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Molecular identification of *bla*SHV, *bla*LEN and *bla*OKP β-lactamase genes in *Klebsiella pneumoniae* by bi-directional sequencing of universal SP6 and T7-sequence tagged *bla*SHV-amplicons

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ABSTRACT

Plasmid encoded *bla*SHV enzymes represent an important sub-group of class A β-lactamases causing an ESBL phenotype which is increasingly found in *Enterobacteriaceae* including *Klebsiella pneumoniae*. The chromosomally encoded β-lactamase *bla*LEN and *bla*OKP enzymes, which so far only have been found in *K. pneumoniae,* do not hydrolyse extended-spectrum cephalosporins. In the present study, multiple displacement amplified DNA derived from 20 *K. pneumoniae* clinical isolates with a *bla*SHV-like genotype was used in a universal SHV PCR assay using SP6 (forward) and T7 (reverse) sequence-tagged primers. Identification and differentiation of *bla*SHV, *bla*LEN and *bla*OKP genes was obtained by bi-directional amplicon sequencing using SP6 and T7-specific primers. Three well characterised *K. pneumoniae* strains having a SHV-genotype were included in the study. The bi-directional amplicon sequencing, covering ~800 bp (~93 %) of the *bla*SHV, *bla*LEN and *bla*OKP enzyme encoding sequences, allowed for an unequivocal discrimination of SHV, LEN and OKP genes. Moreover, sequencing revealed the presence of *bla*SHV allelic variants in six *K. pneumoniae* isolates in which the amplicons had to be cloned accordingly. Based on deduced amino-acid sequences, a dendrogram was constructed. Seventeen out of 20 *K. pneumoniae* isolates with an ESBL-phenotype formed a SHV-like cluster, two were LEN-like, and one isolate was OKP-like. The PCR-based molecular typing method described here enables a rapid, reliable and cost-effective identification and differentiation of *bla*SHV, *bla*OKP and *bla*LEN genes.

1. Introduction

Plasmid encoded *bla*SHV enzymes represent an important sub-group of class-A β-lactamases which hydrolyse broad-spectrum β-lactam antibiotics. They cause an extended spectrum β-lactamase (ESBL) phenotype, which is increasingly found in enterobacterial species, including *Klebsiella pneumoniae* [1]. To date, more than 100 different SHV-type β-lactamases have been described which differ at one or more amino acid positions (www.lahey.org/studies). Originally, the SHV-1 β-lactamase, first described by Pitton in 1972 as Pit-2 [2], was considered to be a plasmid-encoded enzyme, belonging to class A, group 2b β-lactamases [3]. However, using more sensitive nucleic acid-based assays, the chromosomal location of the SHV-1 gene in all *K. pneumoniae* isolates could be demonstrated but not in *Klebsiella oxytoca* isolates [4-6]. Other β-lactamase genes which are unable to transfer ampicillin resistance have been occasionally found in *K. pneumoniae* isolates, e.g. genes encoding LEN-1 which is a class A, group 2a and chromosomally encoded β-lactamase [7, 8].

In a previous study, Haeggman and co-workers [9] identified a new, third group of sequences related to the *bla*SHV and *bla*LEN families, namely the chromosomally encoded *bla*OKP family (for other *K. pneumoniae* β-lactamase). To date, 14 different *bla* OKP-type β-lactamases have been described [10]. Phylogenetic analysis of the three genes revealed parallel evolution, corresponding to the phylogenetic groups KpI, KpII, and KpIII, respectively [9]. It was proposed that the chromosomally encoded *bla*LEN and *bla*OKP enzymes should be grouped in a single new family "*K. pneumoniae*-specific chromosomal β-lactamases," excluding plasmid encoded *bla*SHV enzymes from this family [11]. Furthermore, it has been shown that variants of chromosomally encoded *K. pneumoniae* OKP β-lactamases comprise two main groups, the *bla*OKP-A and *bla*OKP-B β-lactamases [12, 13].

Numerous molecular typing methods have been developed for identification of the growing number of *bla*SHV-genes [4, 14-18], including specific PCR assays of SHV, LEN,

and OKP genes [9, 19]. However, identification of a SHV genotype at the nucleotide level often requires cloning of the amplicons, followed by sequencing. Alternatively, amplicons have been sequenced using a set of gene-specific primers [9, 13, 19]. These methods are labour intensive and time-consuming.

