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Prevalence of piperacillin/tazobactam resistance in invasive *Haemophilus influenzae* in Germany

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Background: *Haemophilus influenzae* (Hi) is a Gram-negative bacterium that may cause sepsis or meningitis, treatment of which mainly includes β-lactam antibiotics. Since 2019 EUCAST breakpoints for piperacillin/tazobactam have been available. Little is known about the prevalence and mechanisms of piperacillin/tazobactam resistance in Hi.

Objectives: To provide reliable prevalence data for piperacillin/tazobactam resistance in Hi in Germany, to evaluate different antibiotic susceptibility testing methods and to examine possible resistance mechanisms.

Methods: According to EUCAST breakpoints, the MIC for piperacillin/tazobactam resistance is >0.25 mg/L. All invasive Hi in Germany from 2019 were examined by gradient agar diffusion (GAD) for piperacillin/tazobactam susceptibility. Piperacillin/tazobactam broth microdilution (BMD), piperacillin GAD on tazobactam-containing agar [piperacillin GAD on Mueller–Hinton agar with horse blood (MH-F)/tazobactam) and piperacillin/tazobactam agar dilution (AD) were used for confirmation. Phenotypic testing was complemented by *ftsI* sequencing.

Results: Piperacillin/tazobactam GAD resulted in 2.9% (21/726) resistant Hi. BMD did not confirm piperacillin/tazobactam resistance. Two strains were found resistant by AD, of which one was also resistant using piperacillin GAD on MH-F/tazobactam. Overall, we found two strains with a piperacillin/tazobactam MIC >0.25 mg/L in at least two different tests (0.3%). Both were β-lactamase-producing amoxicillin/clavulanate-resistant with PBP3 mutations characterized as group III-like+. Relevant PBP3 mutations occurred in six strains without phenotypic piperacillin/tazobactam resistance. These mutations suggest a reduced efficacy of β-lactam antibiotics in these isolates.

Conclusions: Piperacillin/tazobactam resistance prevalence in invasive Hi is low in Germany. Reduced susceptibility was correlated with PBP3 mutations, in particular with group III mutations.

Introduction

Haemophilus influenzae (Hi) is a human pathogen that causes respiratory infections as well as meningitis and sepsis. The incidence of severe invasive infections has been increasing in Germany; especially, rising case numbers of unencapsulated, so-called non-typeable Hi (NTHi) and ampicillin-resistant strains have been reported.[1] Resistance to β-lactam antibiotics is based on β-lactamase expression and alterations in PBP, in particular in PBP3, which is encoded by the *ftsI* gene.[2]

According to international guidelines the acylaminopenicillin/β-lactamase inhibitor combination piperacillin/tazobactam is used besides other β-lactam antibiotics as first-line treatment of sepsis of unknown origin.[3] Even though piperacillin/tazobactam is not a drug of choice for the treatment of invasive Hi infections,[4,5] the susceptibility to this drug may be of clinical relevance.[6,7]

In 2019, EUCAST piperacillin/tazobactam breakpoints became available for Hi. However, little is known about resistance prevalence and mechanisms for piperacillin/tazobactam resistance in Hi. The aim of this study was to apply multiple antimicrobial susceptibility testing (AST) methods to provide robust prevalence data on piperacillin/tazobactam resistance in invasive Hi strains.

Materials and methods

Clinical isolates

Invasive Hi strains isolated from blood and CSF in 2019 were submitted to the German National Reference Laboratory for Meningococci and *Haemophilus influenzae* (NRZMHi) as part of the German laboratory surveillance programme. Patient information received by the NRZMHi included gender, date of birth and place of residence. All Hi strains were cultured on GC II Agar with IsoVitaleX (BD GmbH, Heidelberg, Germany) at 35 ± 1°C and 5% CO_2 overnight and archived at −80°C. All isolates were examined by Gram staining, oxidase test, and factor V- and X-dependent growth on BBL Hemo ID Quad (BD GmbH) for phenotypic species identification. Genetic species confirmation of Hi was carried out as described previously[1,8] by detecting the genes of fuculose kinase (*fucK*),[9] or outer membrane protein P2 (*ompP2*).[10] If both genes were missing, *ompP6* was sequenced to verify Hi.[11] Slide agglutination was done for serotyping. The *bexA* gene was amplified to confirm capsulation.[12] In case of poly- or autoagglutination, serotype PCRs were performed to identify the capsule type genetically.[13]

