Postprint version of article published as: Charnock, C., Samuelsen, Ø., Nordlie, A. L., & Hjeltnes, B. (2017). Use of a Commercially Available Microarray to Characterize Antibiotic-Resistant Clinical Isolates of Klebsiella pneumoniae. *Current Microbiology*, 1-10.

Use of a Commercially Available Microarray to Characterize Antibiotic Resistant Clinical Isolates of *Klebsiella pneumoniae*

Abstract

A commercially available microarray (IDENTIBAC AMR-ve) for the detection of antibiotic resistance determinants was investigated for its potential to genotype 30 clinical isolates and two control strains of *Klebsiella pneumoniae*. Resistance profiles and the production of extended spectrum β-lactamases were determined by disc diffusion and the results were compared with the microarray profiles in order to assess its scope and limitations. Genes associated with resistance to a wide-range of antibiotics, including current first line therapy options, were detected. In addition, the array also detected class 1 integrases. The array is easy to use and interpret, and is useful in providing a general description of the numbers and types of resistance determinants in *K. pneumoniae*. It also provides an indication of the potential for resistance gene acquisition. However, in most instances detected resistance to specific antibiotics could not unequivocally be assigned to hybridization with a specific array probe. We conclude that the microarray is a valuable and rapid means of investigating the presence of resistance gene-classes of therapeutic importance. It can also provide a starting point for selecting analyses of greater resolving power, such as phylogenetic subtyping by PCR-sequencing.

Introduction

Infections caused by drug-resistant bacteria are a major problem worldwide. Resistance to antimicrobial agents by members of the *Enterobacteriaceae* including *Klebsiella pneumoniae* is of particular concern. *K. pneumoniae* is encountered as a saprophyte in humans and other mammals, colonizing the gastrointestinal tract, skin, and nasopharynx [29]. It can cause a wide range of infections including those of the urinary and lower biliary tracts, as well as of surgical wounds and the blood stream. In recent years, *K. pneumoniae* has become established as an important cause of nosocomial infections, acquiring resistance to antibiotics through plasmid-encoded extended-spectrum β-lactamases (ESBLs), along with a variety of genes conferring resistance to other drug classes. The most prevalent ESBLs in *K. pneumoniae* are CTX-M enzymes belonging to Ambler class A [3]. In general, these enzymes have hydrolytic activity against penicillins, extended-spectrum cephalosporins (e.g. cefotaxime and ceftazidime) as well as monobactams (aztreonam), but are generally not effective against cephamycins (e.g. cefoxitin) and carbapenems [25]. The activity against ceftazidime is variable between the different CTX-M types and certain variants also have activity against 4th generation cephalosporins (e.g. cefepime) [10]. Since the initial isolation of CTX-M-1 from a European patient in the late 1980s [8], >170 CTX-M allelic variants have been identified [https://www.ncbi.nlm.nih.gov/projects/pathogens/beta-lactamase-data-resources/]. CTX-M variants can be divided into 5 major phylogenetic groups, CTX-M group 1, CTX-M group 2, CTX-M group 8, CTX-M group 9 and CTX-M group 25 on the basis of their amino acid sequences [32 and references therein].

Tzouvelekis et al., [29] noted that a successive addition of genetic elements encoding resistance to aminoglycosides and ESBLs, coupled with the rapid accumulation of chromosomal mutations conferring resistance to fluoroquinolones, left carbapenems as of around the year 2000, as the first-choice drugs for the treatment of health care-associated infections caused by *K. pneumoniae.* Subsequently there has been a rapid dissemination of multidrug-resistant (MDR) *K. pneumoniae* strains producing carbapenemases encoded by transmissible plasmids. The clinically most important carbapenemases include the class A enzymes of the KPC group, zinc-dependent class B metallo-β-lactamases, represented mainly by the VIM, IMP, and NDM types, and class D carbapenemases of the OXA-48 class [reviewed in 29]. Carbapenem-resistant *K. pneumoniae* (CRKP) have emerged as an important cause of morbidity and mortality from hospital-acquired and long-term care–associated infections [15, 20]. Few antimicrobial therapy options now exist for infections caused by CRKP [26].

The present study evaluates the potential for a commercially available DNA microarray to rapidly and correctly detect resistance genes of clinical importance in *K. pneumoniae.* Furthermore, the scopes and

limitations of the array technique in explaining the results from antimicrobial susceptibility testing were evaluated. The DNA-based assay [IDENTIBAC AMR-ve Genotyping kit Version 05; CLONDIAG/ALERE, GmbH] was developed and validated for the parallel detection of resistance and integrase genes in *Escherichia coli* and *Salmonella* [5], but has not been much used with *K. pneumoniae.* Card et al., [9] report the use of an extended array, containing many of the same probes as AMR-ve, in the typing of *K. pneumoniae* (34 strains distinct from those in the present study), and other *Enterobacteriaceae.* Other studies have screened respectively one [27] and two [31] strains of this species.

