Prevalence of carbapenemase-producing organisms at the Kidney Center of Rawalpindi (Pakistan) and evaluation of an advanced molecular microarray-based carbapenemase assay

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Aim: A DNA microarray-based assay for the detection of antimicrobial resistance (AMR) genes was used to study carbapenemase-producing organisms at the Kidney Center of Rawalpindi, Pakistan. **Methods:** The evaluation of this assay was performed using 97 reference strains with confirmed AMR genes. Testing of 7857 clinical samples identified 425 Gram-negative bacteria out of which 82 appeared carbapenem resistant. These isolates were analyzed using VITEK-2 for phenotyping and the described AMR assay for genotyping. **Results:** The most prevalent carbapenemase gene was *bla*NDM and in 12 isolates we detected two carbapenemase genes (e.g., *bla*NDM/*bla*OXA-48). **Conclusion:** Our prevalence data from Pakistan show that – as in other parts of the world – carbapenemase-producing organisms with different underlying resistance mechanisms are emerging, and this warrants intensified and constant surveillance.

First draft submitted: 13 March 2018; Accepted for publication: 30 May 2018; Published online: 25 June 2018

Keywords: carbapenemases • CarbDetect • CPO • ESBL • hospital • microarray • Pakistan

From the beginning of this century, extended-spectrum β -lactamases (ESBL) have been considered to be a major public health threat, as ESBLs can hydrolyze most cephalosporins and monobactams [1]. More than 700 types of ESBLs have been detected hitherto [2,3]. According to a recent study, the prevalence of ESBLs in Pakistan is alarmingly high, that is, above 80% [4]. One remaining treatment option for ESBL-producing *Enterobacteriaceae* is the use of carbapenems (i.e., imipenem, ertapenem, meropenem and doripenem). However, the overuse of carbapenems to treat ESBL-positive pathogens has been selected for carbapenemase-producing and carbapenem-resistant bacterial pathogens [5,6]. Carbapenemases are β -lactamases that can actively cleave the β -lactam ring of nearly all β -lactam antibiotics used so far in clinical practice. One notable exception is aztreonam, which is stable against metallo- β -lactamases [7].

Carbapenemase-producing organisms (CPOs) cause different types of infections including ventilator-associated pneumonia or urinary tract infections, which consequently are difficult to treat. Infections caused by CPOs are associated with higher in-hospital mortality (up to 70%) related to inefficiency of primary therapeutic regimens [4,8–10].

During the last decade, CPOs that are resistant to almost all other antibiotics have emerged. CPOs expressing NDM-1 carbapenemase are a particular threat, as the *bla*NDM-1 gene is highly promiscuous, being readily transmittable between species and genera [11], with concomitant transfer of up to 14 different antibiotic resistance

genes. There is a lot of evidence that CPOs with NDM-1 are prevalent in the population of the Indian subcontinent and that they are spreading, for example, via international travel [6,12,13].

NDM-1 has been disseminated in different countries and is being reported globally. Because of its association with the Indian subcontinent, a number of studies have included samples from Pakistan, Sri Lanka, Bangladesh and India to evaluate the prevalence and spread of this enzyme. An overall prevalence of 18.5% *bla*NDM-1 positive Enterobacteriaceae has been reported in 2011 for two military hospitals in Rawalpindi, Pakistan [14]. Another study conducted in two tertiary care hospitals in Pakistan from 2013 indicated prevalence rates of 23.0% for *bla*NDM-1, 25.0% for *bla*VIM and 1.5% for *bla*IMP [4].

Treatment options for CPOs are limited to polymyxins, tigecycline, aminoglycosides and fosfomycin [15]. Carbapenem resistance has instigated the use of tigecycline and colistin, and resistance has also been reported against these agents [16]. Until recently, all reported polymyxin resistance mechanisms were chromosomally mediated, involving modulation of two component regulatory systems [17,18], and thus they were non-transferable. However, Liu *et al.* discovered in 2015 a plasmid-borne gene, *mcr-1*, which mediates resistance against colistin, and additional *mcr* alleles have been described later on. It was also shown that *mcr*-associated resistance to colistin is transferable from cell to cell [19].

