogenetic relationship with naturally colistin-resistant genera suggest that *Hafnia* is a naturally colistin-resistant enterobacterial genus.

Introduction

Hafnia spp. are Gram-negative bacilli belonging to the Enterobacteriaceae family.[1] *Hafnia* isolates have been mainly recovered in mammalian guts and from food.[1] They are rarely isolated from human specimens, even though they may be occasionally opportunistic human pathogens. They are responsible for nosocomial infections including gastroenteritis, urinary tract infections, meningitis, pneumonia, wound infections, soft tissue infections, endophthalmitis and septicaemia.[2,3]

Hafnia spp. harbour a chromosomal *ampC* gene, encoding a class C b-lactamase that confers resistance to aminopenicillins and narrow-spectrum cephalosporins.[4] Natural antimicrobial susceptibility patterns of *Hafnia alvei* strains to 69 antimicrobial agents was reported by Stock *et al.*,[4] but polymyxin susceptibility was not tested. Colistin resistance within *Hafnia* isolates has been rarely reported.[2,5–7]

H. alvei was the unique *Hafnia* species described until a novel species, namely *Hafnia paralvei*, was introduced in the taxonomy.[8] A study suggested that *H. paralvei* could be more resistant to colistin than *H. alvei* isolates.[5]

Taking into account the recent subdivision of the *Hafnia* genus into *H. alvei* and *H. paralvei*, our aim was to analyse the distribution of *Hafnia* species among 25 clinical isolates of *Hafnia*, to determine their susceptibility to colistin with the broth microdilution (BMD) reference method, and to compare colistin susceptibility methods for determining colistin resistance in the *Hafnia* genus.

Materials and methods

Bacterial strains and growth conditions

Twenty-five non-duplicated *Hafnia* isolates recovered from human samples between 1996 and 2016 were included in this study. Those isolates

have been collected from private laboratories (community patients) and at the university hospital of Bordeaux (hospitalized patients) (Table 1). They were kept at −80°C because they were recovered from deep samples or because they were resistant to extended-spectrum cephalosporins. They were cultured on bromocresol purple (BCP) lactose agar and UriSelect 4 (URI4) chromogenic medium (Bio-Rad, Marnes-la-Coquette, France). The *Escherichia coli* ATCC 25922 strain, susceptible to colistin, was included in all experiments as a quality control.

Bacterial species identification

Isolates were first identified as *H. alvei* using the MALDI-TOF mass spectrometer version 2.3 Microflex bench-top (Bruker, Champs-sur-Marne, France). As this spectrometer cannot distinguish between the two *Hafnia* species, the 16S rRNA gene of each isolate was partially amplified with primers 8F (5^0 -AGAGTTTGATCCTGGCTCAG-3^0) and 1489R (5^0-TACCTTGTTACGACTTCA-3^0) and sequenced (Eurofins Genomics, Les Ulis, France). Nucleotide sequences were analysed on the LeBiBi website (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi) to identify the bacterial species.[9]

Phylogenetic analysis

A phylogenetic tree based on 16S rRNA gene sequences was constructed for the 25 *Hafnia* isolates and two type strains (*H. alvei* ATCC 13337^T and *H. paralvei* ATCC 29927^T) using the neighbour-joining method.[10] The evolutionary distances were computed using the Kimura two-parameter method[11] and evolutionary analyses were conducted using MEGA6.[12]

Colistin susceptibility testing

Susceptibility testing of colistin was performed using five different methods. Following EUCAST 2017 breakpoints for Enterobacteriaceae,[13] isolates with an MIC of colistin ≤ 2 mg/L were categorized as susceptible although those with MICs . 2 mg/L were categorized as resistant. For the disc diffusion method, EUCAST 2013 disc breakpoints were used, i.e. susceptible if inhibition zone ≥ 15 mm and resistant if , 15 mm.

BMD

The BMD method, which is the reference technique, was performed according to the CLSI/EUCAST common guidelines (www.eucast.org). BMD panels were prepared in untreated polystyrene trays (Sarstedt, Nu¨mbrecht, Germany), and dilutions of colistin sulphate (Sigma–Aldrich, St Louis, MO, USA) were made in CAMHB (Bio-Rad). This procedure was performed in triplicate and the MICs were read after 16–20 h of incubation at 37°C.