Materials and methods

Isolates and control strain

The strain collection consisted of 30 clinical *K. pneumoniae* isolates. Strains were supplied by the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (Tromsø, Norway). *K. pneumoniae* ATCC 700603 (SHV-18-β-lactamase producing) and *K. pneumoniae* ATCC 33495 (non-ESBL-producing) were used for control purposes.

Antibiotic resistance testing

Disc diffusion assays were performed and interpreted according to version 6.0 of the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org). In brief, susceptibility testing was done using Mueller–Hinton agar (Oxoid, Basingstoke UK). The inoculum was made from cells grown at 37 ± 1 °C for 18-24 h on tryptone soya agar (TSA, Oxoid) and adjusted to 0.5 McFarland. After applying antibiotic discs (Oxoid), plates were incubated at 35 ± 1 °C for 16-20 h prior to the reading of zone diameters. Based on zone diameters, strains were recorded as susceptible (S), intermediate susceptible (I) or resistant (R) to each antibiotic according to the guidelines.

The following antibiotics and concentrations were tested: ampicillin (AMP, 10 µg), amoxicillin/clavulanate (AMC, 30 µg), piperacillin/tazobactam (TZP, 30/6 µg), cefotaxime (CTX, 5 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 10 µg), cefuroxime (CXM, 30 µg), meropenem (MEM, 10 µg), ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), trimethoprim-sulphamethoxazole (SXT, 25 µg) and aztreonam (ATM, 30 µg).

Disc-diffusion screening for β-lactamase production

Phenotypic detection of ESBLs was performed on all strains using a double disc synergy test consisting of cefpodoxime 10 µg and cefpodoxime/clavulanic acid 10/1 µg (Combination kit DD0029; Oxoid). An increase of ≥ 5mm between the cefpodoxime and cefpodoxime/clavulanic acid discs was interpreted as a positive test according to the manufacturer's instructions. Based on indications from disc diffusion susceptibility and microarray testing, some isolates were also screened for the presence of metallo-β-lactamases and KPC enzymes (class A) using the KPC/MBL Confirm Kit (ROSCO, Denmark) and the ESBL and AmpC Screen kit (ROSCO) according to the manufacturer's instructions. All tests were performed using Mueller-Hinton agar (Oxoid) as described for the antibiotic susceptibility testing.

AMR-ve Microarray

A DNA-based assay (IDENTIBAC AMR-ve Genotyping kit Version 05; CLONDIAG/ALERE, GmbH) with 89 probes for different genetic markers including resistance gene families and integrases was used. The kit is able to detect ~57 antimicrobial resistance genes in Gram-negative bacteria, including several ESBLs. Basic details of the method and the probes used are given in the original publication [5] and the kit protocol (available at www.identibac.com). In brief, *K. pneumoniae* was grown for 18-24 h at 37 °C on TSA. Genomic DNA was isolated from a 1mm loopfull of cells (about 1×10^9 cells) using the DNeasy® Blood and Tissue set (Qiagen, Venlo, Netherlands) as previously described [12]. The DNA concentration and purity were determined using a Nanodrop-100 spectrophotometer (NanoDrop, DE, USA), and the DNA integrity and absence of intact RNA was checked by agarose electrophoresis. About 0.75 µg DNA was made up in 5 µl ultrapure molecular biology grade water (Sigma Aldrich) [concentration, 0.15 µg/µl]. PCR, biotin labeling, hybridization to arrays, washing and development of spots were performed as described in the AMR-ve protocol. Signal intensities were read within 10–15 min. of the final step (addition of buffer D1). Arrays were aligned in the reader as required by manual setting of array reference marker spots as described in the ATR03 2.0 installation and user guide. Results were exported, and the mean signal value for 3 replicate spots per probe was determined manually. Probes with intensity value ≥ 0.4 were considered positive (P in tables) whilst those <0.3 were considered negative. Values between 0.3 and <0.4 were considered ambiguous (A in tables).

Results

Overview of the results of microarray testing and the most common resistance determinants in *K. pneumoniae*

Fig. 1 provides a summary of the results from the microarray tests. Table 1 shows resistance phenotypes based on disc diffusion assays. All strains were positive for one or more genetic marker in the microarray. Among the 30 clinical isolates tested, hybridization with probes associated with resistance to the following antibiotics was obtained for one of more strains: β-lactams, sulphonamides, trimethoprim, tetracyclines, aminoglycosides, quinolones and chloramphenicol. In addition, hybridization with a probe indicating the presence of a class 1 integrase was obtained with 18 clinical isolates (60%) and both control strains. Thus the assay was able to detect simultaneously resistance markers to a wide-range of antibiotic groups of clinical importance for the treatment of infections caused by *K. pneumoniae*, as well as a genetic element associated with resistance-gene acquisition.

