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Presence of hypervirulence-associated determinants in *Klebsiella pneumoniae* from hospitalised patients in Germany

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ABSTRACT

Background: *Klebsiella* (*K.*) *pneumoniae* is a ubiquitous Gram-negative bacterium and a common coloniser of animals and humans. Today, *K. pneumoniae* is one of the most persistent nosocomial pathogens worldwide and poses a severe threat/burden to public health by causing urinary tract infections, pneumonia and bloodstream infections. Infections mainly affect immunocompromised individuals and hospitalised patients. In recent years, a new type of *K. pneumoniae* has emerged associated with community-acquired infections such as pyogenic liver abscess in otherwise healthy individuals and is therefore termed hypervirulent *K. pneumoniae* (hv*Kp*). The aim of this study was the characterisation of *K. pneumoniae* isolates with properties of hypervirulence from Germany.
Methods: A set of 62 potentially hypervirulent *K. pneumoniae* isolates from human patients was compiled. Inclusion criteria were the presence of at least one determinant that has been previously associated with hypervirulence: (I) clinical manifestation, (II) a positive string test as a marker for hypermucoviscosity, and (III) presence of virulence associated genes *rmpA* and/or *rmpA2* and/or *magA*. Phenotypic characterisation of the isolates included antimicrobial resistance testing by broth microdilution. Whole genome sequencing (WGS) was performed using Illumina® MiSeq/NextSeq to investigate the genetic repertoire such as multi-locus sequence types (ST), capsule types (K), further virulence associated genes and resistance genes of the collected isolates. For selected isolates long-read sequencing was applied and plasmid sequences with resistance and virulence determinants were compared.

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Results: WGS analyses confirmed presence of several signature genes for hv*Kp*. Among them, the most prevalent were the siderophore loci *iuc* and *ybt* and the capsule regulator genes *rmpA* and *rmpA2*. The most dominant ST among the hv*Kp* isolates were ST395 capsule type K2 and ST395 capsule type K5; both have been described previously and were confirmed by our data as multidrug-resistant (MDR) isolates. ST23 capsule type K1 was the second most abundant ST in this study; this ST has been described as commonly associated with hypervirulence. In general, resistance to beta-lactams caused by the production of extended-spectrum beta-lactamases (ESBL) and carbapenemases was observed frequently in our isolates, confirming the threatening rise of MDR-hv*Kp* strains.
Conclusions: Our study results show that *K. pneumoniae* strains that carry several determinants of hypervirulence are present for many years in Germany. The detection of carbapenemase genes and hypervirulence associated genes on the same plasmid is highly problematic and requires intensified screening and molecular surveillance. However, the non-uniform definition of hv*Kp* complicates their detection. Testing for hypermucoviscosity alone is not specific enough to identify hv*Kp*. Thus, we suggest that the classification of hv*Kp* should be applied to isolates that not only fulfil phenotypical criteria (severe clinical manifestations, hypermucoviscosity) but also (I) the presence of at least two virulence loci e.g. *iuc* and *ybt*, and (II) the presence of *rmpA* and/or *rmpA2*.

1. Introduction

Klebsiella (*K.*) *pneumoniae* is an important human pathogen associated with various infections, including pneumonia, urinary tract infections, wound infections and bloodstream infections (Russo and Marr, 2019; Liu et al., 2020). The classical *K. pneumoniae* (c*Kp*) strains cause healthcare associated infections and are often multidrug-resistant (MDR), making treatment challenging. Over the past two decades, the prevalence of c*Kp* producing extended-spectrum beta-lactamases (ESBL) and/or carbapenemases has increased dramatically due to a worldwide spread of distinct clonal lineages and groups (Wyres et al., 2020). The parallel emergence of hypervirulent strains of *K. pneumoniae* (hv*Kp*) has become a further threat to public health. These hv*Kp* strains are usually susceptible to the most antibiotics but exhibit enhanced virulence potential, leading to severe and often life-threatening infections in otherwise healthy individuals. Severe infections with hv*Kp* have been mainly reported in the Asian Pacific region. Infections usually manifest as liver abscess, endophthalmitis, and meningitis, showing metastatic spread causing infections at multiple body sites (Hallal Ferreira Raro et al., 2023; Pavan et al., 2022). hv*Kp* strains have been associated with specific microbiological characteristics, such as hypermucoviscosity, and a variable set of acquired factors, such as, iron acquisition systems, and capsular serotypes. The rapid global spread is mainly caused by hv*Kp* strains of the clonal group 23 (Lam et al., 2018). In the past decade, the emergence of hv*Kp* strains with ESBL and carbapenemase production has been reported as a concerning combination of severe infections with limited treatment options (Pavan et al., 2022; Yang et al., 2020; Zhang et al., 2016).

It is essential to differentiate between c*Kp* and hv*Kp*, as it can guide appropriate therapeutic interventions and infection control measures. However, the delineation of both pathotypes is complicated by the inconsistent definition of hypervirulence. Microbiological methods used for differentiation hypervirulent from classical strains of *K. pneumoniae* include traditional phenotypic tests, such as the string tests (Eisenmenger et al., 2021) and hypermucoviscosity assays (Shi et al., 2018). In addition, molecular techniques, such as PCR (Compain et al., 2014) targeting various virulence associated genes and multilocus sequence typing (MLST) (Lan et al., 2020), have been used for hv*Kp* strain characterisation. Especially, whole genome sequencing (WGS) has emerged as a powerful tool to elucidate the genetic makeup of *K. pneumoniae* strains and to identify virulence associated factors and resistance determinants (Klaper, 2021a,b; Lam, 2021a; Rödel, J., et al.). Comparative genomics, phylogenetic analysis, and genomic epidemiology have further improved the understanding of *K. pneumoniae* strain evolution and transmission dynamics (Yang et al., 2021; Tian et al., 2021; Struve et al., 2015; Pu et al., 2023).

In Germany, PCR-based confirmation of virulence associated genes in *K. pneumoniae* and WGS is not established in clinical diagnostic laboratories and rarely used in reference laboratories. To evaluate the presence of *K. pneumoniae* strains with properties of hypervirulence in Germany we asked diagnostic laboratories in 2016 to send suspicious isolates that show hypermucoviscosity and/or the typical clinical manifestations (e.g. liver abscess) that has been described for hv*Kp*. These isolates and *K. pneumoniae* isolates of further strain collections of Robert Koch Institute were screened for the presence of virulence associated genes encoding capsule synthesis regulators (*rmpA*, *rmpA2*) and capsule type K1 (*magA*); both have been described as being associated with hv*Kp* strains of the clonal group 23 (Yeh, 2007). We selected 62 hv*Kp* suspicious isolates that show at least one of the above-mentioned determinants associated with hypervirulence for further characterisation. This included molecular investigations on the combination of virulence genes, antimicrobial resistance genes, phenotypic traits and phylogeny of the isolates. We evaluated these combinations to identify an optimal combination of phenotypic and/or genetic determinants for characterisation of hv*Kp*. Here we discuss the microbiological and molecular biological approaches commonly used for hv*Kp* characterisation and provide further knowledge in this area, which may help develop future strategies to combat the increasing threat of hv*Kp* infections and ultimately improve patient outcomes.

2. Materials and methods

2.1. Study isolates selection, phenotyping and PCR

Klebsiella spp. isolates included in this study were sent in from clinical diagnostic laboratories throughout Germany since 2016 and showed pre-indications for hypervirulence: clinical manifestation (e.g., liver abscess and further conspicuous infection characteristics such as isolates from blood culture, liquor and wound infections) and/or hypermucoviscosity (string test positive). Furthermore, clinical *Klebsiella* spp. isolates collected at the Robert Koch Institute between 2009 and 2023 that were screened positive by PCR for presence of virulence-associated genes *rmpA* and/or *rmpA2* and/or *magA* (Yeh, 2007) were included in this study. Supplementary Table S1 shows the selected study isolates with the criteria used for selection and sums up the number of fulfilled criteria. The resulting study collection of 62 isolates includes the reference strain *K. pneumoniae* NCTC 14052 from a liver abscess. This strain resembles a typical hv*Kp*. Genome data and assembly information for this strain is available under the NCBI RefSeq assembly accession GCF_002813595.1 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_002813595.1/).