The CDC recommend active screening for CPOs [20–22]. Misidentification of carbapenem resistance could lead to inappropriate antibiotic treatment and dissemination of resistance [23]. There is an urgent need for diagnostic laboratories to introduce methods for a rapid molecular identification of carbapenemase genes in Gram-negative bacteria for infection control and prevention, surveillance, and for epidemiological purposes [5].

Current methods of detecting CPOs in patient samples, such as selective culture media followed by standard susceptibility testing (e.g., VITEK-2 (bioMérieux, Nürtingen, Germany)), are time-consuming and do not provide genotyping data for surveillance and epidemiological questions. Therefore, several PCR-based assays have been developed that are able to detect carbapenemase genes directly in patient samples (e.g., Cepheids Xpert® Carba-R [18] (Cepheid, CA, USA), Check-Points Check-Direct CPE® test [24] (Check-Points, Wageningen, The Netherlands) or to identify carbapenemase genes in cultured bacterial isolates (e.g., Check-Points Check-MDR Carba [25]). However, a multitude of carbapenemase genes and their allelic variants are already known [26] and PCR-based tests normally cover only a small set of carbapenemase genes and alleles thereof.

The microarray-based assay presented herein provides a new option to simultaneously identify 43 globally important carbapenemase genes (including their allelic variants) and other important resistance genes (e.g., those encoding ESBLs, aminoglycoside or fluoroquinolone resistance) using isolated DNA from clonal bacterial culture.

We wanted to address two aims within this study. First, the overall performance of the microarray was to be assessed using genotypic and phenotypic profiles of reference strains and clinical isolates. Second, the newly evaluated array was to be used to study the prevalence of CPOs at the Kidney Center of Rawalpindi, Pakistan.

Materials & methods

Evaluation of the CarbDetect AS-2 kit

The function of the CarbDetect AS-2 kit (Alere Technologies GmbH, Jena, Germany) was evaluated using a set of 97 genotypically characterized reference strains (Table 1, and Supplementary Table 1) from different sources (University Medical Center of Dresden, Dresden, Germany [n = 3], University Medical Center of Jena, Jena, Germany [n = 10], German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany [DSMZ; n = 4], University of Fribourg, Fribourg, Switzerland [n = 72], in-house collection of Alere, Jena, Germany [n = 6] and the German National Reference Laboratory for Multidrug-Resistance Gram-negative Bacteria at Bochum University, Bochum, Germany [NRL, n = 2]). The investigated strain collection comprised *Acinetobacter baylyi* (n = 1), *Acinetobacter junii* (n = 1), *Acinetobacter haemolyticus* (n = 1), *Acinetobacter baumannii* (n = 20), *Acinetobacter lwoffii* (n = 2), *Chryseobacterium gleum* (n = 1), *Chryseobacterium meningosepticum* (n = 1), *Chryseobacterium indologenes* (n = 4), *Citrobacter freundii* (n = 3), *Enterobacter aerogenes* (n = 1), *Enterobacter cloacae* (n = 2), *Escherichia coli* (n = 14), *Klebsiella oxytoca* (n = 1), *Klebsiella pneumoniae* (n = 13), *Proteus mirabilis* (n = 2), *Pseudomonas aeruginosa* (n = 14), *Pseudomonas fluorescens* (n = 3), *Pseudomonas putida* (n = 1), *Pseudomonas stutzeri* (n = 1), *Ralstonia pickettii* (n = 1), *Salmonella enterica* (n = 5), *Serratia marcescens* (n = 2), *Shewanella algae* (n = 1), *Shewanella oneidensis* (n = 1) and *Shigella boydii* (n = 1). All reference strains were used only to analyze confirmed antimicrobial resistance (AMR) genes, which were detected by others methods than microarray (i.e., PCR or sequencing). These confirmed AMR genes are listed in Table 1. Reference strains were analyzed by microarray using the same procedures as described in

Table 1. Evaluation of the CarbDetect AS-2 kit: competitive study using reference strains with confirmed antimicrobial resistance genes.