In this study, we report on the use of a simple, accurate and universal SHV-PCR and bi-directional amplicon sequencing assay well suited for high-throughput identification and differentiation of *bla*SHV, *bla*LEN and *bla*OKP genes.

2. Material and methods

2.1. Susceptibility testing of Klebsiella pneumoniae clinical isolates

Susceptibility testing was performed on all *K. pneumoniae* clinical isolates (roughly 800 isolates/year) at the Department of Clinical Microbiology, University Hospital Linköping, Sweden with agar disk diffusion according to the Swedish Reference Group for Antibiotics (http://www.srga.org). Cefadroxil was used for the screening of cephalosporin resistance, which was followed up by testing of resistant isolates with cefotaxime and ceftazidime or direct testing with cefotaxime and ceftazidime with disk diffusion and Etest (bioMerieux Sverige, Askim, Sweden). All cefotaxime and/or ceftazidime resistant isolates were phenotypically screened by Etest using cefotaxime and ceftazidime with and without clavulanic acid (bioMerieux Sverige). A reduction of MIC by ≥3 twofold dilutions of the cephalosporins in the presence of clavulanic acid, i.e. MIC ratio ≥8 or the presence of phantom or deformation zones was considered indicative for an ESBL-phenotype. Twenty *K. pneumoniae* isolates were selected and stored in glycerol containing Nutrient Broth No 2 (Lab M, Bury, UK) at -70° C until analysis.

2.2 Reference strains

Three *K. pneumoniae* reference strains from the American Type Culture Collection (http://www.lgcstandards-atcc.org), ATCC 11296 (SHV-11), ATCC 13883 (SHV-1) and ATCC 700603 (SHV-18), and three *Escherichia coli* strains containing SHV-genes on conjugative plasmids (1204-SHV-2, J53 SHV-2, J53 SHV-1), provided by Dr. D. Livermore were used in this study. All isolates were kept in glycerol containing Nutrient Broth No 2 (Lab M) at -70ºC until analysed. Isolates were then grown on chromogenic UTI-agar (Oxoid, Basingstoke, UK) and incubated at 35ºC over night.

2.3 Automated DNA isolation

Due to capsule-production in some *K. pneumoniae* clinical isolates, five colonies from each isolate were pooled and harvested for DNA-extraction using a BioRobot EZ1 and a DNA Tissue Kit and DNA Bacteria Card (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

2.4 Multiple displacement amplification of bacterial DNA

To perform multiple sequencing from the same clinical isolate, sufficient amounts of bacterial DNA was produced by means of whole genome amplification using multiple displacement amplification (MDA) [20, 21]. For that purpose, 1 µl isolated bacterial DNA (see previous section) was added to a GenomiPhi V2 DNA amplification-kit cocktail and treated as recommended by the manufacturer (GE Healthcare Bio-Sciences, Uppsala, Sweden). After completition of the reaction, 80 µl ultra pure water (Eppendorf, Hamburg, Germany) was added to each tube. Subsequently, 1 µl of MDA-amplified bacterial DNA was used as template in down-stream applications.

2.5 Universal ^{bla}SHV-gene amplification

For amplification of ^{bla}SHV, ^{bla}LEN and ^{bla}OKP genes, a PCR was carried out using universal sense and antisense primers which were designed based on alignment with all ^{bla}SHV, ^{bla}LEN and ^{bla}OKP DNA sequences (Fig. 1) retrieved from http://www.lahey.org/Studies/ and http://www.pasteur.fr/recherche/genopole/PF8/betalact_en.html, hosted by S. Brisse at the Pasteur Institute and G. Jacoby and K. Bush at the Lahey Clinic, respectivley.