To verify the hypermucoviscous phenotype, all study isolates were subjected to the string test. A string test is defined as positive when the formation of a mucoid string of > 5 mm can be observed by using a bacteriology inoculation loop to stretch a colony on the Müller Hinton sheepblood agar plate (Klaper et al., 2021b; Sanchez-Lopez et al., 2019). Furthermore, all study isolates were tested by PCR presence of virulence associated genes encoding capsule synthesis regulators (*rmpA*, *rmpA2*) and capsule type K1 (*magA*); and for the presence of frequently occurring resistance genes, such as beta-lactamase genes and genes contributing to

fluoroquinolone resistance, were tested by PCR using DreamTaq Polymerase (Thermo Scientific™, Waltham, USA). PCR primers and PCR conditions are listed in Supplementary Table S2.

Species identification and antibiotic susceptibility testing (AST) were performed using the automated system VITEK® 2 (cards GN ID and N248) and broth microdilution; the results were interpreted according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) standards and breakpoints version v13.1 (http://www.eucast.org/clinical_breakpoints).

2.2. WGS and bioinformatic analysis

For WGS, isolates were grown in Brain Heart Infusion (BHI) broth (BD, Heidelberg, Germany). According to manufacturer's instructions, DNA was extracted from overnight cultures using DNeasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands). DNA was quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

The sequencing libraries were prepared using a Nextera XT DNA Library Prep Kit (Illumina®, San Diego, CA, USA). Sequencing was performed according to the manufacturer's protocol on an Illumina NextSeq 550 using a v2.5 chemistry kit (2 × 150 bp) or on an Illumina Miseq using v3 chemistry (2 × 300 bp) according to the manufacturer's protocol.

The generated fastq files were quality-checked and trimmed using Trimmomatic software (version 0.39). The following parameters were used for trimming ILLUMINACLIP:NexteraPE- PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 (Bolger et al., 2014). De novo assembly was performed using Unicycler v0.5.0, including the SPAdes assembler v3.15.5 (Wick et al., 2017a, 2017b; Bankevich et al., 2012).

For long-read sequencing of randomly selected isolates high molecular weight DNA was isolated from 2 mL overnight culture using the Qiagen MagAttract HMW DNA Kit (Hilden, Germany). Sequencing libraries were prepared using the SQK-RKB110.96 Kit according to the manufacturer's instructions and sequenced on a MinION mk1C instrument using a r9.4.1 1D flow cell (Oxford Nanopore Technologies, Oxford, UK). The reads were quality filtered using NanoFilt (De Coster et al., 2018) with default parameters. Unicycler v0.5.0 was used to generate a hybrid assembly based on the filtered long reads in combination with Illumina short reads. Genomes assembled by hybrid assembly were from the six isolates 0236/19–1, 0574/14, 0718/21–1, 0718/21–2, 0736/17, 0005/18 and 0609/17. All sequencing data was submitted to ENA and is accessible under the accession no: PRJEB67454.

In the scope of an additional investigation regarding the plasmid type AA405 we added the plasmid sequence information from hybrid assemblies of six randomly selected carbapenemase producing isolates (0053/23, 0383/22, 0246/22, 0548/22, 0554/22, 0578/22) for further comparison of the plasmid structures.

The generated contigs of the 62 study isolates were further analysed using tools Kleborate v2.3.2 and AMRfinder 3.12.2 (Lam et al., 2021a; Zhou et al., 2020; Feldgarden et al., 2021, 2019). Both tools analyse the genome assemblies for virulence and resistance genes specific gene clusters, to determine sequence types (ST) and capsule types (K-type) of the bacteria. ST classification is based on allelic profiles of specific housekeeping genes in *Klebsiella* species. ST is determined by multilocus sequence typing (MLST) of seven housekeeping genes, and the alleles at each of these genes are assigned numerical identifiers. Combining these numerical identifiers for the seven genes defines the ST of the isolate. Sequence typing is used to trace the genetic relatedness of *Klebsiella* spp. strains. K-type classification is based on the composition and characteristics of capsular polysaccharides in *K. pneumoniae* strains. The K-type is typically determined by the identification of the composition of genes encoding capsular polysaccharide synthesis. Different capsular types can be associated with varying virulence and resistance to host immune responses. These typing methods are valuable tools for studying *K. pneumoniae* in the context of molecular epidemiology and clinical microbiology, and understanding their pathogenicity.

The Kleborate software further calculates a virulence and a resistance score based on the found corresponding genes (https://github.com/klebgenomics/Kleborate/wiki/Scores-and-counts; 09/08/2023). The virulence scoring system is 1: *ybt*, 2: *ybt* + *clb*, 3: *iuc*, 4: *ybt* + *iuc*, and 5: *ybt* + *clb* + *iuc*. Whereas, *ybt* describes the two genes *ybtP* and *ybtQ* coding for yersiniabactin ABC transporter ATP-binding/permease protein (Koh et al., 2016; Fetherston et al., 1999). *Clb* is the locus of colibactin which contains the genes *clbC*, *clbD*, *clbE*, *clbF*, *clbG*, *clbH*, *clbI*, *clbL*, *clbM*, *clbN*, *clbO*, *clbP* and *clbQ* (Lu et al., 2017). Aerobactin is encoded in the *iuc* locus which includes the genes *iucA, iucB, iucC, iucD* and *iutA* (Bailey et al., 2018; Russo and Gulick, 2019). The resistance score is presented as 1: ESBL, 2: carbapenemases, 3: carbapenemases + colistin resistance gene. Both scorings are depicted in the Supplementary Tables S3 and S4.

Contigs belonging to possible plasmids were identified using MOB-suite 3.1.4 using its sub tool MOBrecon to reconstruct and classify plasmids from draft assemblies or contig files (Robertson and Nash, 2018; Robertson et al., 2020). The closed plasmids and all plasmid contigs typed as AA405 and AA406 were compared with plasmid references using the whole genome alignment tool of CLC Genomics Workbench version 23.0.2.

The Ridom software SeqSphere (Ridom SeqSphere+ version 9.0.8 (EULA)) was used for phylogenetic analysis. Core genome(cg)MLST was performed with the respective published schemes (Zhou et al., 2020; Junemann et al., 2013) on the genome assemblies. Genome annotation was performed using PROKKA 1.14.6 (Seemann, 2014). The average nucleotide identity comparison of the *K. pneumoniae* genomes was calculated using fastANI v1.34 (Jain et al., 2018) as implemented in anvio version 7.1 (Eren et al., 2015) and was used for further phylogenetic analysis. The resulting tree was visualised using iTOL (version 6.8) (Letunic and Bork, 2021).

3. Results

In collaboration with diagnostic laboratories throughout Germany, 62 potentially hypervirulent *K. pneumoniae* isolates were collected between 2009 and 2023. The isolates were selected on the basis of either a positive string test and/or clinical manifestation and/or the presence of the virulence-associated genes *rmpA*, *rmpA2*, and *magA*. The list of fulfilled criteria for these isolates is given in Supplementary Table S2. *K. pneumoniae* strain NCTC 14052 from a patient with liver abscess was included in the collection as a hv*Kp* reference strain.

3.1. Phylogenetic analysis and overview

The phylogenetic tree (ANI calculation) for the 62 isolates includes information on sequence type (ST) and capsule type (K-type), virulence and resistance scores, and string test results (Fig. 1). All isolates belonged to the species *K. pneumoniae*, with two exceptions marked with * for *K. oxytoca* and # for *K. quasipneumoniae;* both were string test negative. However, the *K. oxytoca* isolate had a high virulence score (4) and a medium resistance score. The *K. quasipneumoniae* isolate had a score of zero for both but K-type K1 and the respective *magA* gene was present.

Two sequence types were dominant in our study isolates, ST395 (green, including ST395–1LV) with capsule type K2 or K5 and ST23 (blue) with K-types K1, KL107 or KL57 (Fig. 1). The ST395 isolates grouped in one clade, and these 13 isolates had the high virulence score 4, and a medium resistance score (except one isolate with score 3) mainly based on production of NDM carbapenemase. However, only two of the ST395 isolates showed a positive string test result. The 13 isolates were from blood culture, wound swabs, respiratory specimen and urine but also from rectal swabs, mainly isolated in the years 2022 and 2023. Eight ST395 isolates were from an outbreak in one hospital (Nov 2022 – March 2023) (highlighted in dark grey, Fig. 1).