Antibiotic susceptibility testing

All isolates were tested for β-lactamase production by nitrocefin disc test (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany).[14]

Gradient agar diffusion (GAD) was performed using MTS™ (MIC test strips) (Liofilchem SRL, Roseto degli Abruzzi, Italia) on Mueller–Hinton agar with horse blood (MH-F) (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction and EUCAST.[15] All strains were tested for piperacillin/tazobactam (with a fixed tazobactam concentration of 4 mg/L), cefotaxime and ampicillin susceptibility. In case of β-lactamase positivity, amoxicillin/clavulanate susceptibility was additionally examined. Susceptibility was interpreted according to EUCAST breakpoints.[16] Thus, strains were considered resistant with MIC values for piperacillin/tazobactam >0.25 mg/L, cefotaxime >0.125 mg/L, ampicillin >1 mg/L and amoxicillin/clavulanate >2 mg/L.

Isolates with suspected reduced piperacillin/tazobactam susceptibility were additionally tested for susceptibility to amoxicillin, azithromycin, ceftriaxone, doxycycline and ciprofloxacin (Table 1).

Piperacillin/tazobactam susceptibility of selected isolates was verified by broth microdilution (BMD) in Mueller–Hinton II Broth Cation-Adjusted (BD GmbH) with β-NAD (Carl Roth GmbH, Karlsruhe, Germany), lysed horse blood (Thermo Fisher Scientific), a range of ≤0.016 mg/L to 2 mg/L piperacillin sodium salt (Cayman Chemical Europe, Tallinn, Estonia) and a concentration of 4 mg/L tazobactam sodium salt (Cayman Chemical Europe).

Additionally, piperacillin GAD on 4 mg/L tazobactam containing MH-F agar (MH-F/tazobactam) made of Mueller–Hinton Agar (BD GmbH), β-NAD (Carl Roth GmbH), defibrinated horse blood (Thermo Fisher Scientific) and tazobactam sodium salt (Cayman Chemical Europe) was performed.[17]

Agar dilution (AD) on MH-F agar containing 4 mg/L tazobactam (Cayman Chemical Europe) and piperacillin (Cayman Chemical Europe) concentrations from 0.008 mg/L to 8 mg/L was done as a third confirmation method.[18]

As quality control strains *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used. Furthermore, Hi strain H4990, a randomly selected β-lactamase-positive amoxicillin/clavulanic acid resistant (BLPACR) strain of the NRZMHi strain collection, was established as Hi control strain for intra-assay quality control for piperacillin/tazobactam GAD and BMD.

Preparation and testing were performed according to EUCAST and DIN EN ISO 20776-1:2020.

Piperacillin/tazobactam resistance was assumed if GAD and one additional AST method showed piperacillin/tazobactam MIC >0.25 mg/L, as the EUCAST reference standard BMD showed differing results from agar-based methods and reading problems appeared (Figure 1).

Table 1. Antibiotic resistance profile of 21 isolates that showed reduced piperacillin/tazobactam (TZP) susceptibility in gradient agar diffusion testing