For that purpose, 10 pmol of each universal and modified primer, *SP6-*^{bla}SHV.SE 5'-*CATTTAGGTGACACTATAG*ATGCGTTATDTTCGCCTGTG-3' (target position 1 to 20

corresponding to the first nucleotide in amino acid position 5 [22], *T7-*^{bla}SHV.US.AS 5'-*TAATACGACTCACTATAGGG*TGCCAGTGCTCGATCAGCG-3' (target position 854-836), and a HotStarTaq-Master mix (Qiagen, Hilden, Germany) was mixed in a final reaction volume of 25 µl. PCR amplifications were carried out using an Eppendorf Mastercycler gradient thermo cycler (Eppendorf, Hamburg, Germany) and 200 µl thin-walled reaction tubes yielding a PCR-amplicon of ~890 bp (including the SP6 and T7 sequence-tags, Fig. 2) corresponding to ~800 bp (~93 %) of the ^{bla}SHV, ^{bla}LEN and ^{bla}OKP enzyme encoding nucleotide sequences.

```
SHV.SE                  1                   20
                        .                   .
                5'-ATGCGTTATDTTCGCCTGTG-3
SHV 1 (AF148850)        ATGCGTTATATTCGCCTGTG
LEN 1 (X04515)          ---------G----------
OKP A1 (AJ635401)       ---------G----------
OKP B1 (AJ635402)       ---------G----------


US.AS                   836                 854
                        .                   .
                3'-CGCTGATCGAGCACTGGCA-5'
                5'-TGCCAGTGCTCGATCAGCG-3'
SHV 1 (AF148850)        TGCCAGTGCTCGATCAGCG
LEN 1 (X04515)          ------------------
OKP A1 (AJ635401)       ------------------
OKP B1 (AJ635402)       ------------------
```

Figure 1: *Sequence alignment of E. coli SHV-1 [Genbank:AF148850], K. pneumoniae LEN-1 [GenBank:X04515], K. pneumoniae OKP-A1 [GenBank:AJ635401], K. pneumoniae OKP-B1 [GenBank:AJ635402], the universal degenerated SHV.SE primer (forward, position 1 to 20) and the universal US.AS primer (reverse, position 836 to 854). Dashes indicate sequence homologies. The degenerated nucleotide sequence position in the SHV.SE-primer and its corresponding nucleotides in the aligned genes are indicated in bold letters. For clarity, sense and antisense DNA sequences of the universal US.AS-primer are given.*

PCR conditions were as follows: initial denaturation step at 95° C for 15 min; 30 cycles of denaturation at 94° C for 30 s; annealing at 58° C for 30 s; extension at 72° C for 1 min, and a

final extension step at 72° C for 10 min. Prior to sequencing, the quality of the amplicons were analysed electrophoretically on a pre-cast 2% agarose E-gel (Invitrogen, Carlsbad, USA).