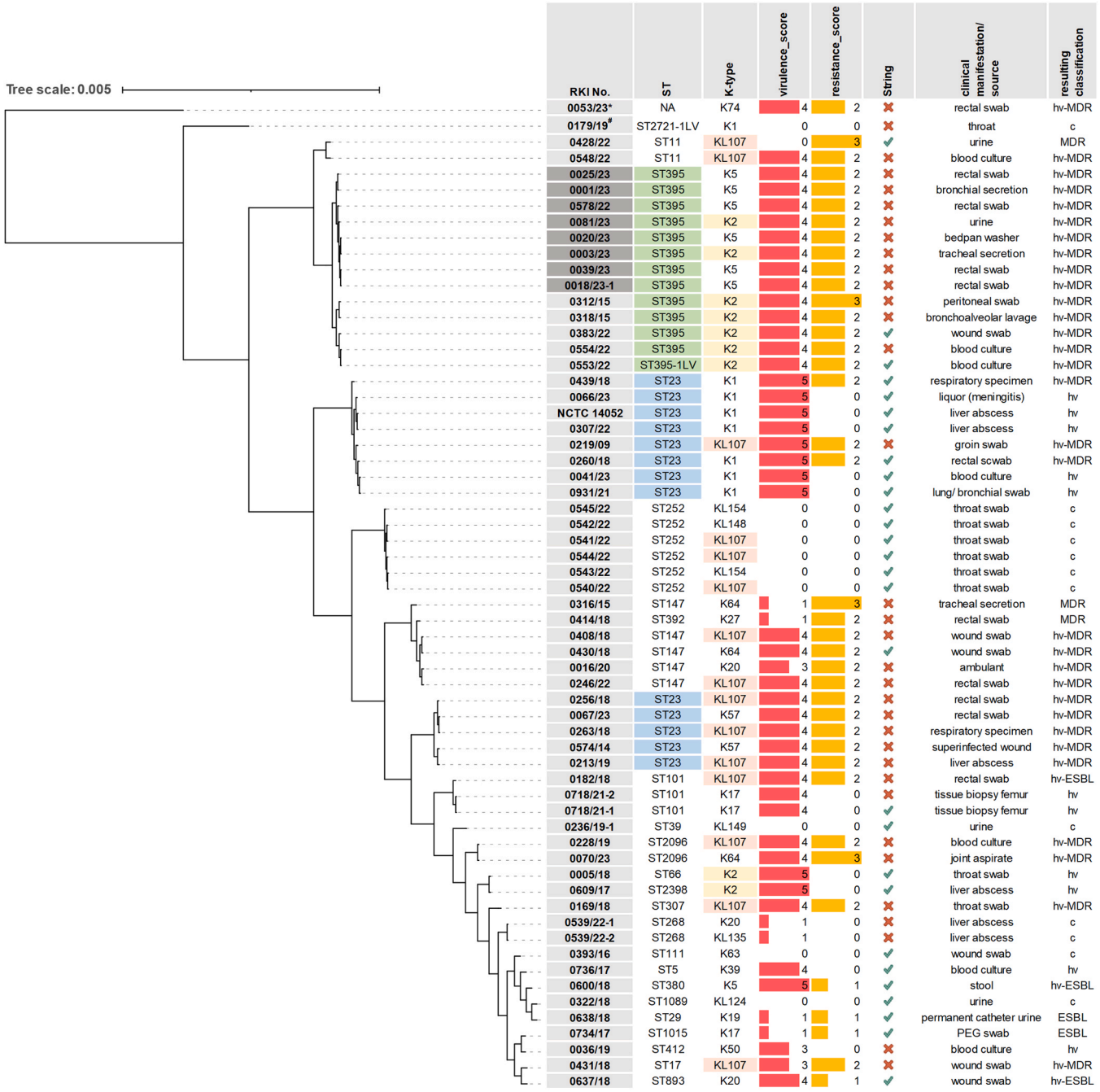

Fig. 1. Phylogenetic tree of 62 potential hv*Kp* isolates from Germany and the reference hv*Kp* isolate NCTC 14052. The tree was created using the tool anvio (v. 7.1) using implemented fastANI for calculation of the average nucleotide identity. It includes information on sequence types (STs), capsule types (K-types), the virulence (red) and resistance scores (orange), shown as bars and numbers. The two most abundant STs and K-types were highlighted with colours (ST in green and blue, K-type in yellow and slight orange). Additionally, results of the string tests and information about clinical manifestation or sample source were given. All isolates belong to the species *K. pneumoniae*, except two strains labelled by * *K. oxytoca* and # *K. quasipneumoniae*. We highlighted in dark grey the ST395 isolates associated with a hospital outbreak in 2022–2023. Criteria for the resulting classification of hypervirulence (last column) are listed in Table 3.

The other dominant ST among the study isolates was ST23 (13 of 62 isolates), present into three clades. The first clade consists of eight ST23 isolates of K-type K1 (except one KL107) isolated between 2009 and 2023 in different hospitals mainly from patients with severe infections (liver abscess, blood stream infection, meningitis). These isolates showed the highest virulence score 5 but mainly low resistance scores (Fig. 1). All ST23 isolates with K-type K1 were string test positive. The second and third clades of ST23 contained only string test negative isolates with KL107 and K57 showing a high virulence score (4) and a medium resistance score (2).

Apart from the dominant ST395 and ST23 other ST with five or more isolates were detected including six ST252 isolates from an outbreak neonatology department in a hospital (colonised patients) showing virulence/resistance scores of zero but a positive string test result. Furthermore, five ST147 isolates with variable virulence and resistance scores were detected (Fig. 1).

Fig. 2 summarises the laboratory results including string test and *rmpA/rmpA2* PCR (for more details see Supplementary Table S5) and the bioinformatic results using software tools AMRfinder and Kleborate to predict resistance genes and virulence associated genes (for more details

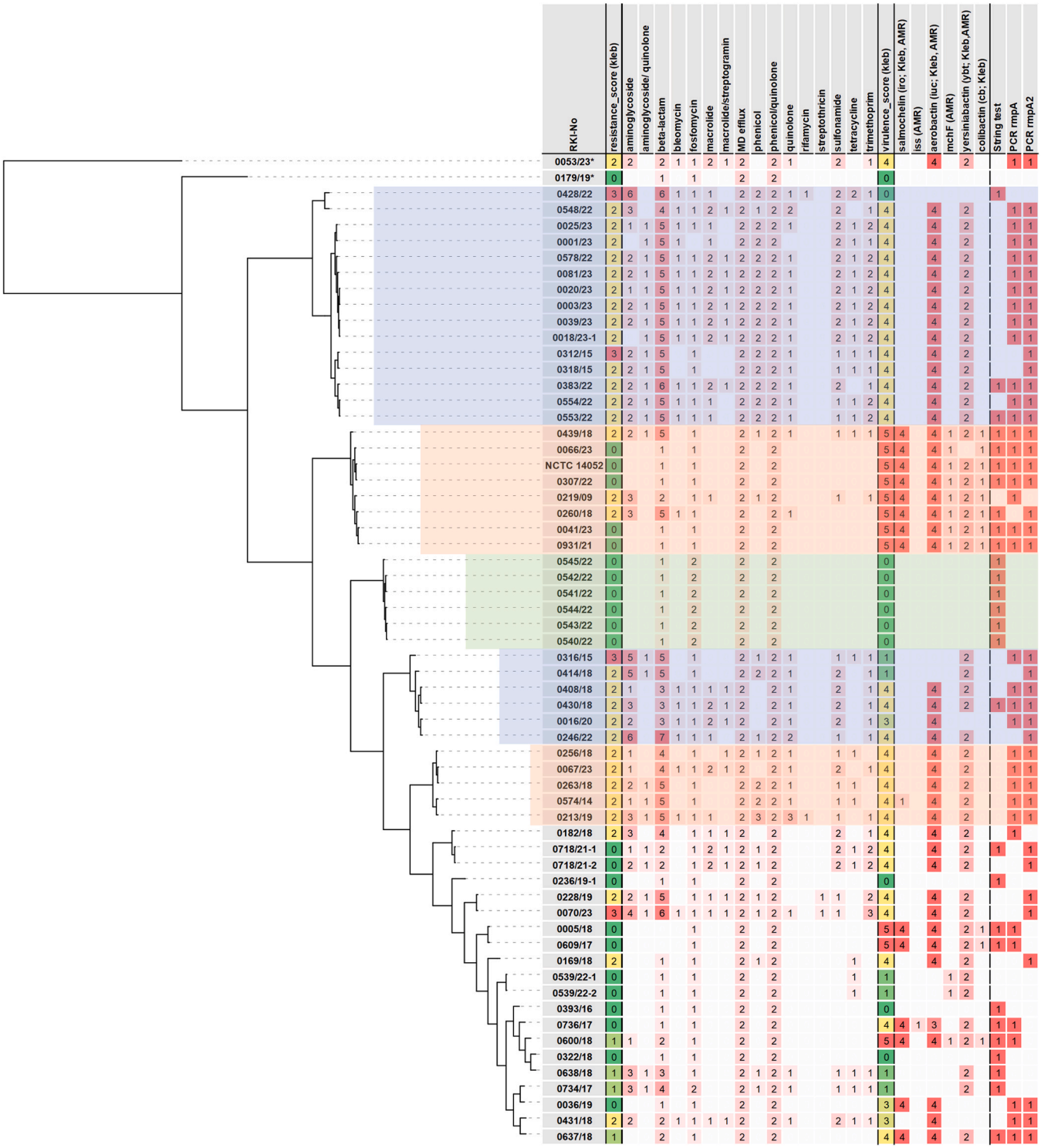

Fig. 2. Phylogenetic overview of 62 investigated isolates with the corresponding resistance, and virulence genes. Antimicrobial resistance (AMR) genes and virulence genes were predicted by software tools Kleborate and AMRfinder. The AMR genes were summarised in categories. The numbers per gene category indicate the number of different genes present in that category. The last three categories show lab results like string test, and PCR results for presence of *rmpA* and *rmpA2*.

