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# Direct Detection and Genotyping of KPC Carbapenemases from Urine using a new DNA Microarray Test

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**Running Title:**

KPC variant genotyping chip

**Abstract:**

*Klebsiella pneumoniae* carbapenemases (KPC) are considered a serious threat to antibiotic therapy as they confer resistance to carbapenems, which are used to treat Extended-spectrum beta-lactamase (ESBL) producing bacteria. Here, we describe the development and evaluation of a DNA microarray for detection and genotyping of KPC genes ($bla_{KPC}$) within 5 hours. To test the whole assay procedure (DNA extraction + DNA microarray assay) directly from clinical specimen, we compared two commercial DNA extraction kits (QIAprep Spin Miniprep Kit (Qiagen), Urine Bacterial DNA Isolation Kit (Norgen)) for the direct DNA

26 extraction from urine samples (dilution series spiked in human urine). A reliable SNP typing
27 from $1x10^5$ CFU/mL urine was demonstrated for *Escherichia coli* (Qiagen and Norgen) and
28 80 CFU/mL urine on average for *K. pneumoniae* (Norgen). The study presents for the first
29 time the combination of a new KPC-microarray with commercial sample preparation for the
30 detection and genotyping of microbial pathogens directly from clinical specimen which paves
31 the way towards tests providing epidemiological and diagnostic data enabling better
32 antimicrobial stewardship.

33 **1 Introduction**

34 Increasing prevalence of carbapenem resistant *Enterobacteriaceae* (CRE) is a growing public
35 health concern (23, 29, 32). Especially resistances conferred by the *Klebsiella pneumoniae*
36 carbapenemase (KPC) are an emerging problem of significant clinical importance (24). KPC
37 enzymes are class A beta-lactamases, which confer resistances to penicillins, cephalosporins,
38 monobactams and carbapenems (16). KPC was first identified in a multidrug-resistant
39 *Klebsiella pneumoniae* isolate from a hospital in the USA in 1996, but has spread since then
40 worldwide and to other Gram-negative species like *Escherichia coli* or *Acinetobacter*
41 *baumannii* (16, 34). Due to a lack of new antibiotics, there is only a limited number of
42 treatment options left against carbapenemase producing bacteria, such as the last-line drugs
43 polymyxin B or colistin, which however have been associated with high rates of
44 nephrotoxicity (15). In addition, recent reports even show the appearance of KPC-producing
45 *K. pneumoniae* that are also resistant to colistin (2, 19, 41).

46 In order to reduce and control further spread of carbapenem resistance, a rapid identification
47 is crucial, so appropriate treatment can be applied (17). Classical microbiological methods are
48 often slow and only give results after an additional cultivation for 24 or even 48 hours (23,
49 33). To address this problem a variety of molecular methods have been developed, such as
50 PCR or real-time PCR assays detecting carbapenemase genes (3, 5, 10, 12, 18, 31). PCR

51 based methods are a lot quicker than microbiological methods and can give results within a
52 few hours. However, these methods lack the possibility to detect single-nucleotide-
53 polymorphisms (SNPs), which is helpful for detailed outbreak investigation and
54 epidemiological studies. There are 11 KPC variants published currently (KPC-2 to KPC-12),
55 which differ only in single point mutations from each other. For each variant slightly different
56 carbapenem MIC values and efficacies of beta-lactam inhibitors like clavulanic acid have
57 been observed. Especially KPC-2 and KPC-6 seem to confer resistance to all carbapenems,
58 whereas other variants show less activity against imipenem or meropenem (35, 45). To
59 identify all different variants from each other, sequencing is the gold-standard, but this
60 method is very time-consuming and yet too demanding for routine clinical diagnostics. An
61 alternative method is the use of DNA microarrays, which allows rapid identification of SNPs
62 and a parallel detection of several resistance genes (4, 6, 13, 14, 21, 22, 40, 44, 47). However,
63 the currently described methods for KPC gene detection (Check-MDR CT101, CT102,
64 CT103 and Check-KPC EBSL Checkpoints health BV Wageningen, Netherlands; hyplex®
65 SuperBug ID test system Amplex Biosystems GmbH, Gießen, Germany) do not allow a
66 differentiation between the different KPC variants. Here we report the development and
67 evaluation of a new DNA microarray, which is capable of SNP detection, allowing an
68 identification of all variants from KPC-2 to KPC-11 directly from urine samples without prior
69 growth in culture.

70 **2 Materials and Methods**

71 The new KPC microarray was designed to run under the same conditions as our previously
72 developed ESBL microarray (14). We evaluated the performance of this new microarray on
73 characterised reference strains and analysed its detection limit. We further tested the
74 performance of the microarray to identify KPC variants directly from urine samples without
75 further cultivation. For this we used two different DNA extraction kits, the QIAprep Spin

76 Miniprep Kit (Qiagen) and the Urine Bacterial DNA Isolation Kit (Norgen) and validated
77 their performance in combination with the KPC microarray. Urine samples which were spiked
78 with different dilutions of *E. coli* or *K. pneumoniae* reference strains, carrying different
79 variants of $bla_{\text{KPC}}$, were used as testing material.

80 **2.1 Reference strains**

81 Twelve well characterized reference strains carrying $bla_{\text{KPC-type}}$ genes were used for the
82 development and validation of the microarray probes and primers: *E. coli* (KPC-2), *K.*
83 *pneumoniae* (KPC-2) and (KPC-3) from the Robert Koch Institute, Wernigerode, Germany
84 (30) and *K. pneumoniae* (KPC-3) from the Health Protection Agency, UK (46). Three strains
85 *K. pneumoniae* VIN, AUB, GOU (KPC-2) were provided by the Hopital Paul Brousse, France
86 (11) and another five strains VA 367 (KPC-3), VA 375 (KPC-3), VA 361 (KPC-2), VA 184
87 (KPC-2), VA 406 (KPC-2) were provided by Prof. Dr. Bonomo from the Louis Stokes
88 Cleveland Department of Veterans Affairs Medical Center (7). All isolates were cultivated at
89 37 °C in Lauria-Bertani (LB) Media.
90