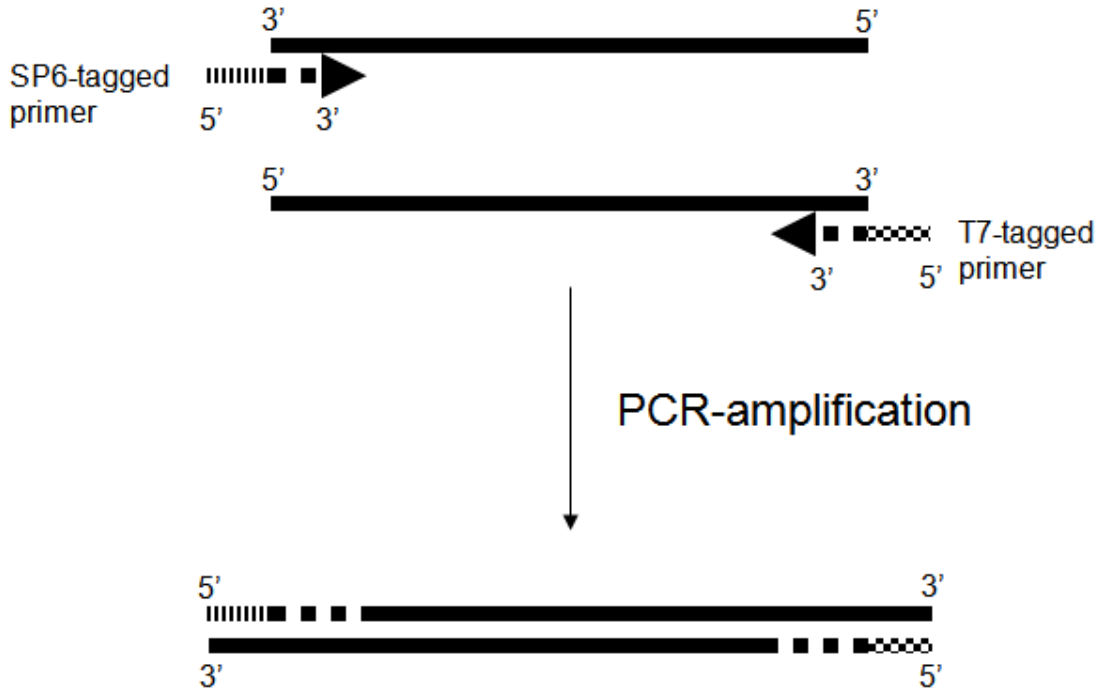

Figure 2: *Schematic diagram illustrating the modified PCR assay used to generate double stranded SP6 and T7 sequence tagged amplicons. In the initial phase, gene-specific target sequences (i. e. SHV, LEN and OKP) are amplified using forward SP6- and reverse T7-linker primers. The incorporation of SP6 and T7-linker sequences during the amplification steps allows for concurrent bi-directional amplicon sequencing using universal SP6 and T7 primers.*

2.6 Sequencing

Bi-directional sequencing of SP6- and T7-sequence-tagged SHV amplicons (Fig. 2) was carried out using a customer sequencing service and SP6 and T7-specific sequencing primers (Eurofins-MWG/Operon, Martinsried, Germany). Prior to sequencing, each amplicon sample was divided in two separate tubes (for SP6 and T7-specific sequencing reactions) and treated with ExoSAP-IT to inactivate excess of oligonucleotide primers following the supplier's protocol (USB, Staufen, Germany). Generated sequences were aligned, edited and compared with all type sequences of ^{bla}SHV, ^{bla}LEN and ^{bla}OKP listed at http://www.lahey.org/Studies/

and http://www.pasteur.fr/recherche/genopole/PF8/betalact_en.html and deposited in Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/nucleotide) using the CLC bioinformatics freeware v.4.6.2 (http://www.clcbio.com).

2.7 Molecular cloning and sequencing of selected universal SHV-amplicons

In 6 of the 20 *K. pneumoniae* isolates, more than one allelic variant of the SHV-gene was present. In theses cases, direct sequencing led to ambiguous results. Therefore, new amplifications were performed using some selected strains and the same universal primer-pair without SP6- or T7-sequence tags. Subsequently, amplicons were cloned using a TOPO-TA Cloning Kit with chemical competent TOP10 cells according to the manufacturer's protocol (Invitrogen, Carlsbad, USA). Five white colonies from a blue/white screening of each isolate were selected and recombinant plasmids were sequenced (Eurofins-MWG/Operon).

2.8 Dendrogram analysis

Partial SHV, LEN and OKP amino-acid sequences deduced from amplicon sequencing and from appropriate SHV, LEN and OKP genes retrieved from Entrez Nucleotide (Fig. 3), were used to construct a dendrogram employing the CLC bioinformatics freeware v.4.6.2. (http://www.clcbio.com) for UPGMA-clustering and bootstrap analysis.

3. Results

3.1 Screening of cephalosporin resistance

During December 2001 until December 2007, twenty *K. pneumoniae* clinical isolates were detected having cefotaxime and ceftazidime resistance. Furthermore, 16 of theses *K. pneumoniae* isolates revealed an ESBL-phenotype as deduced by Etest with cefotaxime and ceftazidime with and without clavulanic acid. Two of the *K. pneumoniae* isolates revealed an ESBL-phenotype only with cefotaxime, and two only with ceftazidime, respectively.

3.2 Bi-directional amplicon sequencing

Bi-directional sequencing of dual SP-6 and T7 sequence-tagged universal SHV-amplicons allowed us to establish partial nucleotide sequences (~800 bp) of the ^{bla}SHV, ^{bla}LEN and ^{bla}OKP genes. Deduced amino-acid sequences revealed the presence of SHV-like enzymes in 17 out of 20 *K. pneumoniae* clinical isolates (Fig. 3). Furthermore, amino-acid sequence alignment of the SHV-like sequences present in *K. pneumoniae* clinical isolates No. 184 and 205 revealed sequence identities with a ^{bla}LEN-11-like enzyme. In the same way, *K. pneumoniae* clinical isolate No. 92 corresponded with a ^{bla}OKP-B6-like enzyme, respectively (Fig. 3).