see Supplementary Table S6, S7, S8). The largest group (top one highlighted in blue, Fig. 2), containing isolates with ST395 and two with ST11, showed mostly high virulence scores (4) due to the presence of aerobactin and yersiniabactin genes; furthermore, in this clade the resistance score was mostly on average at a medium high level (2). The second group contained exclusively strains with ST23. They had low resistance scores, but very high virulence scores, contained genes *rmpA* and *rmpA2* and were mainly string test positive. The third group contained hypermucoviscous ST252 (KL107) isolates from the outbreak in a neonatology department in a hospital without any detection of virulence and resistance genes. The fourth group consisted of ST147 isolates with medium or high resistance scores except one ST392 with very different K-types and virulence scores. A closer look at the different resistance genes found in this group revealed that some isolates harboured up to six genes contributing to aminoglycoside resistance and various genes mediating resistance to sulphonamides and trimethoprim.

In the category of beta-lactam resistances different beta-lactamase genes per isolate were detected including ESBL and carbapenemase genes. The fifth group contained again isolates with the sequence types ST23 with high virulence score but medium resistance scores. They all harboured either *rmpA* or *rmpA2* or both and only one isolate showed a string positive result. Resistance genes in this ST23 group, encoded various beta-lactamases including ESBL (CTX-M-14, CTX-M-55) and carbapenemases (NDM-1, OXA-48). (see extended version of Fig. 2 named as Supplementary Figure 1).

Comparing the results for *rmpA/rmpA2* gained from PCR and Sanger-sequencing with the results from the bioinformatic tools, we found a discrepancy in the number of detected *rmpA/rmpA2* genes (Supplementary Table S9.) Therefore, we decided to use only the data which was supported by Sanger-sequencing, since this resembles most of the results obtained in routine diagnostics.

3.2. Prevalence of sequence and capsule type in relation to hypermucoviscosity

According to the literature hypermucoviscosity has been found to be associated with hypervirulence in *K. pneumoniae* (Sanchez-Lopez et al., 2019; Shon et al., 2013). To analyse whether there is an association between a string test positive result (=hypermucoviscosity) and ST or K-type, we investigated their co-occurrence in our study isolates. The results are summarised in Table 1.

Among the 13 ST395 (including ST395–1LV) isolates two were string test positive (Table 1 | **A**). The seven ST23 isolates with K-type K1 (see Fig. 1) had positive string test results. Furthermore, all ST252 and one of five ST147 isolates were string test positive.

Looking at the capsule types (Table 1 | **B**), KL107 was the most common K-type (15/62) and only four isolates were tested string test positive. The second most common K-type was K2 (9/62) and four string test positive isolates. Further commonly detected K-types were K1 (seven out of eight isolates were string test positive) and K5 (one of seven isolates was string test positive).

In order to analyse the co-occurrence of sequence type and capsule type, we created a matrix counting every possible occurring combination. The results are shown in Fig. 3. Focusing on the prevailing STs and K-types in our study isolates, ST395 and K-type KL107 together was not the most abundant combination. Instead other combinations occurred frequently: ST23 with K1 was detected seven times, and ST395 with K-type K5 and ST395 K2 were detected six times each.

To investigate possible associations between a specific ST or K-type and a preferred virulence and/or antimicrobial resistance gene combination we compared the co-occurrence of detected virulence/resistance score, ST or K-type and also included the results of the string test (Table 2).

Sequence type ST395 occurred mostly in isolates with a virulence score of 4 and resistance score of 2. ST23 also appeared to be associated with high virulence scores. Four ST23 isolates had a virulence score of 4 and eight had a virulence score of 5. In addition, a positive string test result only seems to be associated with ST23, not with ST395. Furthermore, there was an accumulation of positive string test results for *K. pneumoniae*-ST252 outbreak isolates, which were non-virulent and susceptible to antibiotics (Table 2 | **A**).

When comparing the frequency of high virulence scores for K-types, an accumulation of these scores was observed for four types of capsules: K1 with an average virulence score of 4.5, K2 with a score of 3.9 on average, K5 (average virulence score 3.8), and KL107 (an averaged virulence score of 3.0). Furthermore, K-types K1, K2 and KL107 seem to be associated with a positive string test result. Finally, the results showed that the K-type K1 isolates were most frequently hypermucoviscous (seven out of eight isolates with positive string test) (Table 2 | **B**).

Table 1
Prevalence of sequence (A) and capsule types (B) in correlation with positive string test results is depicted for the 62 isolates of this study. The column called "String" shows the number of string positive tested isolates for a distinct ST (A) or K-type (B), respectively. The column "number" shows the number of isolates with the corresponding ST or K-type, respectively. The column "percentage" displays the percentual amount of the corresponding ST or K-type found in the 62 investigated study isolates.

A	string	number	percentage
ST23	7	13	21%
ST395	1	12	19%
ST252	6	6	10%
ST147	1	5	8%
ST101	1	3	5%
ST11	1	2	3%
ST2096		2	3%
ST268		2	3%
ST39	1	1	2%
ST307		1	2%
NA		1	2%
ST1015	1	1	2%
ST1089	1	1	2%
ST111	1	1	2%
ST17		1	2%
ST2398	1	1	2%
ST2721-1LV		1	2%
ST29	1	1	2%
ST380	1	1	2%
ST392		1	2%
ST395-1LV	1	1	2%
ST412		1	2%
ST5	1	1	2%
ST66	1	1	2%
ST893	1	1	2%

B	string	number	percentage
KL107	4	15	24%
K2	4	9	15%
K1	7	8	13%
K5	1	7	11%
K17	2	3	5%
K20	1	3	5%
K64	1	3	5%
K57		2	3%
KL154	2	2	3%
KL149	1	1	2%
K19	1	1	2%
K39	1	1	2%
K50		1	2%
K63	1	1	2%
K74		1	2%
KL124	1	1	2%
KL135		1	2%
KL148	1	1	2%
K27		1	2%

3.3. Resulting hypervirulence classification of the isolates

Taking all study results into account, we made a resulting classification that determined whether an isolate has to be categorised as hypervirulent Table 3). Correspondingly, isolates were named as hypervirulent (hv): (I) with a virulence score of 3 or higher, and (II) with a virulence score of 2 plus a positive string result and/or PCR confirmed presence of *rmpA* and/or *rmpA2*. Adding further information like the antibiotic resistances, we classified isolates as ESBL or MDR according to the suggestion of Magiorakos *et al.* 2012 (Magiorakos et al., 2012) (ESBL with a resistance score of 1, MDR with a resistance score of 2 or higher). The classical (c) *K. pneumoniae* showed a virulence score of 2 or below and a negative string result and no presence of *rmpA/A2* genes and a resistance score of 0. Using those criteria, 45 of the 62 isolates were classified as hypervirulent (73%). Finally, 31 of the 62 study isolates were classified as hv-MDR (Fig. 1).

3.4. Comparison with international K. pneumoniae datasets

For ST and K-types, we compared our study results with the top 10 prevalence of the Global dataset (contains any submitted *K. pneumoniae* with or without resistance or virulence genes) and the top 10 of the EuSCAPE (European Survey on Carbapenemase-Producing Enterobacteriaceae) dataset on the Kleborate-viz platform (Lam et al., 2021a). The

platform was last accessed on 11.09.2023. The comparison is shown in Table 4.