91 **2.2 Spiking of urine samples and DNA extraction**

92 Non infected urine samples (tested by routine microbiological culture) from several patients
93 (New Royal Infirmary, Edinburgh) were pooled and subsequently spiked with reference
94 strains carrying variants of $bla_{\text{KPC}}$. For an accurate determination of the limit of detection
95 (LOD) dilution series of bacteria were produced in urine, covering a range from 1 - $10^9$
96 CFU/mL urine in 11 dilutions steps. The number of bacteria in each dilution step was
97 determined via counting of colonies on LB-Agar plates in duplicates. CFU numbers, which
98 were too large to be counted were extrapolated from the lower concentrations. Dilution series
99 were carried out for all three strains received from the Robert Koch Institute: *E. coli* (KPC-2),
100 *K. pneumoniae* (KPC-2) and *K. pneumoniae* (KPC-3). After spiking of the urine samples each

101 tube was mixed and set aside at room temperature for 30 min. Before DNA extraction
102 procedures were applied 100 µl of each dilution step were used to determine the exact number
103 of CFU/mL urine, by plating onto LB agar.
104 During DNA microarray development, plasmid DNA from each clinical isolate was extracted
105 from 2 ml overnight culture using the QIAprep Spin Miniprep Kit (Qiagen, Hilden,
106 Germany). For the detection study from clinical specimen, plasmid DNA from spiked urine
107 samples was extracted from 1.7 mL of urine using the QIAprep Spin Miniprep Kit (Qiagen)
108 or the Urine Bacterial DNA Isolation Kit (Norgen, Thorold, Canada) both applied according
109 to the manufacturer's instructions.
110

111 **2.3 Target DNA preparation**

112 The target DNA used for the hybridization onto the oligonucleotide microarrays was
113 synthesized via PCR. The primers used for the amplification of the $bla_{KPC}$ gene were the
114 forward primer KPC_PR_F1 (5'-TGTCACTGTATCGCCGTG-3') (48) and the reverse
115 primer KPC_PR_R2 (5'-TTGACGCCCAATCCCT-3'), developed as part of this study. The
116 amplicon was expected to be 871 bp in length. The amplification and labelling of $bla_{KPC}$ took
117 place in a total reaction volume of 30 µl using the following reagents: 0.4 µM of each primer,
118 1x *Taq* buffer, 1 mM $MgCl_2$, 3 U of Hotstar *Taq* polymerase (Qiagen, Hilden, Germany), 0.1
119 mM dATP, dGTP, dTTP, 0.06 mM dCTP and 0.04 mM Cy3-dCTP (Fisher Scientific,
120 Leicestershire, UK). The reactions were carried out on a Techne TC-512 thermocycler
121 (Keison Products, Essex, UK) using the following programme: An initial denaturing and
122 activation step at 95 °C for 15 min followed by 40 cycles consisting of 30 s denaturing at 94
123 °C, 30 s annealing at 54 °C and 1 min elongation a 72 °C, followed by a final extension step
124 at 72 °C for 10 min. The PCR product was purified using the Qiaquick Spin PCR purification
125 kit (Qiagen) following the standard instructions and a final elution in 30 µl $ddH_2O$. The DNA

126 yield and rate of Cy3-dCTP incorporation, expressed as the quotient of the number of
127 nucleotides and the number of incorporated fluorescent dyes (NT/F), was determined by
128 measuring the absorption at 260 and 550 nm (ND-1000 spectrophotometer, Nanodrop
129 Technologies, Rockland, USA). Directly before hybridization the labelled target-DNA was
130 fragmented for 5 min at room temperature using 0.8 mU DNase I (Promega, Mannheim,
131 Germany) for each ng DNA in a total reaction volume of 40 µl containing 1x DNase buffer.
132 The reaction was stopped through addition of 3 mM EGTA and incubation at 65 °C for 10
133 min. The fragmentation efficiency was estimated by capillary gel electrophoresis using a
134 DNA 1000 LabChip kit (Bioanalyser 2100, Agilent, Böblingen, Germany).
135

136 **2.4 Oligonucleotide microarray fabrication**

137 The following protocol is based on our previously published array production methods (14).
138 All oligonucleotide capture probes were purchased from Metabion (Martinsried, Germany)
139 and diluted to a final concentration of 20 µM in spotting buffer (Nexterion Spot I and Spot III,
140 in a 1:3 ratio). Each probe had an 11-thymidine spacer and an amino modification at the 5'-
141 end. Using a contact printer (MicroGrid II, Biorobotics, Cambridge, United Kingdom) with
142 split pins (Biorobotics, MicroSpot 2500) each probe was spotted in triplicates onto epoxy-
143 coated slides (Nexterion Slide E, Schott, Jena, Germany). A total of 4 arrays were printed per
144 slide. In order to immobilize the probes after spotting, the slides were incubated for 30 min at
145 60 °C in a drying oven (Memmert, Schwabach, Germany). At this stage the slides could be
146 stored for several months. Before hybridisation the slides were rinsed 5 min in 0.1% (v/v)
147 Triton X-100, 4 min in 0.5 µL of concentrated HCl per mL of dd$H_2O$, 10 min in 100 mM
148 KCl, and finally 1 min in dd$H_2O$. Subsequently, the slides were blocked for 15 min at 50 °C
149 in blocking solution containing 0.3% (v/v) ethanolamine in 100 mM Trizmabase adjusted to
150 pH9 with HCl. Finally they were rinsed for 1 min in dd$H_2O$ and spun dry at 1300 rpm for 2

151 min in an Eppendorf centrifuge 5810 R (Eppendorf AG, Hamburg, Germany), equipped with
152 swing-bucket rotor adapters for 96-well plates using a metal slide rack (Lipshaw, Detroit,
153 USA). In addition to *$bla_{KPC}$* specific probes several control probes were included on each
154 array. These were: a pre-labelled spotting control (5'-TTTTTTTTTTTT
155 CTAGACAGCCACTCATA-cyanine3 [Cy3]-3'); a positive hybridization control (5'-
156 TTTTTTTTTTTGATTGGACG AGTCAGGAGC-3'), complementary to a labelled
157 oligonucleotide target (5'-Cy3-GCTCCTGACTCGTCCAATC-3'), which was spiked during
158 hybridization; and a negative control (5'-TTTTTTTTTTTCTAGACAGC CACTCATA-3').
159 All control sequences were derived from *Arabidopsis thaliana* and are very distant from any
160 target sequence found in bacteria. Spotting controls were spotted at every corner of each
161 subarray (10 µM), whereas positive and negative controls were distributed alternately along
162 the sides of each sub-array.
163