The most commonly detected resistance determinants

By virtue of a relatively large number of probes representing multiple antibiotic classes, the microarray was able to provide an overview of which resistance gene classes were common among the clinical isolates and control strains. The 5 most commonly detected resistance markers in the 30 clinical isolates were consensus-bla_{SHV} (β-lactams; 87%), *tetA* (tetracycline; 67%), *sul1* (sulphonamides; 60%), consensus-bla_{TEM} (β-lactams; 53%) and $bla_{CTX\text{-}M\text{-}1}$-like (β-lactams; 47%).

Resistance to β-lactams

All strains were resistant to ampicillin, 90% of the clinical isolates were intermediate susceptible or resistant to cefuroxime (2nd generation cephalosporin), and 90% were intermediate susceptible or resistant with respect to cefotaxime (3rd generation cephalosporin). All strains gave hybridization with one or more probes representing classes of resistance determinants to β-lactams. Four clinical isolates and the control strain ATCC 700603 did not produce hybridization with the consensus-$bla_{SHV\text{-}1}$-probe. However, these gave hybridization with other determinants of resistance to β-lactams, variously consensus-bla_{TEM} and/or one or more of the bla_{CTX} and bla_{OXA}-probes. Hybridization to the $bla_{\text{CTX-M-1}}$-like probe was common (Fig. 1), supporting the idea that the class A CTX-M β-lactamases are widespread among clinical isolates of the species. All strains possessing a $bla_{CTX\text{-}M}$ determinant were resistant to cefuroxime and cefotaxime.

Testing for β-lactamase production

Detection of ESBLs:

ESBLs were detected in isolates 5-9, 12-20, 22-26, and 30, but not in 21, 27-29. These results correlate well or are not excluded by the disc-diffusion and microarray-based tests (Table 1; Fig. 1).

KPC/MBL-kit (Rosco) and resistance to meropenems:

Based on the finding of resistance to meropenem, isolates 1-4 (Table 1), were chosen as relevant for screening for KPC (or other class A enzyme) and MBL (Ambler class B) production. Strains 1, 2 and 3 were shown to be MBL producers, whereas strain 4 did not possess an MBL. Strain 4 tested positive for KPC (or other Ambler class A enzyme). The finding of resistance/intermediate susceptibility to meropenem in these strains is thus supported by the confirmatory tests.

ESBL+AmpC-screen test (Rosco):

Isolates 10 and 11 hybridized with respectively probes detecting DHA and FOX/CMY resistance determinants (Fig. 1). These were the only strains where a plasmid-mediated class C β-lactamase was indicated by the array. In a previous array-based study of 29 *K. pneumoniae* clinical isolates, hybridization to the DHA- and FOX-probes was not found, and only 3 instances of hybridization to the CMY-probe was obtained. However, these genes were also only sporadically found in other species of *Enterobacteriaceae* [9]. In the present work, testing of strains 10 and 11 using the ESBL+AmpC screen, confirmed AmpC activity in isolate 10. The results were ambiguous with strain 11 perhaps owing to the presence of multiple β-lactamases (Fig. 1).

Resistance to tetracycline (*tet*)

Disc diffusion tests were not performed for tetracycline resistance as EUCAST zone diameter breakpoints are only validated for *Escherichia coli.* The most common determinant of resistance to tetracyclines detected was *tetA* (detected in 67% of clinical isolates). In addition *tetD* was detected in 3 isolates, whereas *tetB* was only detected in the control strain ATCC 33495.

Resistance to sulfonamides (*sul*) and trimethoprim (*dfr*)

Eighty-seven percent of the clinical isolates were resistant/intermediate susceptible to SXT. Hybridization to *sul1* was obtained for 60% of the clinical isolates and both control strains, whereas the corresponding number for *sul2* and *sul3* was 40% and 3%, respectively (Fig. 1). The four SXT susceptible strains lacked both *sul* and *dfr* (n=3) or only *dfr* (n=1). Consequently, the results of the phenotypic susceptibility testing are in accordance with the microarray findings. However, resistance to SXT was found in 5 strains producing hybridization with *sul* or *dfr* (but not both) and in one which did not hybridize to *sul* or *dfr* (Fig. 1; Table 1).