Reference strain number	Species	Confirmed AMR genes	Detected AMR genes by microarray	Concordance in %
278787	<i>Acinetobacter baylyi</i>	<i>bla</i> PER-2	<i>bla</i> PER-2	100
278788	<i>Acinetobacter junii</i>	<i>bla</i> PER-2	<i>bla</i> PER-2	100
242273	<i>Acinetobacter haemolyticus</i>	<i>bla</i> OXA-58, <i>bla</i> OXA-214	<i>bla</i> OXA-58, <i>bla</i> OXA-214	100
208268	<i>Acinetobacter baumannii</i>	<i>bla</i> OXA-72 (<i>bla</i> OXA-40-like)	<i>bla</i> OXA-40-like	100
219847	<i>A. baumannii</i>	<i>bla</i> GIM	<i>bla</i> GIM (consensus)	100
240618	<i>A. baumannii</i>	<i>bla</i> OXA-40	<i>bla</i> OXA-40-like	100
240734	<i>A. baumannii</i>	<i>bla</i> OXA-97 (<i>bla</i> OXA-58-like)	<i>bla</i> -OXA-58-like	100
240736	<i>A. baumannii</i>	<i>bla</i> OXA-143	<i>bla</i> OXA-143/182/253/255	100
240737	<i>A. baumannii</i>	<i>bla</i> NDM-2, <i>aadB</i> , <i>ant2</i>	<i>bla</i> NDM (consensus), <i>aadB</i> , <i>ant2</i>	100
240741	<i>A. baumannii</i>	<i>bla</i> GES-14	<i>bla</i> GES (consensus)	100
240742	<i>A. baumannii</i>	<i>bla</i> GES-11, <i>bla</i> OXA-23	<i>bla</i> GES (consensus), <i>bla</i> OXA-23	100
240770	<i>A. baumannii</i>	<i>bla</i> NDM, <i>bla</i> OXA-23, <i>bla</i> OXA-69 (<i>bla</i> OXA-51-like)	<i>bla</i> NDM (consensus), <i>bla</i> OXA-23, <i>bla</i> OXA-51-like	100
240785	<i>A. baumannii</i>	<i>bla</i> OXA-23, <i>bla</i> PER-1, <i>aadA1</i> , <i>aphA</i>	<i>bla</i> OXA-23-like, <i>bla</i> PER-1, <i>aadA1</i> , <i>aphA</i>	100
240786	<i>A. baumannii</i>	<i>bla</i> OXA-23, <i>bla</i> PER-7	<i>bla</i> OXA-23-like, <i>bla</i> PER-1	100
240787	<i>A. baumannii</i>	<i>bla</i> OXA-23, <i>bla</i> GES-11	<i>bla</i> OXA-23-like, <i>bla</i> GES-11	100
240788	<i>A. baumannii</i>	<i>bla</i> OXA-24 (<i>bla</i> OXA-40-like)	<i>bla</i> OXA-40-like	100
240789	<i>A. baumannii</i>	<i>bla</i> OXA-51	<i>bla</i> OXA-51-like	100
240794	<i>A. baumannii</i>	<i>bla</i> OXA-69 (<i>bla</i> OXA-51-like), <i>bla</i> VEB-1, <i>bla</i> OXA-10	<i>bla</i> OXA-51-like, <i>bla</i> VEB (consensus), <i>bla</i> OXA-10	100
274215	<i>A. baumannii</i>	<i>bla</i> OXA-107 (<i>bla</i> OXA-51-like), ISABa1	<i>bla</i> OXA-51-like, ISABa1	100
274216	<i>A. baumannii</i>	<i>bla</i> OXA-64 (<i>bla</i> OXA-51-like), <i>bla</i> ACT	<i>bla</i> OXA-51-like, <i>bla</i> ACT	100