164 **2.5 Hybridization and Washing**

165 For the analysis of KPC strains 100 ng target DNA were used for hybridization onto each
166 microarray. In case of the dilution series the total amount of target DNA received from the
167 labelling-PCR was used for hybridization (28 µL), ranging from 1 – 1600 ng DNA. For
168 hybridization the target DNA was supplemented with 0.2 pmol of oligonucleotide
169 complementary to the positive hybridization control in 100 µl with 2xSSPE (20x SSPE: 3 M
170 NaCl, 200 mM $NaPO_4$, 20 mM EDTA, pH 7.4) and 0.01% SDS. The hybridization was
171 performed in an Agilent microarray hybridization chamber using gasket slides to cover the
172 microarray, incubating for 1 h at 47 °C in an Agilent hybridization oven at 6 rpm (Agilent
173 Technologies, USA). After hybridization the slides were washed at room temperature for 10
174 min each in 2xSSC (20xSSC: 3 M NaCl and 0.3 M sodium citrate) with 0.2% SDS, 2xSSC
175 and 0.2xSSC. Subsequently, the slides were dipped in $ddH_2O$ for less than 2 s and spun dry at

176 1300 rpm for 2 min in an Eppendorf centrifuge 5810 R. At this point the slides could be
177 stored at room temperature until scanning.
178

179 **2.6 Image acquisition and data analysis**

180 After hybridization the fluorescent signals were acquired with a Tecan LS Reloaded laser
181 scanner (Tecan Austria GmbH, Grödig, Austria) at 532 nm and a 575 nm Cy3 filter. Each
182 slide was scanned with 3 different photomultiplier tube (PMT) gain settings (150, 180 and
183 200), using a resolution of 10 µm. The quantification of signal intensities was performed
184 using QuantArray (QuantArray Analysis Software, Packard BioChip Technologies, Billerica,
185 USA) followed by data analysis and processing in Microsoft Excel (Microsoft, Redmond,
186 USA). First the local background of each spot was subtracted from the raw spot intensity
187 value, followed by the calculation of the mean net signal intensity (NI) and standard deviation
188 (SD) of the three replicates. Within each probe set (probes interrogating one mutation site) the
189 probe with the highest signal intensity was termed perfect match (PM) whereas the remaining
190 probes were marked as mismatch (MM). In order to evaluate the performance of each probe
191 set, the ratios between the MM and PM signal intensities were calculated. The larger the
192 relative difference between MM and PM signal, the better the discriminative power of the
193 probe set. The MM probe with the highest signal intensity was used for the calculation of the
194 relative signal intensity ($RI_{max(MM)} = NI_{max(MM)} / NI_{PM}$). Only probe sets that showed a
195 performance with $RI_{max(MM)} < 0.7$ were used for the analysis. The use of this threshold has
196 been proven to result in high quality discriminations (9, 14). In addition to the RI value, the
197 limit of detection (LOD) was used to evaluate the performance of probe sets. The LOD was
198 calculated based on the maximum signal intensity ($NI_{max}$) obtained within each probe set
199 based on a no template control (NTC) hybridization plus 3 times the highest standard
200 deviation ($LOD = NI_{max} + 3 \times SD_{max}$). Only probe sets with a perfect match signal intensity

above the limit of detection ($NI_{PM}$ > LOD) were used for analysis. In addition the CV was calculated for each set of replicate probes (CV = SD / $NI_{PM}$). Probe sets with a CV > 30% were flagged and excluded from analysis to ensure that only probe signals with a high reproducibility were used for the analysis. The correct *bla*$_{KPC}$ variant was then identified based on the combination of all valid perfect match signals. The KPC variants and their single nucleotide polymorphisms (SNPs) used for identification correspond to the recently published data (3). The mathematics described above were applied automatically using Excel (Microsoft) using the input of the raw quantification files obtained from QuantArray to identify the correct KPC variant.

# 3 Results

## 3.1 Construction of the KPC microarray

In this study we developed a DNA microarray for the rapid detection of KPC -lactamase (*bla*$_{KPC}$) positive bacteria, which is capable of distinguishing between the different KPC variants. The probes used for the DNA microarray were designed to identify single nucleotide changes in the four mutation hotspots (position 147, 308, 716 and 814) of the *bla*$_{KPC}$ gene, allowing an identification of all known KPC variants (3). For each position of interest two sets of probes were designed, sense and anti-sense probes, resulting in a very robust detection system. Each probe consists of a 16-19 base pair oligonucleotide with a 13-thymidin spacer and a C6-amino modification at the 5'-end. All 32 oligonucleotide probes and the two primers that were used are listed in Table 1. The array layout as shown in Figure 1 can easily be expanded in the future by the addition of new probes to cover potential KPC variants with different hot spots of mutations.

225 ## 3.2 Validation of the DNA microarray using reference strains

226 The performance of the KPC-microarray was validated using 12 well characterized KPC-
227 producing reference strains, which were all identified correctly. In all cases the Cy3 labelling
228 PCR amplification yielded as expected an 871 bp product in a concentration range of 15 to
229 25 ng/µl. The rate of label incorporation, the number of nucleotides per number of
230 incorporated fluorescent dyes (NT/F), varied between 34 and 76, depending on the quality of
231 the template DNA. Best results were obtained using 200 ng labelled DNA product per
232 microarray (2 ng/µl), but as little as 50 ng (0.5 ng/µl) was sufficient in all cases for a correct
233 identification of each variant (equivalent to 870 pmol/L). The performance of each probe set
234 was measured using the (maximum) mismatch to perfect-match ratio ($MM_{max}$/PM). With only
235 one exception, this value was always below 0.7 for all tested reference strains, defining a high
236 level of discrimination for each probe set. In the single exception the antisense probe for
237 position 716 had a ($MM_{max}$/PM) of 0.711 in which case the sense probe was used for
238 discrimination instead with a $MM_{max}$/PM value of 0.54. Based on all reference strain
239 hybridizations the best discrimination for the sense probes was achieved with the probe set
240 SNP-147 having a median relative intensity value ($MM_{max}$/PM) of 0.037, followed by SNP-
241 308 (0.055), SNP-814 (0.377) and SNP-716 (0.526). For the antisense probes the best
242 discrimination was achieved with probe set SNP308 having a median relative intensity value
243 ($MM_{max}$/PM) of 0.041 followed by SNP-814 (0.09), SNP-147 (0.133) and SNP-716 (0.347).
244 Figure 1 (part B and C) shows as an example the relative fluorescent signal intensities of all
245 sense and antisense probes obtained through hybridization with target DNA from *K.*
246 *pneumoniae* carrying variant $\textit{bla}_{\text{KPC-3}}$. The relative intensity values between the maximum
247 mismatch and perfect match signal ($MM_{max}$/PM) are also included in the figure. Both sense
248 and antisense probes identified variant KPC-3 correctly. Results of all other strains are shown
249 in the supplementary material in Figure S1 (KPC-2) and Figure S2 (KPC-3).