Bi-directional amplicon sequencing of both strands revealed the presence of more than one allelic variant of the ^{bla}SHV-gene in 6 out of 20 *K. pneumoniae* isolates (No 19, 33, 10, 137, 138, and 143). A Leu35Gln ^{bla}SHV variant was present in 2 out of 20 isolates, a Pro226Ser ^{bla}SHV variant in three out of 20, a Gly238Ser ^{bla}SHV variant in 6 out of 20, and a Glu240Lys in 5 out of 20 *K. pneumoniae* isolates, respectively (Table 1). *K. pneumoniae* isolates 137, 138 and 143 originated from the same patient. They were collected at different occasions and showed an identical phenotype including the antibiotic susceptibility profile and sequencing profiles.

Table 1: Amino-acid positions with indication of relevant non-silent mutations present in the ^{bla}SHV amino-acid sequence.

K. pneumoniae **clinical isolate No**	**Amino-acid positions**			
	35	**226**	**238**	**240**
19	Leu35Gln	-	Gly238Ser	Glu240Lys
33	-	-	Gly238Ser	Glu240Lys
110	Leu35Gln	-	Gly238Ser	-
137, 138, 143	-	Pro226Ser	Gly238Ser	Glu240Lys

3.3 Sequencing of cloned SHV-amplicons

Amplicons derived from *K. pneumoniae* isolates no 19, 22, 110 and 137 were cloned and sequenced. Recombinant plasmids containing ^{bla}SHV-sequences derived from *K. pneumoniae* isolates No 110 (four colonies), No 137 (four colonies), No 19 (three colonies), and No 33 (two colonies) were sequenced and analysed. The deduced amino-acids revealed the presence of ^{bla}SHV-5-like genes in *K. pneumoniae* isolate No 33 and 137, a ^{bla}SHV-12-like gene in *K. pneumoniae* isolate No 19, a ^{bla}SHV-2-like and ^{bla}SHV-11-like gene in *K. pneumoniae* isolate No 110 (Fig. 3). Cloned amplicon sequences derived from *K. pneumoniae* isolate No 110c and 110e deviated strongly and might represent a cloning artefact (Fig. 3).

3.4 Dendrogram analysis

Construction of an amino-acid sequence-based phylogenetic tree derived from near full-length ^{bla}SHV, ^{bla}LEN and ^{bla}OKP-like genes located between the two universal modified primers revealed the presence of three distinct cluster groups representing SHV-like sequences (17 out of 20 *K. pneumoniae* isolates), LEN-like sequences (2 out of 20 *K. pneumoniae* isolates), and an OKP-like sequence (1 out of 20 *K. pneumoniae* isolates),