274217	<i>A. baumannii</i>	<i>bla</i> OXA-201 (<i>bla</i> OXA-51-like)	<i>bla</i> OXA-51-like	100
274218	<i>A. baumannii</i>	<i>bla</i> OXA-82 (<i>bla</i> OXA-51-like)	<i>bla</i> -OXA-51-like	100
278800	<i>A. baumannii</i>	<i>bla</i> TMB-1	<i>bla</i> TMB-1	100
240796	<i>Acinetobacter lwoffii</i>	<i>bla</i> OXA-134	<i>bla</i> OXA-134/235/284	100
278794	<i>A. lwoffii</i>	<i>bla</i> OXA-134	<i>bla</i> OXA-134/235/284	100
242277	<i>Chryseobacterium gleum</i>	<i>bla</i> IND (CBG-1)	<i>bla</i> IND (consensus)	100
240744	<i>Chryseobacterium meningoseptica</i>	<i>bla</i> CME-2, <i>bla</i> GOB-1	<i>bla</i> CME (consensus), <i>bla</i> GOB (consensus)	100
240766	<i>Chryseobacterium indologenes</i>	<i>bla</i> IND-1	<i>bla</i> IND (consensus)	100
240767	<i>C. indologenes</i>	<i>bla</i> IND-2	<i>bla</i> IND (consensus)	100
240769	<i>C. indologenes</i>	<i>bla</i> IND-4	<i>bla</i> IND (consensus)	100
248612	<i>C. indologenes</i>	<i>bla</i> IND-3	<i>bla</i> IND (consensus)	100
240802	<i>Citrobacter freundii</i>	<i>bla</i> VEB-1, <i>qnrB</i>	<i>bla</i> VEB (consensus), <i>qnrB</i>	100
242269	<i>C. freundii</i>	<i>bla</i> VIM-2, <i>bla</i> TEM-1, <i>bla</i> OXA-9, <i>bla</i> OXA-10	<i>bla</i> VIM (consensus), <i>bla</i> TEM (consensus), <i>bla</i> OXA-9, <i>bla</i> OXA-10	100
242274	<i>C. freundii</i>	<i>bla</i> NDM-1, <i>bla</i> TEM, <i>bla</i> CTX-M15, <i>bla</i> OXA-1, <i>bla</i> OXA-9, <i>bla</i> OXA-10, <i>armA</i> , <i>dfrA12</i> , <i>aadA2</i>	<i>bla</i> NDM (consensus), <i>bla</i> TEM (consensus), <i>bla</i> CTX-M1/15, <i>bla</i> OXA-9, <i>bla</i> OXA-10, <i>armA</i> , <i>dfrA12</i> , <i>aadA2</i>	100
242279	<i>Enterobacter aerogenes</i>	<i>qnrA1</i>	<i>qnrA1</i>	100
240750	<i>Enterobacter cloacae</i>	<i>nmcA</i> , <i>bla</i> MIR	<i>bla</i> IMI-3 (NmcA), <i>bla</i> IMI-R, <i>bla</i> MIR	100
278791	<i>E. cloacae</i>	<i>bla</i> OXA-163 (<i>bla</i> OXA-48-like), <i>dfrA1</i> , <i>sul1</i> , <i>qnrB</i>	<i>bla</i> OXA-48-like, <i>dfrA1</i> , <i>sul1</i> , <i>qnrB</i>	100
157048	<i>Escherichia coli</i>	<i>bla</i> OXA-45	<i>bla</i> OXA-45	100
174396	<i>E. coli</i>	<i>aac</i> (3')-IVa	<i>aac</i> (3')-IVa	100
187734	<i>E. coli</i>	<i>qnrC</i> , <i>dfrA13</i>	<i>qnrC</i> , <i>dfrA13</i>	100
204512	<i>E. coli</i>	<i>bla</i> DHA	<i>bla</i> DHA	100