250

251 ### 3.3 Microarray Limit of Detection

252 Before the limit of detection (LOD) of the whole assay was analysed, the LOD of the
253 microarray to labelled target DNA itself was tested. For this purpose a dilution series of
254 labelled target DNA (2 – 100 ng) was made, amplified from *E. coli* (KPC-2). Before
255 hybridization two different methods of target DNA treatment were applied, one using a
256 DNase concentration adjusted to the actual amount of target DNA and the other using a fixed
257 concentration independent to the amount of target DNA (resembling the clinical test situation
258 where the amount of DNA would be unknown). For the adjusted protocol a DNase
259 concentration of 0.8 mU DNase for each ng DNA was found to be most efficient, whereas for
260 the fixed concentration experiment 16 mU DNase were used, optimized to an average amount
261 of 20 ng target DNA. The first method is more accurate but is also more time consuming, due
262 to additional purification and measuring steps, which are necessary to acquire the exact
263 concentration of the target DNA. The second method using a fixed amount of DNase would
264 be the more practical solution in terms of developing an automated diagnostic tool,
265 contributing to a significant reduction in assay-time. A comparison of the microarray results
266 using both methods is shown in Figure 2. With the adjusted method (A) the correct KPC
267 variant was detected using down to 10 ng of target DNA (equivalent to 170 pmol/L), where as
268 using a fixed amount of DNase (B) the correct KPC variant was identified using down to
269 20 ng target DNA (350 pmol/L). Hybridizations using the adjusted method resulted generally
270 in higher absolute fluorescent signals as well as better (lower) MM/PM ratios. Therefore, this
271 method was applied for all the following experiments.

272

273 ### 3.4 Limit of detection estimated directly from spiked urine samples

274 In order to determine the limit of detection (LOD) of the whole assay, uninfected urine
275 samples were pooled and subsequently spiked with bacteria carrying variants of $bla_{\mathrm{KPC}}$. These

276 samples were diluted in 11 steps resulting in dilution series covering a range of 1 - $10^9$
277 CFU/mL urine, confirmed via colony counts on LB-Agar plates. All dilution series were
278 counted at least in duplicates. Several dilutions series were produced using *E. coli* $bla_{KPC-2}$, *K.*
279 *pneumoniae* $bla_{KPC-2}$ and *K. pneumoniae* $bla_{KPC-3}$. Subsequently, plasmid DNA was extracted
280 from each dilution step using the QIAprep Spin Miniprep Kit (Qiagen) and the Urine
281 Bacterial DNA Isolation Kit (Norgen, Thorold, Canada) in duplicates. None-spiked urine
282 samples were extracted as well and used as no template controls (NTC). The extracted DNA
283 was amplified and analysed using the DNA microarray. As an example, Figure 3 (part A and
284 B) shows the data obtained from analysing a dilution series of *K. pneumoniae* (KPC-3)
285 extracted with the QIAprep Spin Miniprep Kit. KPC-3 was correctly identified down to a
286 concentration of 4 x $10^3$ CFU/mL urine. One dilution step further (360 CFU/mL) the criteria
287 for a correct identification were not fulfilled anymore. At this dilution step the mismatch to
288 perfect match ratio (MM/PM) for one SNP position (SNP-716) was below the threshold of 0.7
289 for both probe sets (sense/antisense) and in addition the limit of detection for more than one
290 probe set was reached. Figure 3 (part C and D) shows the corresponding data obtained with
291 the microarray after extraction using the Urine Bacterial DNA Isolation Kit from Norgen. A
292 correct identification of variant KPC-3 using this method was still possible from a dilution
293 containing 360 CFU/mL urine.
294 Figure 4 shows a summary of all 132 DNA microarray experiments carried out to determine
295 the limit of detection for the whole assay. *E. coli* (KPC-2), which was spiked into urine
296 samples was still identified correctly at a concentration of $1.6x10^4$ CFU/mL urine in all cases.
297 For *E. coli* (KPC-2) the LOD results were the same for all replicates carried out with the
298 Qiagen extraction kit as well as the urine extraction kit from Norgen (Figure 4 A). Cells of *K.*
299 *pneumoniae* (KPC-2) were still identified correctly to a concentration of 120 CFU/mL urine
300 for Norgen and Qiagen. One of the Norgen extractions allowed a correct identification of
301 variant KPC-2 from as little as 40 CFU/mL urine (Figure 4 B). The third tested strain,

302 *K. pneumoniae* (KPC-3), was identified correctly to a concentration of $4x10^3$ CFU/mL urine
303 using the Qiagen extraction kit for both replicates, whereas the Norgen kit allowed an
304 identification from 360 CFU/mL urine (80 CFU/mL in one of the replicates)(Figure 4 C).
305 Over all experiments the LOD seemed to be higher for urine samples spiked with *E. coli*
306 ($1.6x10^4$ CFU/mL urine) and lower for *K. pneumoniae* (40 – 4000 CFU/mL urine). In
307 addition we observed that the Norgen kit gave slightly higher yields than the Qiagen kit when
308 extracting DNA from *K. pneumoniae*, resulting in a lower LOD. A more detailed table
309 containing all absolute PM signal intensities and the corresponding $MM_{max}$/PM ratios can be
310 found in the supplementary material (Figures S3, S4 and S5). When processing 20 urine
311 samples in parallel, the extraction using the Urine Bacterial DNA Isolation Kit (Norgen) took
312 on average 2 hours, resulting in a total time to result of 6 hours after urine sampling. When
313 applying the QIAprep Spin Miniprep Kit (Qiagen) the extraction took on average 1 hour for
314 20 urine samples, resulting in a total time to result of 5 hours.
315

316 ## 4 Discussion

317 A rapid detection of antibiotic resistances in clinical samples is crucial in order to provide
318 appropriate treatment for patients in a timely manner. Especially ESBLs and carbapenemases
319 have become a world wide threat to successful antibiotic therapy. In particular KPC
320 carbapenem resistances have been reported increasingly in recent years, resulting in a need for
321 new and rapid detection methods. Conventional routine methods are mostly based on
322 phenotypical detection procedures. An example is the modified Hodge test, which can
323 confirm the presence of carbapenemases, but can not distinguish between KPC or other
324 carbapenemases (24, 28). To distinguish KPCs from other carbapenem producers boronic disk
325 tests can be used (42, 43), but still an identification of single KPC variants is not possible. In
326 general all phenotypic methods are very time consuming, delivering results often only after

327 one or two days (23). Faster are molecular tests, such as real-time PCR assays, allowing a
328 quick identification of KPC genes (3, 5, 10, 12, 17, 20, 23). Nevertheless, these assays often
329 have only a limited multiplexing capability and also cannot distinguish single KPC variants
330 from each other.