Comparison of the large *K. pneumoniae* datasets and our 62 study isolates with hypervirulence associated determinants revealed the same ST and K-types at higher frequencies. Five of the top 10 STs in our study were also found in the top 10 ranking of the global dataset (ST23, ST147, ST101, ST11 and ST307), and four STs (ST11, ST101, ST307 and ST147) were found in the EuSCAPE dataset (Table 4 | **A**). However, one of the two dominant ST of the present study, ST395 (8 of 13 isolates were outbreak-associated), was not in the top 10 of both data sets. Regarding K-types five of the top 10 in our study are found in the global dataset (KL107, K2, K1, K17 and K64) and 3 in the EuSCAPE dataset (KL107, K2 and K17). Interestingly, KL107 was the most common capsule type in all three data sets (Table 4 | **B**).

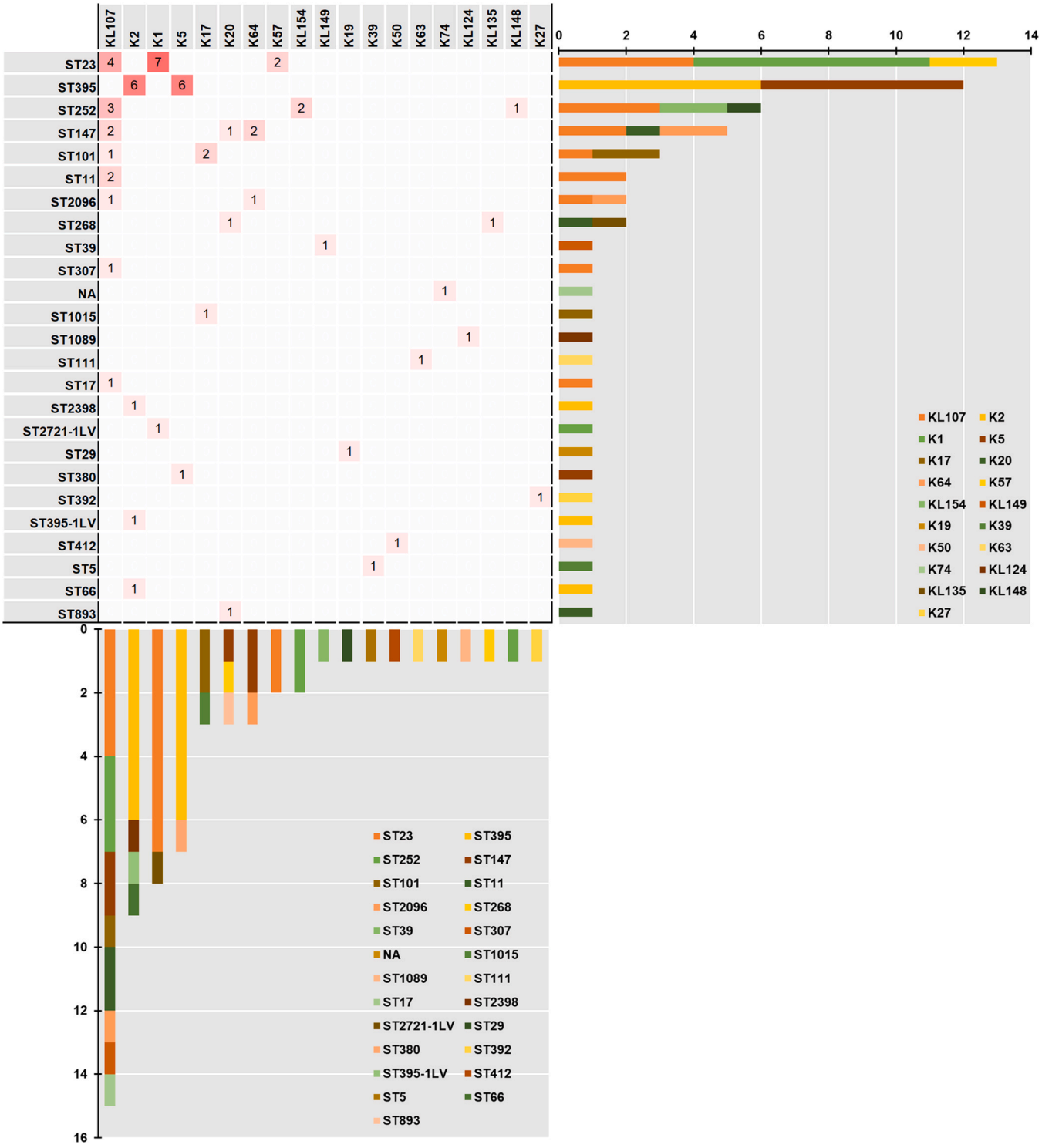

Fig. 3. Co-occurrences of sequence type (ST) and capsule type (K-type). The contingency table shows the number of co-occurring combinations of STs and K-types. The bar plot on the right shows the amount of each ST coloured by variating K-type. The bar plot below the table shows the corresponding amount of each K-type coloured by the variations of STs. The information about ST and K-type for each strain was determined using software tool Kleborate. Horizontally, the capsule types are listed in descending order, and vertically, the sequence types are listed with the most common at the top and the least common at the bottom.

3.5. Co-occurrence of specific plasmids with sequence type

Plasmids are critical factors in the carriage and horizontal transmission of resistance and virulence genes. *K. pneumoniae* is known to carry up to ten different plasmids in a single strain (Shankar et al., 2020). To get a first insight, WGS data of all 62 isolates were analysed using MOBsuite, a bioinformatics tool designed to support the detection and characterisation of plasmids in bacterial genomes. Within the device, a plasmid multilocus sequence typing (pMLST) was performed to classify and track different plasmid types. Virulence and resistance genes on the identified plasmids were detected using Kleborate and AMRfinder. Table 5 summarises the most frequent found types of plasmids in our study isolates and their characteristics concerning incompatibility type (Inc), predicted mobility and resistance and virulence. Furthermore, Supplementary Table S10 shows a complete overview of all detected plasmid types per isolate and additional genome information.

Results revealed the presence of multiple plasmids in the 13 ST395 isolates. The plasmid types AA100, AA941 and AB189 were detected almost exclusively in ST395 isolates but these carried no ESBL genes, carbapenemase genes or virulence genes. Several other plasmids found in ST395 isolates harbour either virulence genes (AA406), resistance genes (AA002, AA275) or both (AA405). In general, plasmids typed as AA405 were the predominant plasmid type in our study isolates (45%). AA405 was present most frequently in isolates of ST395, ST147 and ST101 (Table 5). Kleborate and AMRfinder detected several resistance genes and different *iuc* genes in almost all plasmids of the type AA405. In contrast, all ST23 isolates carried a plasmid of type AA406, which is associated with virulence genes, such as *iuc, iro*, *rmpA* and *rmpA2* (data not shown). Furthermore, five ST23 isolates and three ST395 isolates harboured a plasmid of type AA002 as a resistance plasmid carrying $bla_{\text{OXA-48}}$ or $bla_{\text{OXA-244}}$ carbapenemase genes.

The plasmids typed as AA405, AA406 and AA398 carried multiple

Table 3
Resulting classification after complete molecular and bioinformatic analyses of the 62 *K. pneumoniae* study isolates. Isolates were named as hypervirulent (hv): (I) with a virulence score of 3 or higher, and (II) with a virulence score of 2 plus a positive string result and/or PCR confirmed presence of *rmpA* and/or *rmpA2*. According to Magiorakos *et al.*, 2012 (Magiorakos et al., 2012) multidrug resistant (MDR) isolates show a resistance score of 2 or higher, and extended-spectrum beta-lactamase (ESBL) producing isolates show a resistance score of 1. The classical (c) *K. pneumoniae* showed a virulence score of 2 and smaller, a resistance score of 0, have a negative string results and were negative for *rmpA/A2*.

	c	ESBL	MDR	hv	hv-ESBL	hv-MDR
absolute number	12	2	3	11	3	31
percentage	19%	3%	5%	18%	5%	50%

Table 2
A. Co-occurrence of ST and virulence score/resistance score and hypermucoviscosity (string test positivity). B. Co-occurrence of K-type and virulence score/resistance score and string test positivity. Virulence score and resistance score were calculated by Kleborate, as well as ST and K-type.