Isolates with reduced TZP susceptibility $n=21$	Median MIC [range] (mg/L)	Susceptibility CLSI, *n* (%)	Susceptibility EUCAST, *n* (%)
Ampicillin	0.5 [0.19 to >256]	10 (47.6%)	10 (47.6%)
Amoxicillin	0.75 [0.19 to >256]	9 (42.8%)	9 (42.8%)
Amoxicillin/clavulanate	0.75 [0.25 to >256]	12 (57.1%)	12 (57.1%)
Cefotaxime	0.047 [0.016 to 1.5]	21 (100%)	17 (81.0%)
Ceftriaxone	0.012 [0.006 to 0.25]	21 (100%)	17 (81.0%)
Azithromycin	6 [3 to 24]	5 (23.8%)	5[a] (23.8%)
Doxycycline[b]	0.75 [0.38 to 1.5]	21 (100%)	21 (100%)
Ciprofloxacin	0.012 [0.008 to 0.023]	21 (100%)	21 (100%)

[a]No clinical breakpoints available according to EUCAST. Interpretation according to epidemiological cut-off values.
[b]Interpretation was derived from tetracycline.

Comparability of test results was given within a difference of ≤1 $\log_2$ and interobserver reproducibility was secured by MIC interpretation of at least two independent persons with an acceptable reading difference within 2-fold dilution.

Molecular characterization

To characterize alterations in the transpeptidase domain of PBP3, the *ftsI* gene was amplified by PCR as described previously.[19] This was performed with all piperacillin/tazobactam-resistant strains detected by any method ($n=21$), and 27 randomly selected piperacillin/tazobactam-susceptible strains for comparison. The translated amino acid (AA) sequences were aligned to the corresponding sequence of Hi strain Rd KW20 (ATCC 51907), and PBP3 groups were assigned as recently summarized by Nürnberg *et al.*[19] Briefly, the main AA alterations for group I were R517H, for group II N526K and for group III M377I, S385T and L389F in addition to N526K.

Results

Epidemiology

In 2019, 727 invasive Hi strains were submitted to the NRZMHi. The incidence per 100 000 inhabitants in Germany was 1.1.[20] The highest incidence values appeared in the Federal States of Baden-Württemberg (1.14) and Bavaria (0.92). The sex ratio (male:female) was 1.10. The mean age was 66.9 years (0–100 years; percentiles 25–75: 61–83 years), with women being 7 years older than men. The majority of isolates derived from adult patients ($n=675$; 92.9%); in 52 cases (7.2%) invasive

Hi isolates originated from paediatric patients (aged less than 18 years). Among paediatric cases an increased number of infections occurred under the age of 5 years ($n=39$; average cases per age group = 7.8, 5.4% of all cases), whereas the mean case number in age groups between 5 and 55 was only 1.96.

Most invasive strains were isolated from blood ($n=698$; 96.01%), and only 29 isolates (3.99%) from CSF.

The serotype distribution was: 80.06% ($n=582$) NTHi; 12.52% ($n=91$) Hif; 3.16% ($n=23$) Hie; 2.06% ($n=15$) each Hia and Hib; 0.14% ($n=1$) Hid and no Hic.

Characterization by phenotypic β-lactam susceptibility

A majority of 558 strains (76.75%) were β-lactamase negative and ampicillin susceptible (BLNAS). Among isolates with resistance mechanisms β-lactamase-positive ampicillin-resistant Hi (BLPAR) comprised the largest group ($n=97$; 13.34%). Few strains were characterized as β-lactamase negative and ampicillin resistant (BLNAR; $n=61$; 8.38%) harbouring PBP3 mutations as sole resistance mechanism. Rarely, Hi with β-lactamase production in addition to *ftsI* alterations and consequently resistance to ampicillin and amoxicillin/clavulanate were detected (BLPACR; $n=11$; 1.51%).

Cefotaxime susceptibility testing resulted in 99.04% ($n=720$) susceptible and 0.96% ($n=7$) resistant strains.