AMR: Antimicrobial resistance; n.a: Not available; n.d.: Not detected.

Table 1. Evaluation of the CarbDetect AS-2 kit: competitive study using reference strains with confirmed antimicrobial resistance genes (cont.).

Reference strain number	Species	Confirmed AMR genes	Detected AMR genes by microarray	Concordance in %
208263	<i>E. coli</i>	<i>bla</i> NDM, <i>bla</i> CTX-M1, <i>rmtC</i>	<i>bla</i> NDM (consensus), <i>bla</i> CTX-M1/15, <i>rmtC</i>	100
208270	<i>E. coli</i>	<i>bla</i> OXA-2	<i>bla</i> OXA-2 (consensus)	100
240614	<i>E. coli</i>	<i>bla</i> IMP-1, <i>aac</i> (6')-Ic, <i>dfrA5</i> , <i>sul2</i>	<i>bla</i> IMP (consensus), <i>aac</i> (6')-Ic	100
240615	<i>E. coli</i>	<i>bla</i> KPC-2, <i>aadA4</i>	<i>bla</i> KPC (consensus), <i>aadA4</i>	100
240772	<i>E. coli</i>	<i>bla</i> NDM-1, <i>bla</i> TEM, <i>bla</i> OXA-1, <i>aadA2</i> , <i>aacC2</i> , <i>aacA4</i> , <i>dfrA17</i> , <i>sul2</i> , <i>mph</i> (consensus), <i>mrx</i> (consensus)	<i>bla</i> NDM (consensus), <i>bla</i> TEM (consensus), <i>bla</i> OXA-1, <i>aadA2</i> , <i>aacC2</i> , <i>aacA4</i> , <i>dfrA17</i> , <i>sul2</i> , <i>mph</i> (consensus), <i>mrx</i> (consensus)	100
240773	<i>E. coli</i>	<i>bla</i> NDM-4, <i>bla</i> CTX-M1, <i>bla</i> CMY-6	<i>bla</i> NDM (consensus), <i>bla</i> CTX-M1/15, <i>bla</i> CMY (consensus)	100
242280	<i>E. coli</i>	<i>qnrA1</i>	<i>qnrA1</i>	100
248610	<i>E. coli</i>	<i>bla</i> OXA-204 (<i>bla</i> OXA-48-like)	<i>bla</i> OXA-48-like	100
278798	<i>E. coli</i>	<i>aac</i> (6')-Ib-cr, <i>dfrA7</i>	<i>aac</i> (6')-Ib, <i>dfrA7</i>	50
278799	<i>E. coli</i>	<i>bla</i> CTX-M9-like (<i>bla</i> CTX-M14), <i>qepA</i>	<i>bla</i> CTX-M9, <i>qepA</i>	100
279601	<i>Klebsiella oxytoca</i>	<i>bla</i> VIM, <i>bla</i> ACC	<i>bla</i> VIM (consensus), <i>bla</i> ACC	100
208269	<i>Klebsiella pneumoniae</i>	<i>bla</i> NDM, <i>bla</i> CMY	<i>bla</i> NDM (consensus), <i>bla</i> CMY (consensus)	100
215771	<i>K. pneumoniae</i>	<i>bla</i> OXA-181	<i>bla</i> OXA-181/232	100
215792	<i>K. pneumoniae</i>	<i>bla</i> OXA-9, <i>dfrA14</i> , <i>sul3</i>	<i>bla</i> OXA-9, <i>dfrA14</i> , <i>sul3</i>	100
240610	<i>K. pneumoniae</i>	<i>bla</i> OXA-48	<i>bla</i> OXA-48-like	100
240612	<i>K. pneumoniae</i>	<i>bla</i> VIM-1	<i>bla</i> VIM (consensus)	100
240616	<i>K. pneumoniae</i>	<i>bla</i> KPC-2, <i>bla</i> CTX-M2, <i>dfrA15</i> , <i>sul2</i>	<i>bla</i> KPC (consensus), <i>bla</i> CTX-M2, <i>dfrA15</i> , <i>sul2</i>	100
240749	<i>K. pneumoniae</i>	<i>bla</i> KPC-3, <i>bla</i> CTX-M15, <i>bla</i> TEM-1, <i>bla</i> SHV-11	<i>bla</i> KPC (consensus), <i>bla</i> CTX-M1/15, <i>bla</i> TEM (consensus), <i>bla</i> SHV (consensus)	100