Disc diffusion method

The disc diffusion test was performed according to the EUCAST 2013 guidelines. The diameter of the inhibition zone was measured after an incubation of 16–24 h at 37°C.

Table 1. Origin, susceptibility results and PFGE profile of the *Hafnia* isolates

Isolate	Species[a]	Sample	Year of isolation	Colour on		Disc diameter (mm)	MIC (mg/L)			Rapid Polymyxin NP test	PFGE type
				BCP agar	URI4 agar		Etest	Phoenix	BMD		
1	*H. alvei*	urine	1996	purple	blue	21	0.047	. 4	8	!	A
2	*H. alvei*	sputum	1999	purple	blue	17	3	4	8	!	A
3	*H. alvei*	pleural fluid	2002	purple	blue	18	8	. 4	16	!	A
4	*H. alvei*	respiratory sample	2009	purple	blue	19	4	4	8	!	A
5	*H. alvei*	rectal swab	2016	purple	blue	20	3	4	16	!	A
6	*H. alvei*	rectal swab	2016	purple	blue	20	3	4	8	!	A
7	*H. alvei*	rectal swab	2016	purple	blue	20	4	4	16	!	A
8	*H. alvei*	bile	2013	purple	blue	24	0.016	≤ 0.5	0.12	#	B
9	*H. alvei*	urine	2015	purple	blue	18	8	. 4	8	!	C
10	*H. alvei*	blood culture	2015	purple	blue	17	3	4	8	!	C
11	*H. alvei*	blood culture	2015	purple	blue	18	4	4	8	!	C
12	*H. alvei*	rectal swab	2015	purple	blue	23	0.094	. 4	32	!	C
13	*H. alvei*	blood culture	2015	purple	blue	20	6	. 4	16	!	C
14	*H. alvei*	rectal swab	2016	purple	blue	20	4	4	8	!	C
15	*H. alvei*	rectal swab	2016	purple	light pink	20	2	4	8	!	D
16	*H. paralvei*	drain	1998	yellow	pink	18	3	. 4	8	!	E
17	*H. paralvei*	fluid sample	1999	yellow	pink	17	2	4	8	!	F
18	*H. paralvei*	urine	2002	yellow	pink	20	3	4	8	!	G
19	*H. paralvei*	urine	2006	yellow	pink	20	2	4	8	!	H
20	*H. paralvei*	urine	2009	yellow	pink	21	0.064	. 4	8	!	I
21	*H. paralvei*	urine	2015	yellow	pink	19	6	4	8	!	J
22	*H. paralvei*	rectal swab	2016	yellow	pink	20	3	4	8	!	K
23	*H. paralvei*	rectal swab	2016	yellow	pink	22	4	4	8	!	L
24	*H. paralvei*	rectal swab	2016	purple	light pink	19	3	4	4	!	M
25	*H. paralvei*	rectal swab	2016	yellow	pink	20	3	4	8	!	N

Susceptible results are in bold.
[a]Identification of the species by 16S rRNA partial sequencing.

Etest system

Etest strips (AB bioMe´rieux, La Balme-les-Grottes, France) containing a colistin concentration gradient ranging from 0.016 to 256 mg/L were employed as recommended by the manufacturer's guidelines. MICs were read after an incubation of 16–20 h at 37° C.

Phoenix automated system

The principle of the BD Phoenix automated system (BD Diagnostic Systems, Le Pont de Claix, France) is based on the BMD technique. The bacterial suspension was prepared as recommended by the manufacturer and poured into the Gram-negative panel NMIC-93 (ref. 448282), which contains wells with colistin concentrations ranging from 0.5 to 4 mg/L. The panel was then incubated for 16 h at 37°C in the Phoenix automated system.

Rapid Polymyxin NP test

Susceptibility testing of colistin was also assessed using the Rapid Polymyxin NP test, which is based on the detection of the glucose metabolism due to bacterial growth in the presence of colistin.[14] Formation of acid metabolites from this glucose metabolism is evidenced by a colour change (orange to yellow) of a pH indicator (red phenol). The Rapid Polymyxin NP test was performed as described previously with a bacterial inoculum with a turbidity equivalent to that of a 3 McFarland standard, a final colistin concentration of 3.75 mg/L and a visual reading after 2 h of incubation at 37°C.[14]

Molecular typing by PFGE analysis

The genetic relatedness of the isolates was assessed by PFGE analysis with SfiI-digested genomic DNA.[15]

Nucleotide sequences accession numbers

The nucleotide sequences of the rRNA 16S genes of the *H. alvei* and *H. paralvei* strains were registered in GenBank under accession numbers KY849230 to KY849254.