Resistance to the aminoglycoside gentamicin

Twelve of the 30 clinical isolates (40%) were resistant to gentamicin. The array has 7 probes dedicated to the detection of determinants of aminoglycoside resistance. Of these, Prob_aac3Ia_1, Prob_aac3IVa_1 (3-N-aminoglycoside acetyltransferases) and probe_ant2Ia_1 (aminoglycoside 2''-adenyltransferase) are generally associated with gentamicin resistance [19]. However, none of the clinical isolates produced hybridization with the two first-named probes. Hybridization with probe_ant2Ia_1 was obtained only with the control strain ATCC 700603 which also showed intermediate susceptibility to gentamicin (Table 1). Of the 12 resistant clinical isolates, 10 (83%) produced hybridization with probe Prob_aac6Ib_1 which is associated with an aminoglycoside 6'-N-acetyltransferase. In addition, 3 gentamicin-susceptible strains gave hybridization to this probe.

Resistance to the fluoroquinolone ciprofloxacin.

Sixty-three percent of the clinical isolates showed resistance or intermediate susceptibility to ciprofloxacin (Table 1). Of these 19 strains, only 68% hybridized with one or more of the *qnr*-probes associated with low-level resistance to fluoroquinolones.

Discussion

This study describes the first systematic and extensive application of the commercially available AMR-ve microarray to the determination of resistance determinants and integrases in clinical *K. pneumoniae* isolates. AMR-ve has been used to characterize one [27] and two [31] *K. pneumoniae* in other studies. Data from testing of a non-overlapping set of 29 clinical isolates and 5 control strains of *K. pneumoniae* using an extended version of the array containing many of the same probes has been reported [9] and comparisons are discussed below. The array was able to detect quickly and generally unambiguously, resistance determinants to all the major groups of clinically important antibiotics in the treatment of *K. pneumoniae.* Of the total of 265 individual probe hybridizations recorded for the 32 strains, only 7 reactions (< 3%) were recorded as ambiguous (given as yellow cells in Fig. 1).

The array requires 100 – 400 ng/µl DNA (total volume 5 µl) from a single clone for linear amplification prior to hybridization. This is, of course, less sensitive than a classical PCR approach where typically a few ng of template DNA is required. However, array-based approaches such as AMR-ve have the advantage of being able to detect many more genes (> 50 in the present study) than for example a standard multiplex PCR-analysis. Furthermore, as high as a 98.8% correlation between the array and PCR-data has been reported [5].

Salient details concerning the origins of the array probes and the resistance determinants they may reveal are given in Table 1 in the original publication describing the development of the array [5]. Amr-ve was originally developed for and validated against *Escherichia coli* and *Salmonella spp.* One indication of the applicability of AMR-ve to typing of *K. pneumoniae* comes from the observation in the present and previous work [9], that most of the array probes gave hybridization with one or more *K pneumoniae.* However, the following 13 probes did not hybridize with any strain in either study: *tetE, tetG* (associated with tetracycline resistance); *aadA4, aac(3)-Ia, aac(3)-IV* (associated with aminoglycoside resistance); *dfrA7, dfrA17* (associated with resistance to trimethoprim), bla_{MOX}, bla_{ACC-1}, bla_{OXA-7}. bla_{CTX-M2}, bla_{CTX-M8}, $bla_{CTX-M-26}$ (associated with reistance to β-lactams). This and other concordances between the present and previous work on non-overlapping sets of strains, probably reveals important information on the typical genetic makeup of drug-resistant *K. pneumoniae.* However, in cases of non-hybridiaztion probe suitability might also be an issue. To assess this we compared probe sequences [5] to those in the GenBank® database using the BLAST® search engine [2]. The search was restricted to generally curated *K. pneumoniae* DNA and the resistance gene in question. Of the 13 array-probes which did hybridize to any strain, we found in the database several instances of 100% sequence

identity over the whole probe length for all but *tetE* (probe length 24 bp; sequence identity only over 15 bp). The *tetC*-probe which did not hybridise with any of the strains investigated in the present study gave similar results to *tetE* (probe length 24 bp; sequence identity only over 14 bp). However, in the study of Card et al., [9], 17% of clinical isolates produced hybridization to this probe. More data is desirable in order to evaluate the limitations of these probes in the investigation of resistance to tetracycline in *K. pneumoniae.*

In addition to the detection of a wide-range of resistance determinants to antibiotic classes with relevance to the treatment of infections caused by *K. pneumoniae,* the array also detected class 1 integrases in 60% of the clinical isolates and both control strains. This is similar to the value of 83% for other clinical isolates of the species [9] and 74% reported for multidrug resistant *K. pneumoniae* and *Escherichia coli* in Iran [28]. Furthermore, in a study of 136 clinical isolates of *K. pneumoniae* in India, 52 of 63 ESBL-producing isolates (83%) contained a class 1 integrase [6]. The finding of a significant distribution of class 1 integrases in the present and other studies, supports the notion that a potential for resistance gene acquisition is common in clinical isolates of this species. Some of the studies referenced above [6, 28] used multiplex-PCR-based procedures for the detection of integrases. The present study shows that similar screening can be done effectively with the AMR-ve approach.