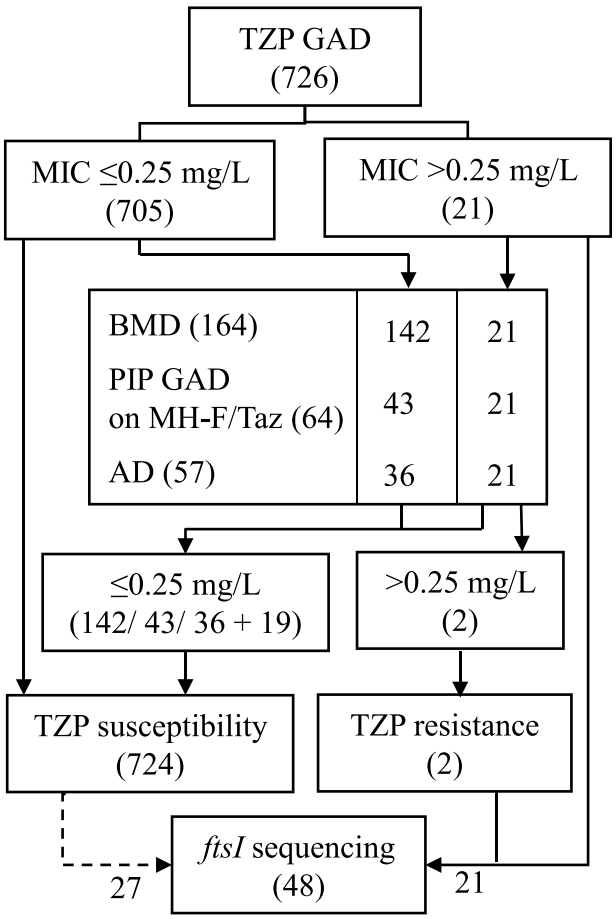

Figure 1. Flowchart of piperacillin/tazobactam (TZP) susceptibility testing. Gradient agar diffusion (GAD) test with piperacillin/tazobactam test strips (piperacillin/tazobactam GAD) was used as primary test. All piperacillin/tazobactam GAD resistant and selected susceptible strains were retested by broth microdilution (BMD), agar dilution (AD) and piperacillin (PIP) GAD on 4 mg/L tazobactam containing Mueller–Hinton agar with horse blood (PIP GAD on MH-F/tazobactam). Piperacillin/tazobactam resistance was assumed to be verified if detected by at least two methods. Sequencing of *ftsI* was done for 48 strains to examine PBP3 mutations that might lead to resistance. Numbers indicate the number of strains tested.

Piperacillin/tazobactam GAD

Of 727 isolates submitted to the NRZMHi, 726 were available for piperacillin/tazobactam susceptibility testing. Piperacillin/tazobactam MICs determined by GAD mostly resulted in susceptible values (Figure 2). Few strains ($n=21$; 2.90%) showed a resistant MIC > 0.25 mg/L, with 0.38 mg/L being the median value among these. Their phenotypic aminopenicillin susceptibility distribution was as follows: BLNAS ($n=10$; 47.62%); BLNAR ($n=5$; 23.81%); BLPAR and BLPACR (each $n=3$; 14.29%).

As already observed by others,[21] ampicillin-resistant strains with PBP3 alterations (BLNAR, BLPACR; $n=39$) showed on average 1.4 $\log_2$ higher MICs than BLNAS and BLPAR in this study.

Piperacillin/tazobactam BMD

All Hi strains with piperacillin/tazobactam GAD MICs >0.125 mg/L ($n=83$) and further randomly selected Hi with lower MICs ($n=81$) were analysed by BMD, the EUCAST reference method, to verify the piperacillin/tazobactam GAD results. Here all strains were tested susceptible (Figure 2). Thus, none of the 21 piperacillin/tazobactam GAD resistant strains was confirmed as resistant by BMD. Although overall categorical agreement was high,