240757	<i>K. pneumoniae</i>	<i>bla</i> IMP-8, <i>bla</i> SHV-12, <i>dfrA19</i> , <i>sul2</i>	<i>bla</i> IMP (consensus), <i>bla</i> SHV (consensus), <i>dfrA19</i> , <i>sul2</i>	100
240781	<i>K. pneumoniae</i>	<i>bla</i> VIM-4, <i>bla</i> TEM-1, <i>bla</i> CTX-M1/15, <i>qnrS</i>	<i>bla</i> VIM (consensus), <i>bla</i> TEM (consensus), <i>bla</i> CTX-M1/15, <i>qnrS</i>	100
240798	<i>K. pneumoniae</i>	<i>bla</i> OXA-232, <i>bla</i> SHV-1, <i>bla</i> TEM-1, <i>bla</i> CTX-M1/15, <i>bla</i> OXA-1	<i>bla</i> OXA-181/232, <i>bla</i> SHV (consensus), <i>bla</i> TEM (consensus), <i>bla</i> CTX-M1/15, <i>bla</i> OXA-1	100
240799	<i>K. pneumoniae</i>	<i>bla</i> OXA-232, <i>bla</i> NDM-1, <i>bla</i> SHV-1, <i>bla</i> TEM-1, <i>bla</i> CTX-M1/15, <i>bla</i> OXA-1, <i>dfrA1</i> , <i>dfrA14</i> , <i>sul1</i> , <i>sul2</i>	<i>bla</i> OXA-181/232, <i>bla</i> NDM (consensus), <i>bla</i> SHV (consensus), <i>bla</i> TEM (consensus), <i>bla</i> CTX-M1/15, <i>bla</i> OXA-1	100
242275	<i>K. pneumoniae</i>	<i>bla</i> OXA-162 (<i>bla</i> OXA-48-like)	<i>bla</i> OXA-48-like	100
280640	<i>K. pneumoniae</i>	<i>rmtB</i>	<i>rmtB</i>	100
240777	<i>Proteus mirabilis</i>	<i>bla</i> NDM-1, <i>bla</i> CMY-16, <i>bla</i> OXA-10, <i>armA</i>	<i>bla</i> NDM (consensus), <i>bla</i> CMY (consensus), <i>bla</i> OXA-10 (consensus), <i>armA</i>	100
242267	<i>P. mirabilis</i>	<i>bla</i> VEB-6, <i>bla</i> NDM-1, <i>qnrD</i>	<i>bla</i> VEB (consensus), <i>bla</i> NDM (consensus), <i>qnrD</i>	100
204517	<i>Pseudomonas aeruginosa</i>	<i>bla</i> VIM-1, <i>aac</i> (3')-Ic	<i>bla</i> VIM (consensus), <i>aac</i> (3')-Ic	100
227120	<i>P. aeruginosa</i>	<i>bla</i> IMP-31, <i>aac</i> (3')-Ic	<i>bla</i> IMP (consensus), <i>aac</i> (3')-Ic	100
227126	<i>P. aeruginosa</i>	<i>bla</i> IMP-2	<i>bla</i> IMP (consensus)	100
240613	<i>P. aeruginosa</i>	<i>bla</i> VIM-2	<i>bla</i> VIM (consensus)	100
240617	<i>P. aeruginosa</i>	<i>bla</i> IMP-1, <i>aac</i> (6')-31	<i>bla</i> IMP (consensus), <i>aac</i> (6')-31	100
240740	<i>P. aeruginosa</i>	<i>bla</i> GES-2, <i>aac</i> (3')-Ia	<i>bla</i> GES (consensus), <i>aac</i> (3')-Ia	100
240760	<i>P. aeruginosa</i>	<i>bla</i> IMP-13	<i>bla</i> IMP (consensus)	100
240761	<i>P. aeruginosa</i>	<i>bla</i> IMP-15	<i>bla</i> IMP (consensus)	100
240762	<i>P. aeruginosa</i>	<i>bla</i> IMP-19	<i>bla</i> IMP (consensus)	100
240783	<i>P. aeruginosa</i>	<i>aac</i> (6')-Ib, <i>bla</i> VIM-2, <i>bla</i> PER-1, <i>strA</i> , <i>strB</i>	<i>aac</i> (6')-Ib, <i>bla</i> VIM-2, <i>bla</i> PER-1, <i>strA</i> , <i>strB</i>	100