The most commonly indicated resistances were bla_{SHV} (87% clinical isolates), *tetA* and *sul1*. The bla_{SHV} gene is chromosomally located in *K. pneumoniae* [13] and it has been suggested that SHV enzymes approach ubiquity in *K. pneumoniae* [4]. The finding that the majority of clinical isolates produced hybridization with bla_{SHV} is thus in keeping with previous work. However, the bla_{SHV}-probe included on the array is a consensus sequence for bla_{SHV}-genes and this limitation of scope is discussed further below. It has been reported that *tetA* encoded efflux pumps are widespread among *Enterobacteriaceae* [32], but there is limited data for *K. pneumoniae*. In an analysis of 30 *K. pneumoniae* causing urinary tract infections in Iran [7], all of the isolates tested positive for *tetA* and *tetB*. This is in line with the present array results. However, in contrast to the Iranian strains, the present study strongly suggests that *tetA* (67% Norwegian clinical isolates) is more common than *tetB* (0%) in Norway. In their study of 29 isolates, Card et al., [9] found that only 21% of *K. pneumoniae* hybridized with *tetA*, again suggesting significant clonal differences with respect to this gene. Alternatively the low rate of detection of *tetB* might be due to mutations/mismatches in the area of the probe. This could be investigated further by sequencing studies.

β-lactam antibiotics are the most important agents in the therapy of *K. pneumoniae* infections, and the array's standard set of probes is suitably dominated by those detecting determinants of resistance to these

antibiotics. The assay has only a single, consensus probe for *bla*$_{SHV}$ genes. SHV-enzymes can, however, be both narrow spectrum and extended-spectrum (ESBLs), depending on the subtype in question [17, 18]. Concordances between hybridization with Prob_shv1_11 and measured resistances to cephalosporins and aztreonam illustrate some of the limitations of the AMR-ve system: ten of the strains tested gave hybridization with only Prob_shv1_11 from among the array's set of probes for β-lactam resistance genes (Table 2). If SHV-enzymes are responsible for the β-lactam resistance phenotypes shown in Table 2, the array is unable to distinguish between what is probably a classical narrow-spectrum enzyme in the control strain ATCC 33495, and the broad-spectrum enzymes present in for example strains 8 and 14. Such a differentiation (subtyping) which is most useful to the researcher can be accomplished by the PCR-sequencing approach, but is a limitation of the array in its present form. In contrast to the single determinant for SHV-enzymes, the array contains several probes hybridizing to *bla*$_{CTX-M}$-genes. For 14 clinical isolates, positive signals were obtained with Prob_ctxM1_11. This probe detects among others the phylogenetic group CTX-M-15 which is currently the most widely disseminated CTX-M type in the world [21]. Antibiotic resistances assayed by the disc diffusion assay were in general agreement with the expected effects of the presence of *bla*$_{CTX-M}$- genes (Table 1; Fig. 1). CTX-M-type ESBLs can exhibit activity against monobactams and extended spectrum cephalosporins, including cefotaxime and ceftriaxone, but are classically less effective against ceftazidime, cephamycins (e.g. cefoxitin) or carbapenems [3]. Thus, the finding that the majority of clinical isolates (87%) were resistant or intermediately susceptible to ceftazidime (Table 1) is somewhat surprising. However, the classical pattern of resistance conferred by CTX-M is not universal since mutations can alter the specificity of CTX-enzymes [14]. Some CTX-M sub-types (e.g. CTX-M-15) have greater catalytic efficiency against ceftazidime [8]. This sub-type is for example detected along with others by the array ctxM1_11 probe. Given this, a more stringent probe, if practically feasible, directed at ceftazidime resistance related CTX-M sub-types would be useful. Incidences of ceftazidime resistance/ intermediate susceptibility in 11 clinical isolates (Table 1) not showing hybridization with the ctxM1_11 probe, could potentially also be explained by mutations in common plasmid-mediate SHV (or TEM) genes producing ESBLs [8]. Over-production of SHV-enzymes (all 11 isolates produced hybridization with the *bla*$_{SHV}$-probe) and/or changes in outer-membrane protein changes have also been associated with reduced susceptibility to ceftazidime in *K. pneumoniae* [22, 23].