Results and discussion

Fifteen *H. alvei* and 10 *H. paralvei* isolates were identified by using 16S rRNA gene sequencing (Table 1). The subsequent phylogeny analysis revealed that *H. alvei* and *H. paralvei* were clearly separated (two main branches highly stable to bootstrap resampling, with bootstrap values of 99 and 97 for *H. alvei* and *H. paralvei*, respectively) (Figure 1). It should be noted that *H. paralvei* isolates are more distant from each other than those of *H. alvei*.

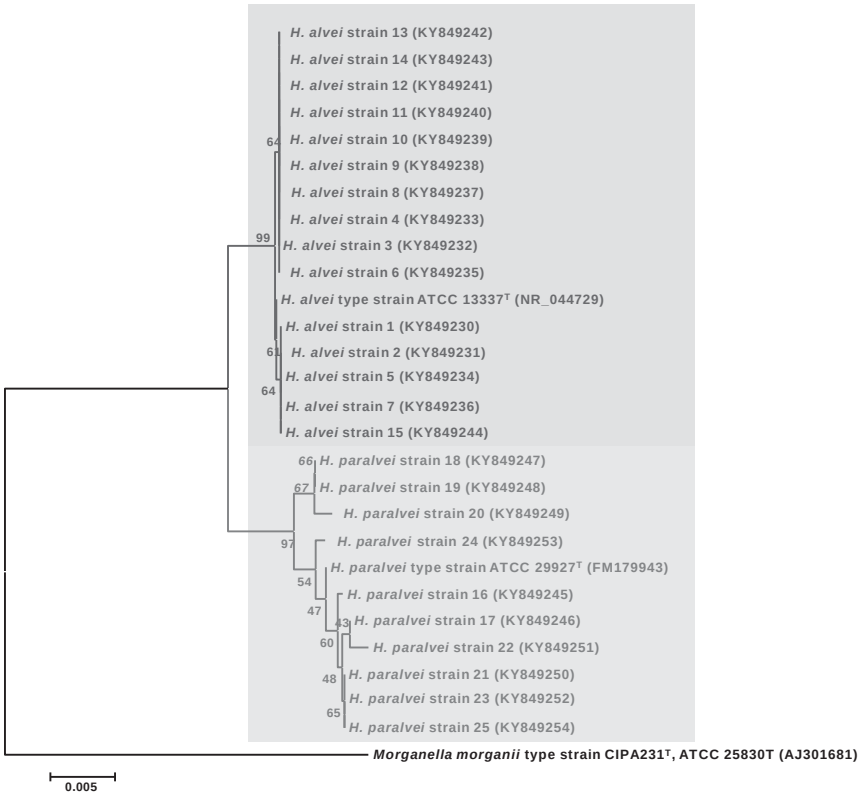

Figure 1. Phylogenetic tree derived from 16S rRNA sequence comparisons of the *Hafnia* isolates. Phylogenetic analysis of approximately 1400 nt of the 16S rRNA gene of the 25 *Hafnia* isolates and two type strains was performed using MEGA software version 6.06[12] and the neighbour-joining method. The optimal tree with the sum of branch length " 0.06351829 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is not rooted and is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method and are represented in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1398 positions in the final dataset. The 16S rRNA gene of *Morganella morganii* was used as an unrelated outgroup sequence. All sequences are labelled by species, strain name and/or collection number and accession number in brackets. T, type strain.