AMR: Antimicrobial resistance; n.a: Not available; n.d.: Not detected.

Table 1. Evaluation of the CarbDetect AS-2 kit: competitive study using reference strains with confirmed antimicrobial resistance genes (cont.).

Reference strain number	Species	Confirmed AMR genes	Detected AMR genes by microarray	Concordance in %
242278	<i>P. aeruginosa</i>	<i>bla</i> SPM-1, <i>rmtD</i>	<i>bla</i> SPM-1, <i>rmtD</i>	100
248608	<i>P. aeruginosa</i>	<i>bla</i> IMP-29	<i>bla</i> IMP (consensus)	100
278789	<i>P. aeruginosa</i>	<i>bla</i> GES-1, <i>aac</i> (3')-Ib, <i>aac</i> (6')-Ib	<i>bla</i> GES (consensus), <i>aac</i> (3')-Ib, <i>aac</i> (6')-Ib	100
278792	<i>P. aeruginosa</i>	<i>bla</i> OXA-18	<i>bla</i> OXA-18	100
202104	<i>Pseudomonas fluorescens</i>	<i>rmtA</i>	<i>rmtA</i>	100
248793	<i>P. fluorescens</i>	<i>bla</i> BIC-1	<i>bla</i> BIC-1	100
278793	<i>P. fluorescens</i>	<i>bla</i> BIC-1	<i>bla</i> BIC-1	100
240784	<i>Pseudomonas putida</i>	<i>bla</i> VIM-5	<i>bla</i> VIM (consensus)	100
278797	<i>Pseudomonas stutzeri</i>	<i>bla</i> DIM-1	<i>bla</i> DIM (consensus)	100
240801	<i>Ralstonia pickettii</i>	<i>bla</i> OXA-60	<i>bla</i> OXA-60	100
240764	<i>Salmonella enterica</i>	<i>bla</i> IMP-4	<i>bla</i> IMP (consensus)	100
254995	<i>S. enterica</i>	<i>grm</i>	<i>grm</i>	100
255023	<i>S. enterica</i>	<i>aac</i> (3')-Ie	<i>aac</i> (3')-Ie	100
278790	<i>S. enterica</i>	<i>bla</i> PER-1	<i>bla</i> PER-1	100
281793	<i>S. enterica</i>	<i>bla</i> CTX-M8	<i>bla</i> CTX-M8	100
240751	<i>Serratia marcescens</i>	<i>bla</i> SME-1	<i>bla</i> SME (consensus)	100
240765	<i>S. marcescens</i>	<i>bla</i> IMP-11	<i>bla</i> IMP (consensus)	100
240791	<i>Shewanella algae</i>	<i>bla</i> OXA-55, <i>qnrA3</i>	<i>bla</i> OXA-55	100
278796	<i>Shewanella oneidensis</i>	<i>bla</i> OXA-54	<i>bla</i> OXA-54	100
227453	<i>Shigella boydii</i>	<i>bla</i> CTX-M2	<i>bla</i> CTX-M2	100
n.a.			<i>aac</i> (6')-II	n.d.
n.a.			<i>aac</i> (6')-IIa	n.d.
n.a.			<i>aac-aph</i>	n.d.
n.a.			<i>bla</i> FOX	n.d.
n.a.			<i>bla</i> KHM (consensus)	n.d.
n.a.			<i>bla</i> MOX (consensus)	n.d.
n.a.			<i>bla</i> MOX-CMY9	n.d.
n.a.			<i>bla</i> OXA-279	n.d.
n.a.			<i>bla</i> OXA-292	n.d.
n.a.			<i>bla</i> PAM-1	n.d.
n.a.			<i>bla</i> SFH-1	n.d.
n.a.			<i>bla</i> SMB-1	n.d.
n.a.			<i>npmA</i>	n.d.
Overall concordance (mean)				98.8

AMR: Antimicrobial resistance; n.a.: Not available; n.d.: Not detected.

Braun *et al.* [27]. The concordance C was determined using following formula (n = number):

$$C = \frac{100 \cdot n_{\text{detected AMR-genes}}}{n_{\text{confirmed AMR-gene}}}$$

A calculation of the true-positive and true-negative rate is difficult for the herein-described multiparameter assay (which detects 134 targets simultaneously), as the only reference method which could be used for that is whole genome sequencing. The costs to analyze all reference strains and clinical isolates using whole genome sequencing are far too high. Therefore, only strains with confirmed AMR genes were used to calculate the quality of the CarbDetect AS-2 kit.