Carbapenems are in some instances the last therapeutic options for treatment of infections by *K. pneumoniae.* It is, therefore, pertinent to look more closely at the results for the 4 strains (strains 1-4; Table 1) which showed meropenem resistance/intermediate susceptibility. Although it includes probes detecting various

classes of Ambler A and C enzymes, the AMR-ve system is not equipped to detect determinants of class B enzymes. Thus the array is not able to provide support for the finding of MBLs (detected phenotypically) in strains 1, 2 and 3. Furthermore the array does not detect bla_{KPC} which would be useful given that KPC activity was detected for strain 4.

Plasmid-mediated resistance to sulphonamides and trimethoprim is normally due to the acquisition of dihydropteroate synthases for sulphonamides and dihydrofolate reductases for trimethoprim. Three resistance genes, *sul1*, *sul2* and *sul3* encoding dihydropteroate synthases and more than 20 dihydrofolate reductase (*dfr*) genes have been described [16]. Both groups of genes are associated with class 1 integrons residing on plasmids and/or the chromosome [16]. The array shows 100% correlation between susceptibility to SXT and the absence of hybridization to *dfr* and/or *sul* probes. However, 5 SXT-resistant strains did not produce hybridization with either one or both of *sul* and *dfr* probes, possibly indicating the presence of other resistance determinants/mechanisms not detected by the microarray. In summary, the microarray was able to explain sensitivity but not always resistance to SXT.

The present study suggests that an aminoglycoside 6'-N-acetyltransferase may be associated with gentamicin resistance in the current panel of strains: thirteen clinical isolates produced hybridization with this probe (Fig. 1). Of these, 77% showed resistance to gentamicin (Table 1). Similarly, in a previous study of *K. pneumoniae* isolates [9], it was found that 76% of strains producing hybridization to the probe were also resistant to gentamicin. However, although AAC(6')-Ib is the most prevalent aminoglycoside-modifying enzyme known, and is present in over 70% of AAC[6']-producing Gram-negative isolates [30], group I type 6'-N acetyl transferases are not typically agents of resistance to gentamicin [19, 30]. In one study of carbapenem-resistant *K. pneumoniae*, 98% of strains tested possessed *aac(6')-Ib* [1]. However, this gene was associated with resistance to tobramycin and not to gentamicin. In addition, about 94% of the strains that carried *aac(6')-Ib* as the only determinant of an aminoglycoside-modifying enzyme were susceptible to gentamicin [1]. The authors thus proposed that *aac(6')-Ib* in association with other aminoglycoside resistance genes could explain the high gentamicin MIC values found for some strains. Furthermore, they suggest that the widespread presence of *aac(6')-Ib* in *K. pneumoniae* could be a consequence of it being on the same plasmids as those carrying ESBL genes [1]. In the present study, the four clinical isolates which did not produce ESBLs (phenotypic tests) also failed to give hybridization to Prob_aac6Ib_1, whereas every strain producing hybridization with Prob_aac6Ib_1 was also an ESBL producer. Thus the array test provides some support for the proposed presence of *aac(6')-Ib* and ESBL-genes on the same plasmids [1]. Notwithstanding, it should be noted that some *aac(6')-Ib* subvariants