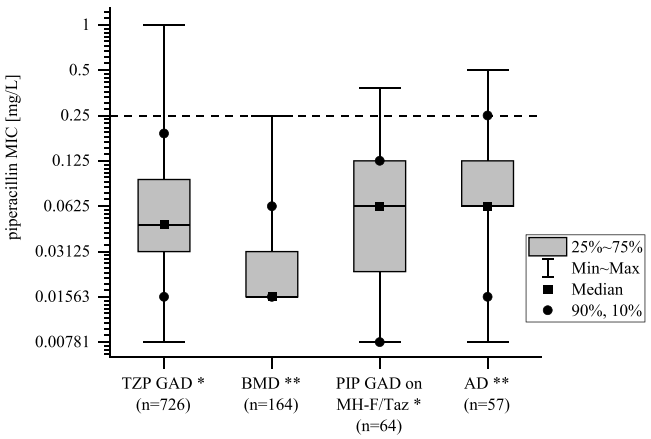

Figure 2. Summary of piperacillin/tazobactam (TZP) MIC of all applied testing methods: piperacillin/tazobactam gradient agar diffusion (TZP GAD), broth microdilution (BMD), piperacillin GAD on 4 mg/L tazobactam-containing Mueller–Hinton agar with horse blood (PIP GAD on MH-F/tazobactam) and agar dilution (AD). MIC percentiles are shown as dots (P10, P90), squares (P50) and boxes (P25, P75). Piperacillin/tazobactam breakpoint is marked by the broken line. *Values <0.016 mg/L and ≤0.00781 mg/L are shown as 0.00781 mg/L; **values ≤0.016 mg/L for BMD are all shown as 0.01563 mg/L).

especially among piperacillin/tazobactam GAD sensitive strains, the average difference between MICs of piperacillin/tazobactam GAD and piperacillin/tazobactam BMD was 2.3 $\log_2$ with a low correlation (Table 1). Due to these inconsistent results, further susceptibility tests were carried out.

Piperacillin GAD on MH-F/tazobactam

Piperacillin GAD was performed to analyse whether the use of tazobactam-containing agar has an impact on MIC values compared with tazobactam diffusion from gradient strips.

To verify that the homemade MH-F agar was comparable to commercial agar, ampicillin susceptibility was tested by GAD on both, with no differences in MIC results. Furthermore, BLNAR and BLNAS strains were tested using piperacillin test strips on homemade MH-F agar with and without tazobactam, to see whether the β-lactamase inhibitor had an influence on agar quality and MICs. The results were compared with MICs from piperacillin/tazobactam GAD on commercial MH-F agar and no differences were detected. Therefore, the homemade agar was rated equivalent to commercial media.

Applying piperacillin GAD on MH-F/tazobactam on 64 Hi, including the 21 piperacillin/tazobactam GAD resistant strains, only one of the latter was resistant (0.38 mg/L) (Figure 2). Piperacillin GAD MICs on MH-F/tazobactam showed a good correlation to piperacillin/tazobactam GAD MICs ($r=0.83$), but the absolute MIC values differed by 1.7 $\log_2$ on average (Table 2). Especially among β-lactamase-positive strains the difference was high (2.1 $\log_2$). Categorical changes from resistant to susceptible appeared in 95.2% (20/21) of all piperacillin/tazobactam GAD resistant Hi.

Piperacillin/tazobactam AD

To check the influence of different piperacillin applications, piperacillin/tazobactam susceptibility of 57 Hi, including the 21 piperacillin/tazobactam GAD resistant isolates, was examined by piperacillin/tazobactam AD. Two of the piperacillin/tazobactam GAD resistant strains showed a piperacillin/tazobactam MIC >0.25 mg/L. One of them was also tested resistant by piperacillin GAD on MH-F/tazobactam. All 36 piperacillin/tazobactam GAD susceptible strains were also piperacillin/tazobactam AD susceptible (Figure 2). Compared with the other methods, the best correlation ($r=0.85$), best categorical agreement (95.0%) and lowest average MIC difference (0.03 $\log_2$) were identified between piperacillin/tazobactam AD and piperacillin GAD on MH-F/Taz.

In summary, among the German Hi strains from 2019 two piperacillin/tazobactam-resistant strains (2/726 = 0.3%) were identified by at least two methods (Figure 1).

According to the EUCAST reference method BMD, the resistance prevalence was 0%, with few strains showing a categorical change in agar-based methods.