331 Therefore, DNA microarrays are a good alternative offering a high multiplexing capability
332 and furthermore allow an identification of SNPs, which is necessary to distinguish between
333 single variants. The possibility to identify single variants from each other using a DNA-
334 microarray has been demonstrated for the ESBL relevant genes $bla_{\mathrm{TEM}}$, $bla_{\mathrm{SHV}}$ and $bla_{\mathrm{CTX\text{-}M}}$
335 (9, 14). The commercially available microarray assays from Check-Points enable only
336 identification of genes and mutation hotspots relevant to resistances caused by ESBLs and
337 carbapenemases, including the detection of $bla_{\mathrm{KPC}}$ (4, 6, 8, 21, 22, 40, 44, 47). However the
338 Check-Points system can be used as a reliable screening tool to guide PCR-sequencing,
339 allowing this way an indentification of single variants (13).

340 The capability to identify single variants of the KPC gene might not have been a requirement
341 in the past with only a very limited number of reported KPC variants, showing very similar
342 phenotypes. However, recent studies suggest that an increasing number of different KPC
343 variants confer different resistance profiles. Knowing which variant is present might open
344 new treatment options in the future especially under strict antibiotic stewardship. The
345 difference in resistance profiles and their effect on beta-lactam inhibitors were demonstrated
346 directly in clinical samples, in transformants with KPC variants, as well as through
347 comparisons of hydrolytic activities (1, 26, 27, 36, 45). Robledo et al. reported a variation of
348 antimicrobial susceptibility to carbapenems depending on the KPC variant during a 1 year
349 study based on KPC-producing isolates taken from 6 Puerto Rico Medical Center Hospitals.
350 All isolates were resistant to ertapenem irrespective of the KPC variant. Isolates with KPC-2
351 and KPC-6 were resistant to all carbapenems tested. Isolates with KPC-4 were susceptible to
352 imipenem and meropenem, while those with KPC-3 demonstrated variable susceptibility (35).

353 Therefore, knowing the exact KPC variant might allow a more target driven use of individual
354 carbapenems or beta-lactam inhibitors. However, the most benefit of SNP detection in $bla_{\text{KPC}}$
355 genes is the application for epidemiological studies, allowing to understand if the resistance
356 found is a single case or a pandemic spread (9).
357 The here described KPC microarray is able to identify and distinguish all KPC variants,
358 which were published at the time of design (KPC-2 to KPC-11). These variants differ from
359 each other in 4 SNP positions (nucleotide 147, 308, 716 and 814). The recently reported
360 variant KPC-12 (www.lahey.org/Studies/) differs from KPC-2 by a single mutation at SNP
361 position 502, a new position, which is not covered in the current version, but could easily be
362 added to future versions of this microarray. Therefore KPC-12 would currently be identified
363 as KPC-2 using the microarray. Due to the selected melting temperatures of the probes and
364 primers the KPC microarray could be used together with our existing ESBL microarray (14),
365 or both could be spotted onto one new microarray in the future, applying the same reaction
366 conditions.
367 The KPC microarray was tested successfully on 12 different reference strains carrying either
368 variant KPC-2 or KPC-3. These are the most frequently found KPC variants. During the
369 course of the project we had no access to any other KPC variants. But nevertheless all probe
370 sets could still be validated, due to the fact that each probe set is covered by the amplicon
371 used. Each probe set gave a clear positive hybridization signal with a high level of
372 discrimination between perfect match and mismatch probes when being tested with KPC-2 or
373 KPC-3. Consequently, there are no untested probes on the array. We would consider this as
374 being sufficient at this stage as the method of allele-specific-hybridization for SNP detection
375 using microarrays is well established and all probe sets were tested positive in over 160
376 separate hybridization experiments. Although theoretically possible, we did not design
377 synthetic targets to test all possible hybridization patterns (all variants for each position) as
378 this would have gone beyond the scope of the study while giving only a limited scientific

379 benefit due to the difference in PCR amplicon and synthetic targets. The limit of detection for
380 labelled target DNA was found to be 10 ng per assay when using a DNase amount that was
381 adjusted to the target DNA concentration. When using a fixed DNase amount, optimized for
382 20 ng target DNA, the limit of detection also turned out to be 20 ng. Lower DNA amounts
383 were probably over-digested and could therefore not be detected anymore. Higher fluorescent
384 signals and better discrimination values (MM/PM) were obtained using the adjusted method.
385 With the adjusted method a total assay time of 3.5 hours after DNA extraction was possible,
386 which is significantly shorter than conventional PCRs followed by sequencing or
387 phenotypical methods that require 1 or 2 day over-night cultivation (25, 38, 39). By using a
388 fixed amount of DNase before hybridization this assay time could even be reduced by at least
389 30 min, which would otherwise be necessary for DNA purification, concentration
390 measurements and final digestions. This microarray has therefore the potential to be used as a
391 rapid KPC resistance test.
392 Disregarding the much faster time to result, the introduction of molecular assays into routine
393 diagnostics depends on the cost. In general molecular assays are still more costly than culture
394 based tests. Commercially available molecular assays currently have prices in the order of $19
395 (RT-PCR) to $40 (microarrays) per sample. For our KPC microarray we calculated a price of
396 $38 per sample, which includes array production, DNA extraction from urine samples and
397 consumables for running the assay. Sequencing is already cheaper with prices around $6 per
398 sample, but prior overnight cultivation and DNA extraction is still necessary in addition.
399 Therefore sequencing is still too demanding for routine clinical diagnostics.
400 This study most importantly demonstrates, possibly for the first time, the direct identification
401 of KPC variants from urine samples, without prior cultivation. Two different DNA extraction
402 kits (Qiagen and Norgen) were tested for the extraction of bacterial DNA from urine followed
403 directly by the microarray analysis. Urine samples spiked with dilution series of different
404 reference strains were used as testing material. In total 132 extractions and microarray