389 *are* known to be associated with gentamicin resistance. Among the recent variants of this enzyme with altered
390 specificity are AAC(6')-Ib11, which confers simultaneous resistance to gentamicin and amikacin [11] and
391 AAC(6')-Ib-cr, which has a unique extension of its substrate specificity from aminoglycosides to structurally
392 unrelated fluoroquinolones [24]. If feasible, it would be useful to include additional probes in order to ascertain
393 if the apparent correlation between gentamicin resistance and hybridization to Prob_aac6Ib_1 - probe aac (6')-Ib
394 in the present study, indicates the presence of gene sub-types conferring gentamicin resistance
395 Of the 30 clinical isolates tested, 19 showed resistance or intermediate resistance to ciprofloxacin. Of
396 these, 32% showed resistance to ciprofloxacin without giving hybridization with probes associated with
397 resistance to quinolones. However, *qnr* genes are known to only give low-level resistance to quinolones.
398 Quinolone resistance is mainly caused by chromosomal mutations in *gyrA* and *parC*, and the contribution of a
399 *qnr*-gene can be additive to other resistance factors. The microarray is thus useful in its ability to detect *qnr* but
400 unsurprisingly it is not predicative of resistance to ciprofloxacin.
401 Susceptibility to chloramphenicol was not investigated in the present study. AMR-ve hybridizations
402 indicated that the most common determinants of resistance to this antibiotic were chloramphenicol
403 acetyltransferases (CatB3; CatA1). Genes connected with efflux proteins (FloR; CmlA) were also detected, but
404 in fewer strains (Fig.1). The same tendency was seen in a previous study of clinical isolates [9]. In the referenced
405 work, all strains producing hybridization with the *catA1*-probe and/or the *cmlA*-probe were also resistant to
406 chloramphenicol. Thus hybridization with these probes seems to be a good indicator of the resistance phenotype.
407 Summing up the results, the present study evaluates the usefulness of a commercially available
408 microarray, AMR-ve, for the detection of clinically important resistance determinants and integrases. The assay
409 was able to detect genetic determinants of integrases and a wide-range of antibiotic classes of relevance for
410 treatment of infections caused by *K. pneumoniae.* Furthermore the test was easy to perform and the results were
411 only rarely difficult to interpret ('ambiguous'). AMR-ve functions well in the genotyping of clinical isolates of
412 *K. pneumoniae* and provides a useful start point for further testing. However, the assay was shown to be too
413 restricted in scope to explain many important measured resistances to β-lactams (e.g. to carbapenems) and
414 aminoglycosides in *K. pneumoniae*. The producer of the AMR-ve has recently launched a new CarbDetect AS-1
415 Kit for multidrug-resistant Gram-negative bacteria which runs on the same platform. This test allows DNA-
416 based detection of amongst others the most important carbapenemase genes (e.g. $bla_{\text{OXA-23}}$, bla_{KPC}, bla_{NDM},
417 bla_{VIM}) as well as other prevalent β-lactamases (e.g. $bla_{\text{CTXM-1/15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$). It also contains species-
418 specific probes for identification of *K. pneumoniae.* Thus this new system supplies some of the information

needed to bridge gaps between genotyping and resistance testing reported as weaknesses of the AMR-ve in the present work. Additionally, whole genome sequencing studies are becoming an option for more laboratories and are offered commercially. These can reveal allelic forms of genes and point mutations which are often specifically associated with resistance to a particular antibiotic. The current price for the AMR-ve Genotyping kit is about 1500 EUR for 50 tests (about 30 EUR per bacterium). A single analyst in our laboratory was able to test about 5-10 strains per day with the AMR-ve. There are now many commercial agents offering whole genome sequencing at about 300-400 EUR for a single strain with lower prices for bulk orders (including DNA purification from supplied cells, libraries and bioinformatics) with a turnaround of 3-4 weeks or less. Thus the total prices for the two approaches are near parity.

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Table 1 Antibiotic resistance profiles of thirty clinical isolates and two control strains of *Klebsiella pneumoniae*

Antibiotic	Strain designation and resistance phenotype[a]																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	ATCC 700603	ATCC 33495
AMP	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
AMC	R	R	R	R	R		R		R	R	R	R		R							R	R		R					R		
TZP	R	R	R	R	R		I		R	R	R	I		R							R			I			I		R		I	
CTX	R	R	R	R	R	R	R	R	R	I	R	R	R	R	I	R	R	R	R	R		R	R	R		R		I	R	R	I	
FOX	R	R	R	R							R						R	R					R				R	R	R		R	
CAZ	R	R	R	R	R		R	R	R	R	R	R		R	I	R		R	R	R	I	I	R	R	I	I		I		I	R	
CXM	R	R	R	R	R	R	R	R	R	R	R	R	R	R		R	R	R	R	R		R	R	R	R	R		R	R	R	R	
MEM	R	R	I	R																												
ATM	R	R	R	R	R	I	R	R	R	I	R	R	I	R		R	R	R	R	R		R		R		R			R	R	R	
CIP	R	R	R	R	R		I		R	R	I	R	I		I	I			R	I		R		I		R	R					
CN	R	R			R	R	R		R	R	R	R	R									R		R							I	
STX	R		R	R	R	R	R		R	R	R	R	R	R	R	R	I	R	R	R		R	R	R	R	R	R		R	R	I	

[a] (R) indicates clinical resistance and (I) indicates intermediate susceptibility. A blank entry in the table indicates full susceptibility (S) to a particular antibiotic,

Table 2 Overview of resistance phenotypes of strains producing hybridization with either the SHV-1 or the TEM-1 probe and no other determinant of resistance to β-lactams (derived from Table 1)