PBP3 sequence analysis

Among the resistant Hi in piperacillin/tazobactam GAD, eight BLNAS and two BLPAR strains showed a PBP3 WT sequence as expected by ampicillin phenotype. One BLNAS strain showed alterations in PBP3 classified as group IIa whereas another BLNAS strain, one BLPAR strain and four BLNAR strains were categorized as group IIb. One BLNAR strain was categorized as III-like, and all three BLPACR strains, of which two were verified piperacillin/tazobactam resistant, harboured group III-like+ PBP3 mutations (Figure 3).

Among 27 randomly selected piperacillin/tazobactam-susceptible Hi, all BLNAS and BLPAR were PBP3 WT strains. All but two BLNAR strains and one BLPACR strain were categorized as group IIa and IIb (Figure 3).

Discussion

To our knowledge, this is the first study to provide systematic piperacillin/tazobactam susceptibility data for invasive Hi strains in Europe. The strains are representative in regard to patient age, serotype and ampicillin susceptibility for the epidemiology in Germany.[1] The incidence of invasive Hi infections per 100 000 inhabitants has increased in Germany since 2010, mainly due to rising NTHi cases, a growing proportion of ampicillin-resistant Hi and to an increasing extent of PBP3 mutations especially in BLNAR.[1] Results from Japan showed that BLNAR strains have the tendency to develop MDR.[22]

Our data show that among German invasive Hi strains the prevalence of piperacillin/tazobactam resistance was at a very low level (0.3% or even 0%). Piperacillin/tazobactam resistance is similarly rare in other countries.[23,24] A Japanese study with mainly non-invasive Hi showed no increase in piperacillin/tazobactam resistance over a time period of 9 years.[25]

In 2015, EUCAST published a warning regarding the reliability of piperacillin/tazobactam GAD, which was partly removed for

Table 2. MIC differences and categorical agreements between piperacillin/tazobactam gradient agar diffusion and verification methods

Verification method	Number of isolates	Differences to TZP GAD MIC (indicated as differences in $\log_2$ dilution steps)						Agreement within 1 $\log_2$ (%)	Categorical agreement (%)	Correlation coefficient (r)
		<−2	−2	−1	Same	1	>1			
BMD	164	86	40	23	15			23.17	87.20	0.65
PIP GAD	64	11	36	13	3		1	25.00	68.75	0.83
AD	57	9	22	19	6		1	43.86	64.91	0.70

All categorical changes were from resistant to susceptible. AD, agar dilution; BMD, broth microdilution; PIP GAD, piperacillin gradient agar diffusion on 4 mg/L tazobactam-containing agar; TZP GAD, piperacillin/tazobactam gradient agar diffusion.

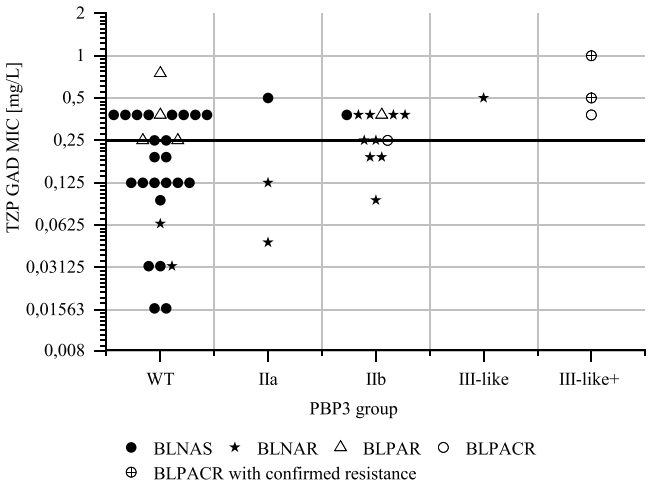

Figure 3. MICs of piperacillin/tazobactam gradient agar diffusion (TZP GAD) and PBP3 groups respectively WT of 48 *Haemophilus influenzae* strains subdivided into phenotypic characteristics: β-lactamase-negative ampicillin-sensitive (BLNAS); β-lactamase-negative ampicillin-resistant (BLNAR); β-lactamase-positive ampicillin-resistant (BLPAR); and β-lactamase-positive amoxicillin/clavulanic acid-resistant (BLPACR). The horizontal line marks the piperacillin/tazobactam breakpoint of 0.25 mg/L according to EUCAST. Among piperacillin/tazobactam-susceptible strains no PBP3 group III-like or III-like+ strain was found.