A	virulence score						resistance score				String
	0	1	2	3	4	5	0	1	2	3	+
ST23					5	8	5		8		7
ST395					12				11	1	1
ST252	6						6				6
ST147		1		1	3				4	1	1
ST101					3		2		1		1
ST11	1				1				1	1	1
ST2096					2				1	1	
ST268		2					2				
ST39	1						1				1
ST307					1				1		
NA					1				1		
ST1015		1						1			1
ST1089	1						1				1
ST111	1						1				1
ST17				1					1		
ST2398						1	1				1
ST2721-1LV	1						1				
ST29		1						1			1
ST380						1		1			1
ST392		1							1		
ST395-1LV					1				1		1
ST412				1			1				
ST5					1		1				1
ST66						1	1				1
ST893					1			1			1

B	virulence score						resistance score				String
	0	1	2	3	4	5	0	1	2	3	+
KL107	4			1	9	1	3		11	1	4
K2					7	2	2		6	1	4
K1	1					7	6		2		7
K5					6	1		1	6		1
K17		1			2		2	1			2
K20		1		1	1		1	1	1		1
K64		1			2				1	2	1
K57					2				2		
KL154	2						2				2
KL149	1						1				1
K19		1						1			1
K39					1		1				1
K50				1			1				
K63	1						1				1
K74					1				1		
KL124	1						1				1
KL135		1					1				
KL148	1						1				1
K27		1							1		

virulence genes such as *iuc* encoding aerobactin. Furthermore, in about 28% of the plasmids typed as AA405, *rmpA* seems to be present and in about 38% of the plasmids typed as AA406, *rmpA* appears to be present. Genes encoding for aerobactin (*iuc*) were present in almost all AA406 plasmids, and in 66% of this plasmid type also salmochelin encoding genes (*iro*) was detected. In contrast, genes encoding for yersiniabactin (*ybt*) were mainly found on contigs sorted as chromosomal. 77% of all isolates encode for *ybt* genes on chromosomal contigs. Genes encoding colibactin (*clb*), as an additional siderophore were mostly found on chromosomal associated contigs and were present in 17% of the isolates (data not shown).

In summary, data from Table 5 and Supplementary Table S11 **|A**, indicate that some plasmid types occurred in combination with certain STs (AA406 and ST23); others seem to be ubiquitous (AA405). Occurrence of plasmid types in isolates of certain K-types was not so clear (Supplementary Table S11 **| B**).

We were able to close six of the plasmids grouped into plasmid type AA405 and compared those to a plasmid reference sequence suggested by MOBsuite (closest relative). This reference sequence CP040726.1 (pKvST147B) and a second one that we selected (pKvST147L) describe so called convergence or hybrid plasmids in *K. pneumoniae* containing both resistance genes and virulence genes (Turton et al., 2019). Results show a high degree of similarity with a lot of homologous sequence regions despite differences in predicted plasmid sizes (339 - 396 kbp) (Fig. 4). For example, the big purple section of the plasmids contains the virulence genes (*iuc)*. Carbapenemase genes $bla_{\text{NDM-1}}$ or $bla_{\text{NDM-5}}$ and virulence genes *iuc* and in part *rmpA/A2* were detected on these convergence plasmids detected in our study isolates that belong to different ST. Additionally, we compared all plasmids of this study typed as AA405 with plasmid references pKvST147B and pKvST147L (Turton et al., 2019) using the contig files of the plasmids for a whole genome alignment by CLC Genomics Workbench tool (Supplementary Fig. S2). Using the same method, we compared the second most abundant plasmid type AA406 that was nearly exclusively detected in ST23 isolates with the closest relative, a classical virulence plasmid pK2044 (Wu et al., 2009) with virulence genes *iuc*, *iro* and *rmpA/A2* (Supplementary Fig. S3). The AA406 plasmid sequences varied remarkably in size but show also several identical gene arrangements. Among the six isolates randomly selected for long read sequencing and hybrid assembly only one ST23 isolate was a carbapenemase producer ($bla_{\text{OXA-48}}$) and contained the abundant virulence plasmid type AA406 (no. 0574/14, Supplementary Fig. S3) but no convergence plasmid. But this carbapenemase was encoded on another plasmid typed as AA190 which contained further resistance genes: $bla_{\text{OXA-1}}$, *aac(3)-IIa* (encodes an aminoglycoside N(3)-acetyltransferase), *aac(6′)-Ib-cr* (encodes an aminoglycoside N(6′)-acetyltransferase) and *catB4.v1* (encodes a chloramphenicol acetyltransferase).

4. Discussion

The number of reports of infections attributed to hv*Kp* isolates is increasing worldwide (Liu et al., 2020; Wyres et al., 2020; Hallal Ferreira Raro et al., 2023). However, the definition of hv*Kp* varies considerably and comprehensive data regarding their prevalence in Europe remains limited, as highlighted by previous studies (David et al., 2019; Russo and Marr, 2019; Struve et al., 2015; Lee et al., 2017; Fazili et al., 2016). Recently, a study in one German hospital based on isolate selection by hypermucoviscosity and additional PCR for several virulence genes (*magA, rmpA/A2, iuc*) showed a low prevalence (<1%) for these putative hv*Kp* (Neumann et al., 2023). We characterised 62 isolates that were pre-selected based on clinical manifestations and/or the presence of a hypermucoviscous phenotype and/or presence of virulence associated genes (*rmpA/rmpA2/magA*). The further analyses focused on identification of reliable markers or a meaningful combination of features to define hypervirulence in *K. pneumoniae* and to describe a population snapshot of these hypervirulent and multidrug-resistant *K. pneumoniae* from hospitalised patients in Germany collected over a period of 14 years. The 62 isolates exhibited a remarkable genetic diversity, as evidenced by the identification of 19 distinct capsule types and 24 different sequence types (Fig. 1). Interestingly, certain combinations of ST and K-type (ST23 and K1, ST395 and K2/K5) were common, and we detected a distinct set of virulence-associated genes, resistance genes and plasmids, within these common combinations. Based on the results we made a resulting classification of hypervirulence for our isolates that might facilitate the detection of these pathogens in future.

ST23 was one of two dominant ST in our study (13 of 62 isolates). ST23 has been described as the most prevalent *K. pneumoniae* lineage causing liver abscesses and has been mainly reported from the Asian region (Li, 2023; Marr and Russo, 2019; Shanthini, 2023; Shi, 2018; Struve, 2015). In our study ST23 isolates clustered in three subgroups with three different K-types. The eight ST23-K1 strains were mainly from severe infections, showed a positive in the string test, the highest virulence score (5) and presence of capsule regulator genes *rmpA/A2*, in

Table 4
Comparison of this study's most prevalent sequence and capsule types with the most prevalent on the Kleborate-viz Global and EuSCAPE datasets. A. Top 10 of the most abundant sequence types (STs). B. Top 10 of the most abundant capsule types (K-types). Kleborate-viz data was accessed last on 2023/11/9; the datasets were restricted to *K. pneumoniae* species only. The Global dataset includes 11259 *K. pneumoniae* isolates and the EuSCAPE dataset 1624. The Global dataset includes publicly available *Klebsiella* genomes with matched isolate metadata, and the EuSCAPE dataset (pan-European genomic surveillance data) contains data from Nov 2013 to May 2014 (David et al., 2019). STs and K-types shared in all three groups are highlighted. STs and K-types shared only in two groups are in bold type.

A

top 10 sequence type		
Global dataset	this study	EuSCAPE dataset
ST258	**ST23**	**ST512**
ST11	ST395	ST11
ST15	ST252	**ST15**
ST512	ST147	ST101
ST307	ST101	**ST258**
ST101	ST11	ST45
ST16	ST2096	ST405
ST23	ST268	ST437
ST147	ST39	ST307
ST14	ST307	ST147

B

top 10 K-type		
Global dataset	this study	EuSCAPE dataset
KL107	KL107	KL107
K64	K2	**K24**
K106	**K1**	K17
K2	K5	**K15**
K24	K17	KL105
K51	K20	K2
KL102	**K64**	KL112
K1	K57	KL151
K17	KL149	**KL102**
K15	KL154	**KL106**

Table 5
Co-occurrence of most abundant plasmid types with ST. A cut-off was set for plasmid types present in at least 10% of the investigated 62 isolates and only four most abundant ST are shown. Higher numbers of combinations were highlighted with more intense red colours. Plasmids were analysed with MOBsuite and separately from chromosomal contigs with Kleborate and AMRfinder. For more details see Supplementary Table S10 and Supplementary Table S11.