405 experiments were carried out to determine the limit of detection (LOD). In all experiments the
406 correct KPC variant was still identified from urine samples with as low as $1.6x10^4$ CFU/mL.
407 This LOD was obtained analysing urine samples spiked with *E. coli*, whereas for *K.*
408 *pneumoniae* an even lower limit of detection was observed ($4x10^3$ CFU/mL for Qiagen and
409 360 CFU/mL for Norgen). For the DNA extraction of *K. pneumoniae* the Norgen kit seemed
410 to be slightly more sensitive than the Qiagen kit. On average only 80 CFU/mL urine were
411 identified using the Norgen kit. If such level of sensitivity is not required the Qiagen kit
412 seemed to be a lot more practicable for routine extractions, with a much shorter handling
413 time. Phenotypic tests have a lower detection limit (e.g. $4x10^1$ - $9x10^2$ CFU/mL for
414 CHROMagar KPC test) but results can only be obtained after 24 - 48 h or even later (25, 38).
415 Bacterial loads in urine of more than $10^6$ CFU/mL are considered to be a clear indication for a
416 urinary tract infection (UTI) (37). Therefore, the KPC microarray test presented in our study
417 would be sensitive enough to identify bacteria with KPC resistances from patients with UTIs.
418 Since only 1.7 mL urine was used for the analysis, the limit of detection for both extraction
419 methods could still be further improved by increasing the amount of urine used for DNA
420 extraction. This would be especially interesting for the analysis of symptomatic patients,
421 where the presence of 100 CFU/mL is enough to diagnose bacteriuria (37). The technology is
422 in principle suitable for direct testing of patient samples. However, the performance ability in
423 terms of sensitivity and specificity needs to be further investigated in a separate study.
424

425 **5 Conclusion**

426 This study demonstrates the possibility to identify single KPC variants directly from urine
427 samples without prior cultivation, using a new DNA microarray. The total assay time of 5
428 hours (Qiagen extraction + DNA microarray) or 6 hours (Norgen extraction + DNA
429 microarray) is a lot faster than classical methods to analyse antimicrobial susceptibilities from

urine samples. The bacteria could be analysed directly from urine samples without further cultivation plus the identification of the exact KPC variant, allowing direct information towards possible treatment options and epidemiology. A larger study on urine samples carrying KPC variants would further confirm the performance of this test.

## 6 Acknowledgements

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620

621

**Tables and Figures:**

**Table 1: *bla*$_{KPC}$ primer and oligonucleotide probe sequences**

| Oligonucleotide name[a] | 5'-3' sequence[b,c] | Position / SNP in *bla*$_{KPC}$ | $T_m$ (°C)[d] | Ref.[e] |
|---|---|---|---|---|
| KPC_SNP1_s | TGTACGCGATNGATACCGG | 147 | 55.4 | ‡ |
| KPC_SNP1_as | CCGGTATCNATCGCGTACA | 147 | 55.4 | ‡ |
| KPC_SNP2_s | GCTGGTTCNGTGGTCAC | 308 | 54.9 | ‡ |
| KPC_SNP2_as | GTGACCACNGAACCAGC | 308 | 54.9 | ‡ |
| KPC_SNP3_s | TGCGGAGNGTATGGCA | 716 | 55.2 | ‡ |
| KPC_SNP3_as | TGCCATACNCTCCGCA | 716 | 55.2 | ‡ |
| KPC_SNP4_s | GATGACAAGNACAGCGAGG | 814 | 54.5 | ‡ |
| KPC_SNP3_as | CCTCGCTGTNCTTGTCATC | 814 | 54.5 | ‡ |
| KPC_PR_F1 | TGTCACTGTATCGCCGTC | 2-20 | 54.5 | |
| KPC_PR_R2 | AGGGATTGGGCGTCAA | 857-872 | 53.8 | ‡ |

[a] Every probe was spotted as sense (s) and antisense (as).

[b] For each single nucleotide polymorphism (SNP) position four probes were designed that differ only at their central base (N = A, G, C or T). The relevant nucleotide triplet is underlined.

[c] Every probe was modified with a 13-thymidine spacer and a C6-amino modification at the 5'-end.

[d] The melting temperatures ($T_m$) were calculated with the OligoAnalyzer using default parameters (www.idtdna.com).

[e] Reference: ‡: this study, :(48)

636

637 **Figure 1: A - Left:** An example of a typical fluorescent image of a DNA microarray

638 hybridized with $bla_{KPC}$ target DNA from *Klebsiella pneumoniae* (HPA isolate) carrying

639 variant KPC-3. **A - Right:** KPC DNA microarray layout. All relevant SNP positions are

640 covered by a set of 8 probes (all four bases as sense and antisense) spotted in triplicates. The

641 perfect match positions are marked with black circles corresponding to $bla_{KPC}$ variant 3. The

642 bottom images represent the resulting relative fluorescent signal intensities of sense (A) and

643 antisense (B) probes hybridized. The corresponding perfect match signal patterns from sense

644 (GCTT) and antisense (CGAA) probes identified variant KPC-3 correctly. The combined

645 analysis of sense and antisense strand increases the robustness of the system. The numbers

646 represent the mismatch to perfect-match ratios (MM/PM), a measure for the discriminative

647 power of each probe set. In general, probe sets with MM/PM ratios larger than 0.7 where

648 omitted from the analysis. The respective SNP was then covered by the corresponding

649 sense/antisense probe set.

650

**Figure 2: Limit of detection of the DNA microarray using dilutions of target DNA.** A dilutions series of labelled target DNA (2 - 100 ng) was hybridized onto the KPC microarray to identify its LOD. **(A)** Represents the absolute fluorescent signal intensities obtained after the hybridization of target DNA, which was digested with 0.8 mU DNase/ng DNA. The identification of KPC-2 was possible down to 10 ng target DNA. In **(C)** the DNA was digested with a fixed amount of DNase (16 mU), which is equivalent to the amount used for 20 ng in experiment A. Here down to 20 ng target DNA / microarray were still correctly identified. Part **(B and D)** show the corresponding MM/PM ratios. At 20 ng target DNA / microarray the SNP 814 sense probe was out of range (MM/PM > 0.7), therefore the antisense probe was used for discrimination instead, which still correctly identified variant KPC-2. For all following experiments, the method presented in figure **A** and **B** (0.8 mU DNase/ng DNA) was used due to its higher reproducibility and sensitivity.