Strain	Probe hybridizations	Penicillins	Cephalosporins/ Cephamycins	Monobactams (Aztreonam)	Carbapenems (Meropenem)
3[a]	Prob_shv1_11	AMP, AM/C TZP	FOX, CXM CTX, CAZ,	ATM	MEM (I)
8	Prob_shv1_11	AMP	CXM, CTX,CAZ	ATM	-
14	Prob_shv1_11	AMP, AM/C, TZP	CTX, CAZ, CXM	ATM	-
15	Prob_shv1_11	AMP	CTX(I), CAZ(I)	-	-
23	Prob_shv1_11	AMP	FOX, CXM CTX, CAZ,	-	-
25	Prob_shv1_11	AMP	CXM, CAZ(I)	-	-
26	Prob_shv1_11	AMP	CTX, CAZ(I), CXM	ATM	-
27	Prob_shv1_11	AMP, TZP(I)	FOX	-	-
28	Prob_shv1_11	AMP	FOX, CTX(I), CAZ(I) CXM,	.	.
ATCC 33495	Prob_shv1_11	AMP	-	-	-
6	Prob_tem1_1	AMP	CTX CXM	ATM(I)	-

[a] MBL detected by phenotypic tests

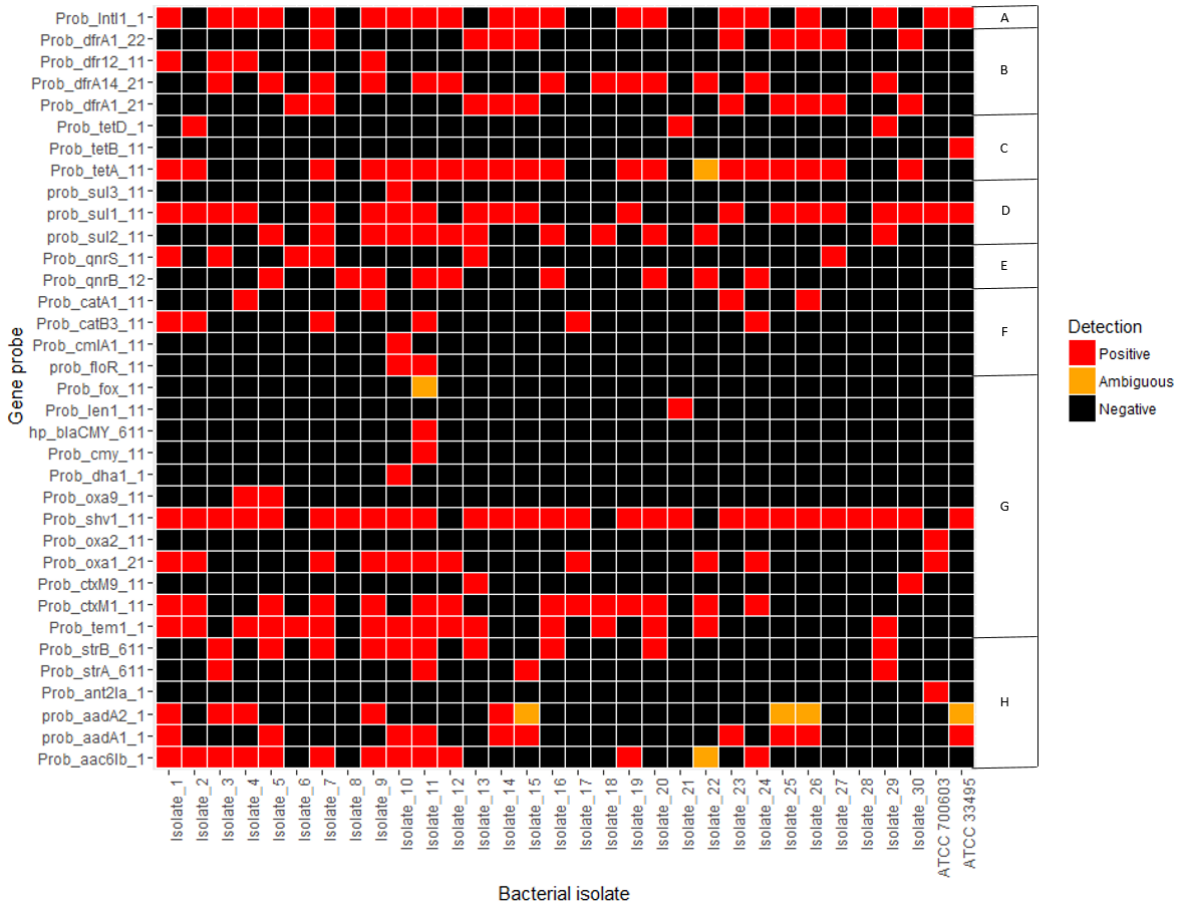

Fig 1. Heat map showing the pattern of probe hybridizations for 32 strains of *Klebsiella pneumoniae*. Only probes giving hybridization to one or more strain are shown.
Key: A – probe associated with class 1 integrases. B – trimethoprim. C – tetracyclines. D – sulphonamides. E – quinolones/fluoroquinolones. F – chloramphenicol. G – β-lactams. H - aminoglycosides