	ST395	ST23	ST252	ST147	sum plasmids	% of isoloates	Inc types	predicted mobility	suggested reference (closest relative)	resistance scores	virulence scores	frequent resistance genes	frequent virulence genes
AA405	12	1		4	28	45%	IncFIB, IncHI1B, IncR	conjugative (2 mobilisable)	CP040726 (21/28)	2 (19/28)	3 (27/28)	*bla* $_{CTX-M-15}$ (15/28), *bla* $_{NDM-1}$ (17/28)	*iuc* (27/28)
AA406		13			15	24%	IncFIB, IncHI1B	non-mobilisable (14/15), conjugative (2/15)	CP025081 (5/15), CP018338 (3/15)	0	3	-	*iuc* (15/15), *iro* (10/15)
AA002	3	5		2	15	24%	IncL/M, 2x IncFIA, IncFIC	conjugative	diverse	2	0	*bla* $_{OXA-48}$ (14/15), *aadA** (3/15), *ant(2")-1a* (3/15), *bla* $_{CTX-M-14}$ (2/15), *sul1* (2/15)	-
AA103		4	2	4	14	23%	ColRNAI_rep_cluster_1987	non-mobilisable (13/14), mobilisable (1/14)	CP024435 (7/14)	0	0	-	-
AA100	12				13	21%	-	non-mobilisable	CP034042 (13/13)	0	0	-	-
AA275	1		5	1	13	21%	IncFIB,IncFII,rep_cluster_2183	conjugative	CP020499 (5/13),	1 (1/13), 2 (1/13)	0	*bla* $_{CTX-M-15}$, *bla* $_{TEM-1}$, *dfrA14*, *tet(A)* (2/13); *bla* $_{NDM-1}$ (1/13)	-
AA941	8	1		2	12	19%	ColRNAI_rep_cluster_1857	mobilisable	CP024884	0	0	-	-
AA277			6		10	16%	IncFIA,IncFII,IncFIB,IncFII	non-mobilisable (7/10), conjugative (5/10)	MH056209 (4/10), LR134219 (3/10)	1 (3/10)	0	*dfrA14*, *tet(A)* (4/10); *qnrB1*, *sul2*, *bla* $_{OXA-1}$, *bla* $_{TEM-1}$, *bla* $_{CTX-M-15}$ (3/10)	-
AB189	5				6	10%	IncR	non-mobilisable	CP039972	0	0	*dfrA1* (4/6)	-

agreement with the literature characterising this ST as a "prototype" hv*Kp* with high virulence score and low resistance score (Choby et al., 2020). Four ST23 isolates (three from rectal/groin swabs one from a liver abscess) showed K-type KL107 characterised by high virulence (4) and medium resistance scores (2), presence of *rmpA/rmpA2* but a negative string test result. KL107 isolates have been described previously as having a non-hypermucoviscous phenotype and being non-hypervirulent (Wanford et al., 2021). According to our results the ST23-KL107 strains can be assigned as hypervirulent (due to presence of different virulence genes or clinical manifestation) but they are not hypermucoviscous. Two ST23-K57 from rectal swab and a superinfected wound were string test negative, too, despite the presence of *rmpA/rmpA2*, a high virulence score (4) and a medium resistance score (2), classifying them as hypervirulent plus carbapenemase- and

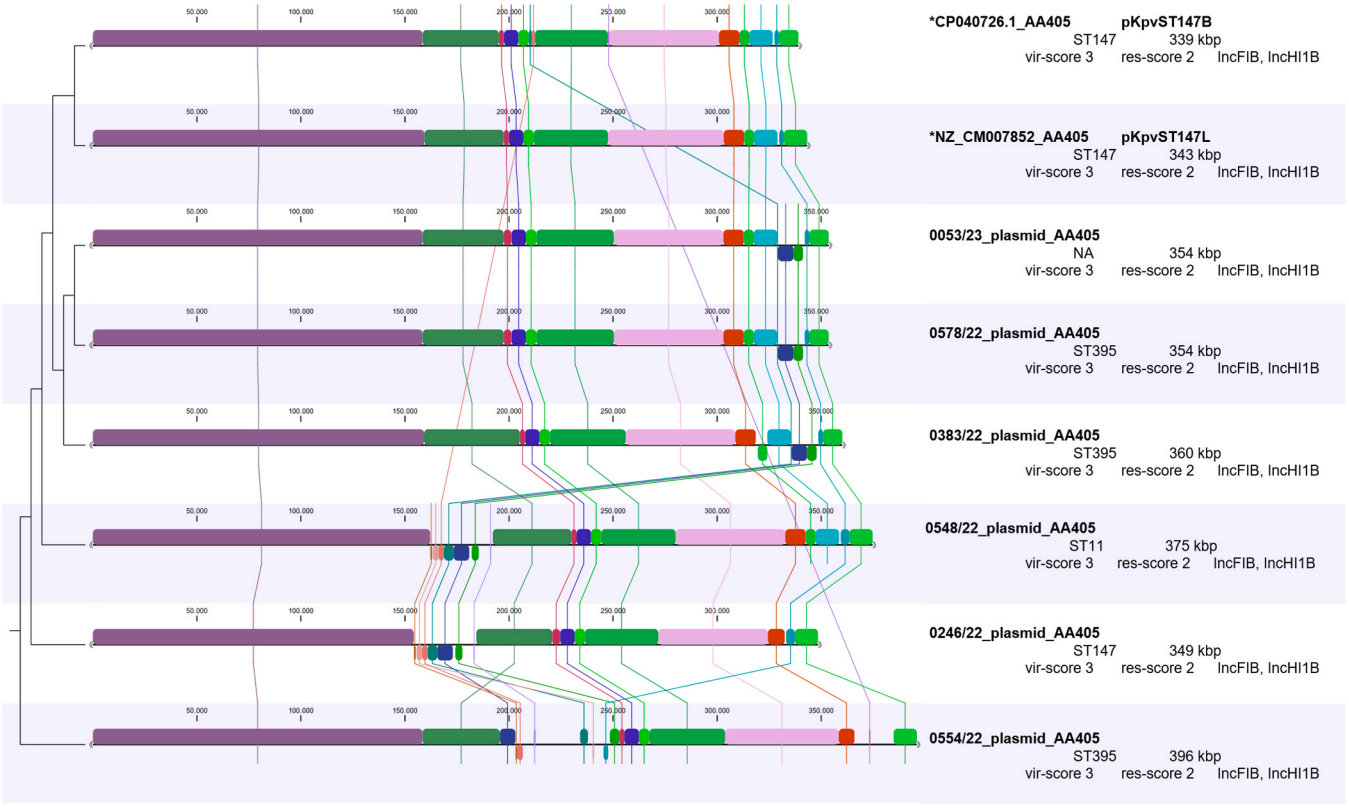

Fig. 4. Comparison of closed plasmids typed as AA405 with the reference plasmid pKvST147B_virulence (CP040726.1) (Turton et al., 2019). As a further reference pKpvST147L (Turton et al., 2019) was added, both reference sequences are indicated with an asterisk (*). Additionally, the ST of the corresponding isolate is added, the plasmid size in kbp, as well as, the Inc-types and the virulence and resistance scores for the plasmids. Comparison was created using CLC Genomics Workbench.

ESBL-producing. K57 *K. pneumoniae* isolates have been described to be associated with liver abscesses (Fung et al., 2002; Pan et al., 2008) but have so far not been linked to antibiotic resistance.

The second dominant sequence type in our study was ST395, accounting for 13 isolates that were all carbapenemase producer (mainly due to presence of carbapenemase gene bla_{NDM-1}) with a high virulence score (4) and presence of one or both of the capsule regulator genes *rmpA* and *rmpA2*. However, only two isolates exhibited a positive string test. This underscores the complex relationship between these hypervirulence-associated genes and expression of a hypermucoviscous phenotype that needs to be investigated future studies. ST395 isolates have already been described as combined MDR-hv*Kp* pathotype emerging in Europe (Hallal Ferreira Raro, 2023; Rödel et al., 2023), mostly in combination with capsule type K2 (Khrulnova et al., 2022). In our study seven of the 13 ST395 isolates had K-type K2 and six isolates had K5. K-type K5 has also been described before to be associated with hypervirulence like capsule types K2 or K1 (Shon et al., 2013). In a recent study by Shaidullina *et al.* (2023), 297 *K. pneumoniae*-ST395 isolates, mainly from Russia, were genomically characterised (Shaidullina et al., 2023). These isolates, like ours, showed genetic characteristics for hypervirulence, ESBL and carbapenemase production.