666

667

668 **Figure 3: Limit of Detection (LOD) Analysis directly from urine samples.** Overnight

669 cultures of *Klebsiella pneumoniae* carrying $bla_{KPC-3}$ were spiked into urine samples in a

670 dilution series from $10^7$ to 1 CFU/mL urine. The samples were then left for 30 min at room

671 temperature before the DNA was extracted. In this example the QIAprep Spin Miniprep Kit

672 (Qiagen) was used for extraction. **(A)** This figure represents the absolute fluorescent signal

673 intensities of 2 sense and 2 antisense perfect match probes obtained after DNA microarray

674 analysis of the extracts from each dilution. In part **(B)** the mismatch to perfect match ratios of

675 the same probes are presented showing until which dilution step a good discrimination

676 (MM/PM < 0.7) was possible. In this case KPC-3 was still identified correctly to a dilution

677 step of 4,000 CFU/mL urine. The identified variant is shown underneath the concentration.

678 Part **(C)** and **(D)** represent the data obtained from the same dilution series after extraction

679 using the Urine Bacterial DNA Isolation Kit (Norgen). With this method the correct KPC

680 variant was still identified from a dilution containing 360 CFU/mL urine. (NTC: no template

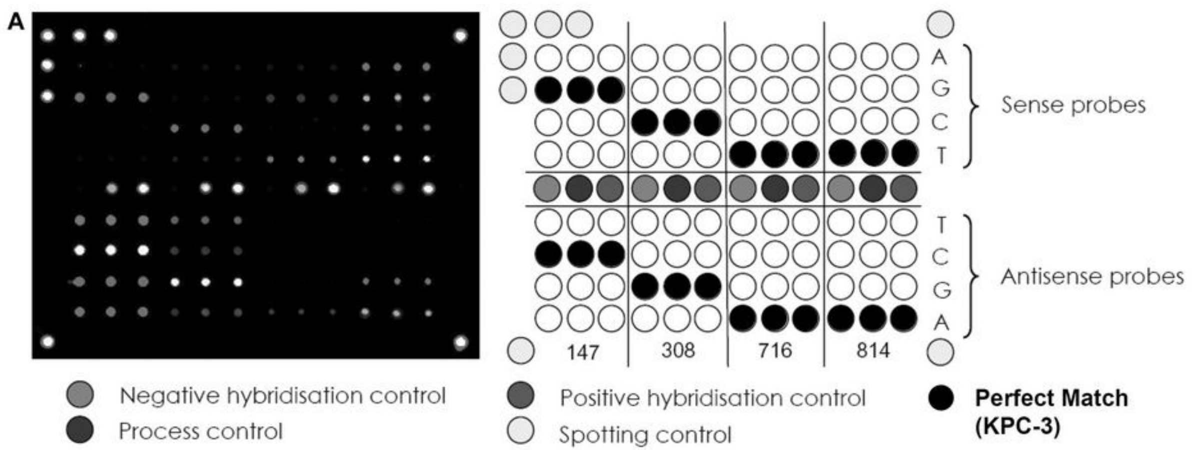
681 control).

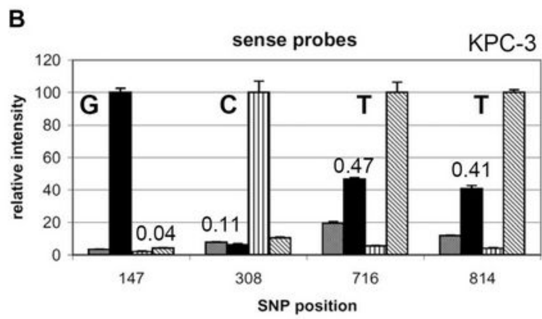
682

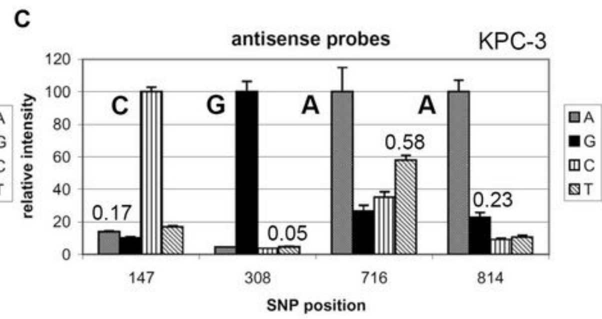
683

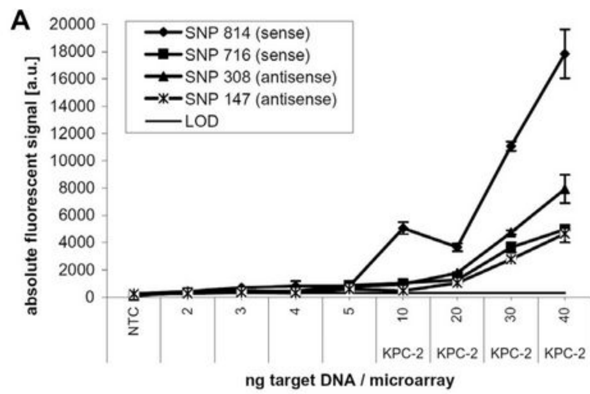
684

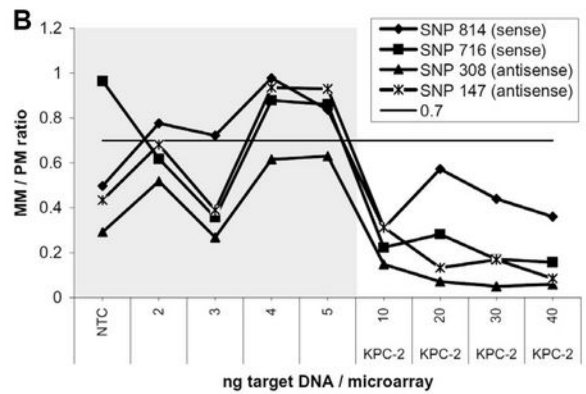
685 **Figure 4: Summary - Limit of Detection (LOD) from urine samples.** This figure
686 summarizes the results obtained from 132 microarray hybridizations carried out to determine
687 the limit of detection of the whole assay. Two extraction kits (Norgen / Qiagen) were used to
688 isolate DNA from urine samples spiked with 3 different strains carrying variants of $bla_{KPC}$.
689 Fields marked with an “x” represent array experiments that did not fulfil all mathematical
690 criteria for a correct analysis and therefore were beyond the limit of detection (MM/PM < 0.7,
691 PM > LOD). The numbers represents the KPC variants, which were identified. (NTC: no
692 template control).