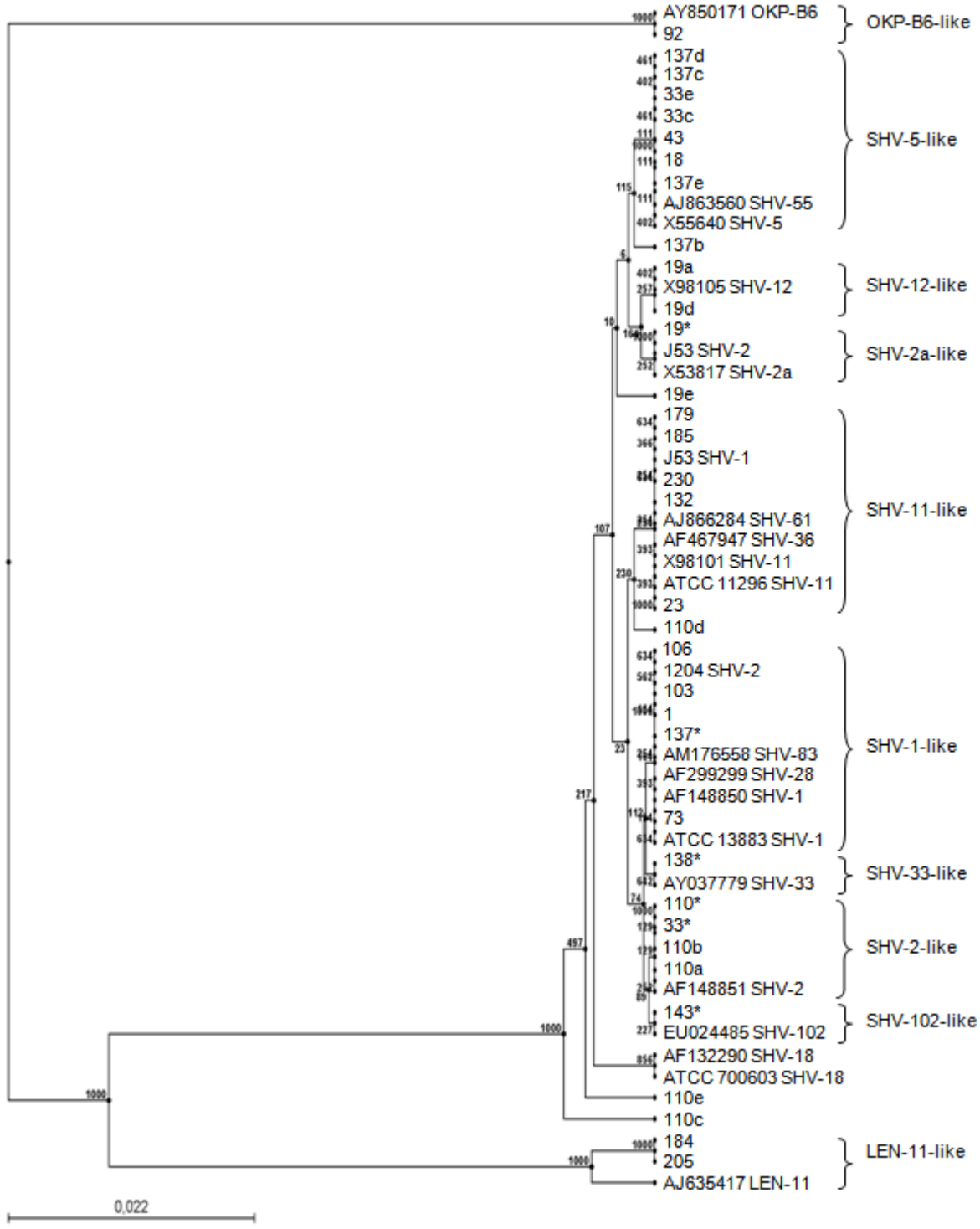

Figure 3: *Phylogenetic tree based on amino-acid sequences deduced from sequencing derived from 20 K. pneumoniae clinical isolates, reference strains (J53 SHV-2, J53 SHV-1, 1204 SHV-2, ATCC 11296 SHV-11, ATCC 13883 SHV-1, ATCC 700603 SHV-18) and retrieved SHV, LEN and OKP sequences (SHV-1 [Genbank:AF148850], SHV-2 [Genbank:AF148851], SHV-2a [Genbank:X53817], SHV-5 [Genbank:X55640], SHV-11 [Genbank:X98101], SHV-12 [Genbank:X98105], SHV-18 [Genbank:AF132290], SHV-28 [Genbank:AF299299], SHV-33 [Genbank:AY037779], SHV-36 [Genbank:AF467947], SHV-55 [Genbank:AJ863560], SHV-61 [Genbank:AJ866284], SHV-83 [Genbank:AM176558], SHV-102 [Genbank:EU024485], LEN-11 [Genbank:AJ635417] and OKP-B6 [Genbank:AY850171]) from Entrez Nucleotide forming distinct SHV, LEN and OKP-like cluster groups. In isolates marked with an asterix (*), the position of the allelic variation could not be defined and is indicated with X.*

respectively. Moreover, the SHV-like cluster group could be further divided into SHV-5, SHV-12, SHV-21, SHV-11, SHV-1, and SHV-2-like subgroups, respectively (Fig.3).

4. Discussion

Rapidly and easily acquired information on bacterial antimicrobial resistance genes is becoming increasingly important for diagnosis and decision-making when choosing suitable antimicrobial therapies for human infectious diseases. Due to the growing number of *Klebsiella* species that possess plasmid and chromosomally encoded ^{bla}SHV-enzymes, the traditional iso-electrofocusing technique appears not to be the method of choice for discriminating between a *K. pneumoniae* ^{bla}SHV, ^{bla}LEN and ^{bla}OKP-genotype any longer [9]. This creates new demands on clinical research and routine laboratories to develop molecular methods that can accurately identify and characterise ^{bla}SHV, ^{bla}LEN and ^{bla}OKP-genotypes. Moreover, it might be desirable to perform concurrent, multiple bacterial genotyping analysis from the same bacterial DNA pool, omitting tedious bacterial culture and DNA isolation procedures.