Separating our dataset into chromosomal and plasmid contigs reveals that the genes associated with virulence and resistance are mainly located on plasmids, lesser on the chromosomal backbone (data not shown). Plasmids are typed or grouped according to their incompatibility type (Inc-type). Plasmids of the same Inc-type are not compatible and cannot coexist in the same bacterial cell. The Inc-typing system groups plasmids sharing similar replication and maintenance mechanisms (Carattoli, 2011; Carattoli et al., 2005). In literature it has been described for some plasmid types that they are the main carrier for virulence and/or resistance genes (Hallal Ferreira Raro et al., 2023; Shaidullina et al., 2023; Zeng et al., 2022; Yang et al., 2022a, 2022b). In the present study we used the MOBsuite typing to classify and track different plasmid types. All ST23 isolates carried plasmids of type AA406, closely related to the classical virulence plasmid pK2044 with virulence genes *iuc*, *iro* and in part *rmpA/A2*. This virulence plasmid has been nearly exclusively found in this ST (Wu et al., 2009).

In contrast, the 13 ST395 isolates had three plasmids in common, typed as AA100, AA405 and AA941. According to the database used by MOBtyper, AA100 appears to be restricted to *Klebsiella* species. AA405 and AA941 were also found in other species such as *Citrobacter freundii*, *C. koseri*, *Enterobacter cloacae* or *Escherichia coli* (Robertson and Nash, 2018; Robertson et al., 2020). Plasmids of type AA405 are associated with lncFIB, lncHI1B, rep_cluster_1254 and lncR and are phylogenetically very close related to plasmid CP040726 (Turton et al., 2019) (Table 4). This so-called hybrid virulence and resistance plasmid was first described 2019, it also contained several virulence associated genes, e.g., *rmpA*, *rmpA2* and *iuc*, as well as resistance genes, like $bla_{CTX-M-15}$, *sul1* and *sul2*. Plasmids typed AA405 seem to be able to "collect" virulence and resistance genes, an observation previously described them as convergence plasmids (Wagner et al., 2017; Lam et al., 2019; Lan et al., 2021; Lam et al., 2023). The occurrence and spread of these hybrid plasmids within ST395 and other ST need to be further investigated in more detail with respect to plasmid evolution and mechanisms of maintenance. This multidrug-resistant and hypervirulent lineage is an emerging threat, that needs to be closely monitored (Sandfort et al., 2022).

Further frequently occurring STs in this study were ST252, ST147, ST101, ST11, and ST39. While ST147, ST101, ST11 and ST39 have been often described as epidemic or high risk clones with carbapenemase production (Hallal Ferreira Raro et al., 2023; Yang et al., 2020; Papagiannitsis et al., 2013; Zou et al., 2022; Falcone et al., 2022; Poulou et al., 2013; Lam et al., 2021b; Bolourchi et al., 2022; Wei et al., 2022; Martin et al., 2021), ST252 is less mentioned (Papagiannitsis et al., 2015; Toledano-Tableros et al., 2021; Yang et al., 2023). Our six study isolates of ST252 (KL107 and KL154) showed a positive string test result and represented an outbreak. However, these isolates showed no virulence or resistance factors and were from throat swabs of neonates without any clinical sign of infection. A study describes that ST252 strains have been transferred from domesticated animals (dogs, cats) to humans but without severe health issues (Marques et al., 2019). It is unknown, whether the present outbreak was caused by such a transmission.

In the present study were the detected remarkable discrepancies between the hypermucoviscous phenotype and presence of capsule regulator genes *rmpA/A2*. 28 of 62 isolates showed a positive string test result but only 16 of these isolates harboured *rmpA and/or rmpA2*. In contrast, out of the 34 isolates with a negative string test result 31 carried *rmpA/rmpA2*. Hypermucoviscosity had been described as a marker for hypervirulence and is strongly associated with the *rmpA/rmpA2* genes but several studies described these pathotypes as being distinct (Bolourchi et al., 2022; Tang et al., 2020; Catalan-Najera et al., 2017). Our results support these studies revealing that a positive string test result (hypermucoviscosity) cannot be used solely as an indicator for hypervirulence and a negative result cannot exclude hypervirulence (Catalan-Najera et al., 2017; Altayb et al., 2022; Dey et al., 2022). In contrast to the positive string test result, the presence of *rmpA/rmpA2* genes seems to be a more reliable indicator for hypervirulence. 17 of our 62 study isolates showed a virulence score below 2 but only in two isolates *rmpA* and/or *rmpA2* were present, respectively. The 45 isolates with a virulence score above 2 all harboured at least one of the two genes. Therefore, a positive string test alone as well as a positive PCR for *rmpA/rmpA2* alone does not seem to be a reliable determination for hypervirulence. To use a combination of two and more marker genes or further traits for hypervirulence was already suggested by Russo *et al.* (2018) (Russo et al., 2018).

It is noteworthy, that discrepancies between PCR results and bioinformatic tools were detected in in our study regarding the presence/absence detection of *rmpA/rmpA2* genes (Supplementary Table S9). However, the sequencing raw data and constructed assemblies were in general of high quality, all PCR detected resistance genes were confirmed by Kleborate/AMRfinder analysis and for the closed genomes generated by hybrid assembly, the concordance between the PCR results and assembly data was observed. For some isolates, a contig break in the *rmpA* region lead to the negative results in the Kleborate/AMRfinder analysis, while positive PCR results were observed. An additional factor might be the presence of a STOP-codon in *rmpA* that influence the chance of prediction of both tools. For the resulting definition of hypervirulence in the present study the virulence score of the Kleborate tool (does not contain *rmpA/A2*) was included and the PCR-based *rmpA/A2* detection. However, for future studies that are only based on Illumina short read WGS it should be considered that the raw data quality (read coverage) and the assembly process (remaining number of contigs) may influence the prediction for single determinants.

Using the resulting definition of hypervirulence of our study (Table 3) we categorised half of our isolates (31/62) as hv-MDR. One clear limitation was the bias of the used strain collections for selection of study isolates. These strain collections mainly contained MDR clinical isolates that are currently the most relevant in Germany. This may cause the overrepresentation of MDR isolates among the hv*Kp* in the present study. Furthermore, the inclusion of outbreak isolates in our isolate collection influences the prevalence of certain STs (such as ST395 or ST252). Finally, the decision for the marker genes *rmpA/A2 and magA* as the only selective marker genes in the present study may also influence the number of detected hv*Kp*.

In conclusion, our study confirmed *K. pneumoniae*-ST23 and ST395 as the most abundant clonal lineages with hypervirulence-associated genes. Of concern is the co-presence of virulence genes and resistance genes encoding ESBL and carbapenemases on the same plasmid, which can potentially be transferred. In future studies long read sequencing should be preferred for a more detailed and precise isolate and plasmid characterisation. The non-uniform definition of hypervirulence remains

a problem for exact diagnostic of these pathogens.

Our data show clearly that the presence of only one characteristic is not meaningful enough to determine hypervirulence, as there are too many exceptions for single traits. We recommend to test a combination of genetic virulence markers (*iuc, ybt, rmpA/rmpA2*) in combination with phenotypic traits (hypermucoviscosity) and documented severe clinical manifestations of the *K. pneumoniae* infection.

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CRediT authorship contribution statement

Wahl Anika: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Fischer Martin A.:** Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. **Klaper Kathleen:** Investigation, Validation, Visualization, Writing – original draft. **Müller Annelie:** Formal analysis, Investigation, Methodology, Validation, Visualization. **Borgmann Stefan:** Investigation, Validation, Writing – original draft. **Friesen Johannes:** Investigation, Validation, Writing – original draft. **Hunfeld Klaus-Peter:** Investigation, Validation, Writing – original draft. **Ilmberger Arkadius:** Investigation, Validation, Writing – original draft. **Kolbe-Busch Susanne:** Investigation, Validation, Writing – original draft. **Kresken Michael:** Investigation, Validation, Writing – original draft. **Lippmann Norman:** Investigation, Validation, Writing – original draft. **Lübbert Christoph:** Investigation, Validation, Writing – original draft. **Marschner Matthias:** Investigation, Validation, Writing – original draft. **Neumann Bernd:** Investigation, Validation, Writing – original draft. **Pfennigwerth Niels:** Investigation, Validation, Writing – original draft. **Probst-Kepper Michael:** Investigation, Validation, Writing – review & editing. **Rödel Jürgen:** Investigation, Validation, Writing – original draft. **Schulze Marco H.:** Investigation, Validation, Writing – original draft. **Zautner Andreas E.:** Investigation, Validation, Writing – original draft. **Werner Guido:** Conceptualization, Project administration, Supervision, Validation, Writing – original draft. **Pfeifer Yvonne:** Conceptualization, Data curation, Formal analysis, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Transparency declarations

All authors declare no conflict of interest.

Appendix A. Supporting information

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