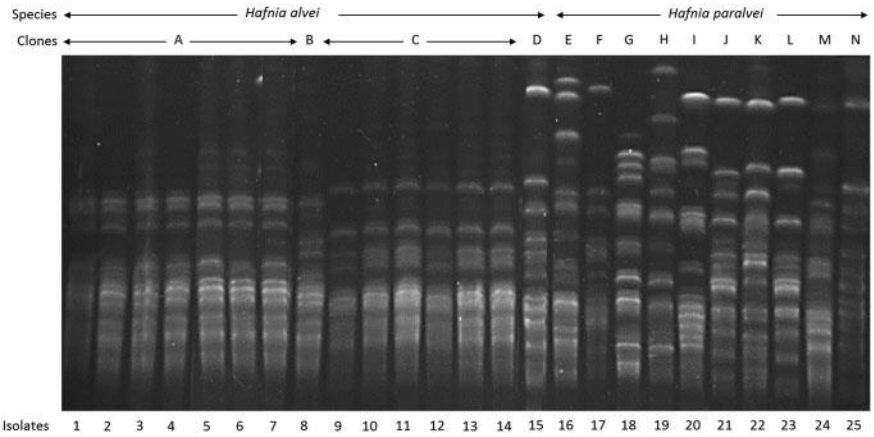

Figure 2. PFGE analysis of *H. alvei* (n = 15) and *H. paralvei* (n = 10) isolates. Isolates are numbered from 1 to 25 and clones are numbered from A to N.

Features of the colonies on URI4 and BCP agar plates showed discrepant results between *H. alvei* and *H. paralvei* isolates, respectively (Table 1). The combination of those features seems therefore to be sufficient to distinguish *H. alvei* and *H. paralvei* species in routine laboratory testing.

The BMD reference method revealed a high rate of colistin resistance (96%) among the 25 *Hafnia* isolates recovered over a 20 year period (Table 1). Only a single isolate of *H. alvei* was susceptible to colistin (MIC 0.125 mg/L). The other isolates had colistin MIC values ranging from 4 to 32 mg/L with no *Hafnia* species-dependent variation in resistance level. This high rate of colistin resistance was not related to the spread of a clonal strain as the PFGE analysis identified a diversity of clones (14 clones) in our collection (Figure 2 and Table 1). Surprisingly, several isolates of *H. alvei* seemed to be clonally related (clone A and clone C, Table 1). Considering that isolates belonging to clone A have been recovered over a 20 year period in various laboratories in the Bordeaux area, it is unlikely that they correspond to a single disseminated strain. We therefore suppose that the genetic diversity of the *H. alvei* isolates is intrinsically less important than that of the *H. paralvei* isolates (none of the *H. paralvei* isolates was clonally related). Those results are in agreement with the 16S rRNA gene sequences analysis, which indicated a greater distance between the *H. paralvei* isolates compared with those of *H. alvei*.

As expected, a high rate of false susceptibility results (25%) was observed with Etest strips, compared with the BMD reference method. Moreover, none of the isolates was identified as colistin resistant with the disc diffusion method. Those resistance rates were similar to those reported in previous studies using the same diffusion methods.[2,5] This further highlights that those methods are not reliable for evaluating the colistin susceptibility (www.eucast.org) and may explain why resistance to colistin within the *Hafnia* genus has been previously underestimated. On the contrary, the results obtained with the Phoenix automated system and the new Rapid Polymyxin NP test fully correlated with the BMD reference method.

Analysis of the 16S rRNA gene sequences on the LeBiBi web site revealed that *H. alvei* and *H. paralvei* species belonged to the same cluster, which is closely related to the cluster of the naturally colistin-resistant *Serratia* genus (data not shown), thus suggesting a natural resistance of *Hafnia* spp. The DNA relatedness of *Hafnia* spp. with genera naturally resistant to colistin has already been reported by Sproer *et al.*[16] (relatedness of *Hafnia* spp. and *Serratia* spp.) and Dauga[17] (relatedness of *Hafnia* spp. with the *Proteus–Providencia–Morganella* cluster).

This colistin-resistant trait may be related to the structural features of the lipopolysaccharide of *Hafnia* spp. as already described for *Serratia* spp.[18] and *Proteus* spp.[19] The unique strain determined as colistin susceptible with the BMD method might be a mutant not expressing its resistant phenotype. Ongoing investigations will contribute to understand the mechanism of intrinsic resistance in *Hafnia* genus and to identify to what extent the expression of colistin resistance can be modulated in colistin-susceptible strains.

Conclusions

Hafnia spp. may be added to the list of enterobacterial species that are naturally resistant to colistin, along with *Proteus* spp., *Providencia* spp., *Morganella* spp. and *Serratia* spp.

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Transparency declarations

None to declare.

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