some species, but not for Hi, after the material in question was improved.[26] Therefore, our results acquired by different testing methods confirm the warning and suggest that inhomogeneous diffusion of tazobactam from test strips might result in elevated MICs. On the other hand, piperacillin diffusion from the test strips seems to be adequate. The observation that particularly MICs of BLP Hi strains were reduced by more than half on piperacillin GAD on MH-F/tazobactam supports this hypothesis. When testing piperacillin/tazobactam susceptibility by AD and GAD, different antimicrobial activity with just 25% of categorical agreement between the two methods was already suspected in other bacteria.[27] An intrinsic antibacterial activity of tazobactam by binding PBP2 leading to a deficient cell wall and a change of cell shape, seen in phase-contrast microscopy, has been suggested for other species.[28] This may explain higher MIC results of BLN Hi strains in piperacillin/tazobactam GAD compared with piperacillin GAD on MH-F/tazobactam. However, further research is needed, as varying opinions on this topic exist.[27,29]

Different diffusion characteristics of piperacillin and tazobactam in MH-F agar compared with MH-F broth may also have influenced the testing results, which showed great differences in MICs between the methods. In addition, Hi may not cause unambiguously visible turbidity in the BMD medium, whereas single colonies in the inhibition area of GAD can be easily detected. Furthermore, heterogeneity of resistance expression and single colonies on the agar are frequently used arguments for inconsistent piperacillin/tazobactam MICs determined by different susceptibility testing methods for other bacteria,[30] and have also been observed in Hi tested with other β-lactams.[21]

Most studies focus on PBP3 sequences to analyse β-lactam resistances, even though other PBPs, in particular PBP4, have also been suggested to play a role in resistance.[31] Apart from a few exceptions, group III-like and III-like+ mutations of PBP3 were usually found in strains with higher MICs. The role of PBP3 in raising MICs of ampicillin and other β-lactams has been shown in several studies.[2,19] It has been suggested that piperacillin differs from other β-lactams in its interaction with PBP3 in Hi, as AA alterations of group IIa-d, III and III+ (N526K and additionally M377I, S385T and L389F) did not affect its antimicrobial activity.[25] Consistently, ampicillin, amoxicillin/clavulanate and cefotaxime GAD MICs of the NRZMHi collection correlated poorly with piperacillin/tazobactam GAD MICs. This is in line with previous findings.[32] However, there are contradicting reports that the AA substitutions M377I and R517H, the defining mutation for group III-like and III-like+, were strongly correlated with elevated piperacillin/tazobactam MIC.[25] It is noteworthy in this context that both confirmed piperacillin/tazobactam-resistant BLPACR strains in our study showed this mutation.

A limitation of this study is that only 76.2% of the cases reported by the Robert Koch Institute for 2019 ($n=954$) were submitted to the NRZMHi ($n=727$).[20] Furthermore, not all strains were examined by BMD, the EUCAST reference standard.

In conclusion, our study resulted in a very low piperacillin/tazobactam resistance prevalence in invasive Hi strains in Germany. PBP3 group III-like and III-like+ mutations may play a role in piperacillin/tazobactam resistance. Because resistance remains dynamic, further surveillance is warranted to monitor Hi piperacillin/tazobactam resistance development in the future. Our own previous studies[33] and data from other countries[34,35] have shown that ampicillin resistance was common and had an increasing trend.[1] However, reduced susceptibility to drugs used for the treatment of invasive Hi infections is still rare.[19,34] These findings underline the importance of continued antimicrobial susceptibility surveillance for Hi to monitor trends and mechanisms of resistance.

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Transparency declarations

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