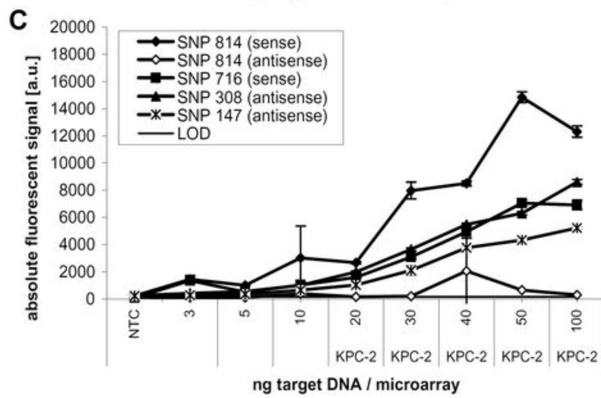

A
A
G
C
T
Sense probes
T
C
G
A
Antisense probes
147
308
716
814
Negative hybridisation control
Process control
Positive hybridisation control
Spotting control
Perfect Match (KPC-3)

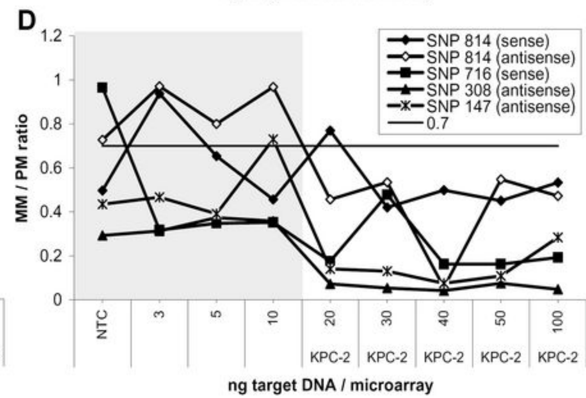

B
sense probes
KPC-3
relative intensity
SNP position
G
C
T
T
0.04
0.11
0.47
0.41
147
308
716
814
A
G
C
T

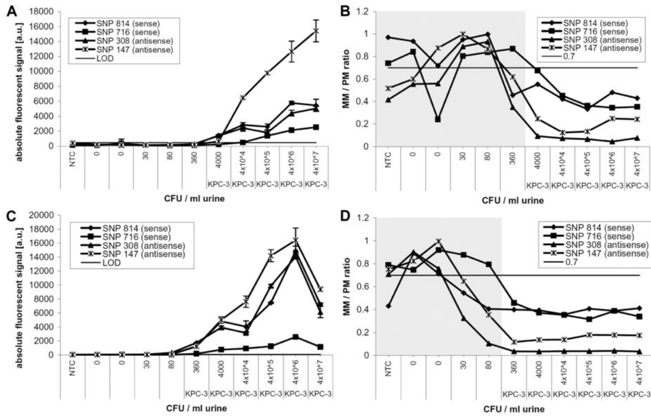

C
antisense probes
KPC-3
relative intensity
SNP position
C
G
A
A
0.17
0.05
0.58
0.23
147
308
716
814
A
G
C
T


A
SNP 814 (sense)
SNP 716 (sense)
SNP 308 (antisense)
SNP 147 (antisense)
LOD
absolute fluorescent signal [a.u.]
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
NTC
2
3
4
5
10
20
30
40
KPC-2
KPC-2
KPC-2
KPC-2
ng target DNA / microarray


B
SNP 814 (sense)
SNP 716 (sense)
SNP 308 (antisense)
SNP 147 (antisense)
0.7
MM / PM ratio
1.2
1
0.8
0.6
0.4
0.2
0
NTC
2
3
4
5
10
20
30
40
KPC-2
KPC-2
KPC-2
KPC-2
ng target DNA / microarray


C
SNP 814 (sense)
SNP 814 (antisense)
SNP 716 (sense)
SNP 308 (antisense)
SNP 147 (antisense)
LOD
absolute fluorescent signal [a.u.]
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
NTC
3
5
10
20
30
40
50
100
KPC-2
KPC-2
KPC-2
KPC-2
KPC-2
ng target DNA / microarray


D
SNP 814 (sense)
SNP 814 (antisense)
SNP 716 (sense)
SNP 308 (antisense)
SNP 147 (antisense)
0.7
MM / PM ratio
1.2
1
0.8
0.6
0.4
0.2
0
NTC
3
5
10
20
30
40
50
100
KPC-2
KPC-2
KPC-2
KPC-2
KPC-2
ng target DNA / microarray



A

| | cells/ml urine (counted) | NTC | 0 | 0 | 1.6x10^1 (30) | 1.6x10^2 (170) | 1.6x10^3 (1660) | 1.6x10^4 | 1.6x10^5 | 1.6x10^6 | 1.6x10^7 | 1.6x10^8 | LOD (cells / ml urine) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Escherichia coli* , KPC-2 | Qiagen 1 | x | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 16000 |
| | Qiagen 2 | x | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 16000 |
| | Norgen 1 | x | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 16000 |
| | Norgen 2 | x | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 16000 |

B

| | cells/ml urine (counted) | NTC | 0 | 0 | 1.4x10^2 (40) | 1.4x10^2 (0) | 1.4x10^2 (120) | 1.4x10^3 (1400) | 1.4x10^4 | 1.4x10^5 | 1.4x10^6 | 1.4x10^7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Klebsiella pneumoniae* , KPC-2 | Qiagen 1 | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 2 | 120 |
| | Qiagen 2 | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 2 | 120 |
| | Norgen 1 | x | x | x | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 40 |
| | Norgen 2 | x | x | x | x | x | 2 | 2 | 2 | 2 | 2 | 2 | 120 |

C

| | cells/ml urine (counted) | NTC | 0 | 0 | 4x10^0 (30) | 4x10^1 (80) | 4x10^2 (360) | 4x10^3 (4000) | 4x10^4 | 4x10^5 | 4x10^6 | 4x10^7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Klebsiella pneumoniae* , KPC-3 | Qiagen 1 | x | x | x | x | x | x | 3 | 3 | 3 | 3 | 3 | 4000 |
| | Qiagen 2 | x | x | x | x | x | x | 3 | 3 | 3 | 3 | 3 | 4000 |
| | Norgen 1 | x | x | x | x | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 80 |
| | Norgen 2 | x | x | x | x | x | 3 | 3 | 3 | 3 | 3 | 3 | 360 |