Bacterial DNA isolation is commonly carried out manually using time-consuming cartridge or magnetic bead-based techniques. These methods do not allow for an efficient high-throughput analysis of clinical isolates and significant variations of DNA yield and purity can be observed. By contrast, an automated nucleic extractor combined with multiple displacement amplification (MDA) of bacterial DNA yields DNA of high purity that can be used in multiple downstream applications. In recent years, MDA has been tried out for microbial and total DNA (bacterial and cellular) isolated from human biopsy specimens [20, 21, 23]. The present study shows the feasibility of using MDA-amplified bacterial DNA as a source in ^{bla}SHV, ^{bla}LEN and ^{bla}*OKP* genotyping analysis of *K. pneumoniae* clinical isolates having an ESBL-phenotype.

The use of M13-sequence tagged amplicons in combination with M13-specific primers has been described for sequencing of *Staphylococcus aureus* protein A-gene (Spa-typing) amplicons [24] Our results convincingly demonstrate that this approach when employing a

similar technique using SP6 and T7-specific sequencing primers for bi-directional sequencing of SP6 and T7-sequence tagged universal *bla*SHV-amplicons allowed for an unequivocal discrimination between *bla*SHV, *bla*LEN and *bla*OKP genes. The sequencing approach also confirmed that the apparent heterozygosity occurring at certain positions in the DNA-sequences were due to heterozygosity caused by the simultaneous presence of *bla*SHV allelic variants.

Based on PCR amplification alone it would be difficult, not to say impossible to distinguish between *K. pneumoniae* *bla*SHV, *bla*LEN and *bla*OKP genotypes if sequencing had not been performed. The necessity of obtaining complete sequences of *bla*SHV, *bla*LEN and *bla*OKP genes with appropriate primer-pairs was pointed out [10, 11, 19]. Moreover, the phylogenetic tree that was established is in agreement with a previous report describing three major groups of sequences corresponding to the *bla*SHV, *bla*LEN and *bla*OKP families of β-lactamase genes [9, 12, 13].

A different approach for the identification of *bla*SHV β-lactamases in nosocomial infection-associated *K. pneumoniae* has been described by Howard and co-workers [15]. In their study, a minisequencing-based discrimination assay was developed targeting polymorphism at the first bases of codons 238 and 240, allowing a reliable discrimination between *bla*SHV-2a and *bla*SHV-12 genes [15]. Later on, it was shown that sequencing of *bla*SHV amplicons gave a double peak in the electropherograms, suggesting the presence of more than one *bla*SHV-gene. Subsequently, fluorescence resonance energy transfer real-time PCR was used to detect single-nucleotide polymorphisms and the presence of two *bla*SHV-genes in the same isolate [25].

It is generally accepted that the great majority of *K. pneumoniae* isolates possess a chromosomal copy of either *bla*SHV-1 or *bla*SHV-11 genes, encoding non-ESBL enzymes, which differ from each other only at residue 35. In the SHV β-lactamase family, plasmid-

encoded ESBL activity is most frequently associated with a Gly238Ser substitution, and frequently increased ESBL activity is commonly associated with a Glu240Lys substitution [26]. The co-occurrence of chromosomal and plasmid-born β-lactamases may hamper their accurate molecular identification. To overcome this obstacle, a pyrosequencing-based technique was developed that allows typing of isolates carrying more that one *bla*SHV-gene. For that purpose, a set of PCR assays using specific primers encompassing amino-acid positions 35, 238 and 240 had to be performed [27]. Similarly, our study shows that that bi-directional amplicon sequencing allows rapid typing of *bla*SHV-genes differing at corresponding amino-acid positions 35, 238 and 240 in a single amplification and sequencing step. Moreover, we were able to detect a Pro226Ser substitution in the same amplicon (Table 1).

The conclusion to be drawn from the present study is that dual SP6 and T7-sequence tagged amplicon sequencing provides a reliable and powerful tool to distinguish between *K. pneumoniae* *bla*SHV, *bla*LEN and *bla*OKP genotypes which also signals co-occurrence of chromosomal and plasmid-born β-lactamases in *K. pneumoniae* clinical isolates having an ESBL-phenotype. The principle used could also be applied in any other situation where microbial resistance or virulence genes are to be sequenced.

Acknowledgments

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