Clinical isolates: growth conditions, susceptibility testing & genomic DNA extraction

We investigated non-duplicate, microbiological samples ($n = 7857$) taken from patients admitted to the Kidney Center (Al-Sayed Hospital, with 150 beds), Rawalpindi, between January and May in 2016. The Kidney Center is a tertiary/quaternary care hospital that is mainly specialized in the treatment of renal ailments and in kidney, liver and bone marrow transplantations. Most of the patients included in the study suffered from chronic renal failure or received kidney transplants.

Out of all samples tested, 425 Gram-negative bacteria (i.e., *Enterobacteriaceae* and non-fermenter) were isolated. These isolates were subjected to susceptibility tests by the standard Kirby–Bauer disc diffusion method and those strains that were resistant to carbapenems were selected for further characterization. The sample sites from which carbapenem-resistant isolates were obtained included urine ($n = 24$), Foley catheter ($n = 16$), pus ($n = 13$), throat swab ($n = 9$), endotracheal tube ($n = 7$), bronchoalveolar lavage ($n = 5$), wound swab ($n = 3$), double-J stent ($n = 2$), blood culture ($n = 1$), central venous pressure tube ($n = 1$) and nasogastric tube ($n = 1$). Species identification of carbapenem-resistant isolates was performed using the API 20 E assay and VITEK-2 (both from bioMérieux, Nürtingen, Germany).

All carbapenem-resistant isolates were cultivated on tryptone yeast agar and subsequently retested using an automated microdilution technique (VITEK-2, card: AST-248, bioMérieux) that included the following antibiotics, imipenem, meropenem, cefepime, cefotaxime, ceftazidime, piperacillin, piperacillin/tazobactam, aztreonam, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxacin, co-trimoxazole, tigecycline, fosfomycin and colistin. The interpretation of antimicrobial susceptibility tests was performed automatically by the VITEK-2 Expert software (bioMérieux, VITEK-2 system version: 07.01, MIC guidelines: EUCAST 2014 + CLSI 2014 D) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and/or Clinical and Laboratory Standards Institute (CLSI) breakpoints. All carbapenem-resistant isolates were additionally tested with a modified Hodge test [28] to identify isolates with overexpression of both AmpC β -lactamases in combination with porin loss and efflux pumps [29,30] using a 10 μ g meropenem susceptibility disk (Oxoid Ltd, Basingstoke, UK).

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. When necessary, DNA was concentrated to at least 100 ng/ μ l using a SpeedVac centrifuge (Eppendorf, Hamburg, Germany) at room temperature for 30 min/1400 r.p.m. Recovered genomic DNA (5 μ l) was used directly for internal biotin labeling and subsequent hybridization.

Clinical isolates: antimicrobial resistance genotyping

For all isolates, the carbapenemase, ESBL, narrow-spectrum β -lactamase (NSBL), *ampC*, aminoglycoside, macrolide, fluoroquinolone and co-trimoxazole genotypes were detected using the CarbDetect AS-2 kit. The data were automatically summarized by the 'result collector', a software tool developed by Abbott (Alere Technologies GmbH). Detailed results to all detected AMR genes are given in the Supplementary Tables 2 and 3. The *mcr-1* and *mcr-2* genes, mediating colistin resistance, were determined by PCR [19]. No genetic markers for tigecycline and fosfomycin resistance were included because resistance to these compounds is related to mutations in ubiquitous genes rather than to an acquisition of dedicated genes [31].

An AMR-genotype was defined as a group of genes that have been described to confer resistance to a family of antibiotics (e.g., the AMR-genotype '*bla*NDM' confers resistance to all tested β -lactam antibiotics or '*aac6*, *aac6ib*, *aadA1*' confer resistance to aminoglycosides).

The concordance described in Table 3 was calculated between the resistance pattern as predicted by the genotypic profile and the actual phenotypic profile as determined by VITEK. For a better comparison between the different AMR families, a mean concordance within each family was also calculated. An overall concordance was calculated as a mean to all calculated values.

Multiplex labeling, hybridization & data analysis

Array experiments were performed using the CarbDetect AS-2 kit. This test is an advanced version of the original CarbDetect AS-1 kit (Abbott [Alere Technologies GmbH], Jena, Germany). Some of the hybridization probes and labeling primers have been described previously [27]. In a direct comparison between both kits, 104 new target genes could now be detected with the advanced version. All target genes of the CarbDetect AS-2 kit and the differences to the original AS-1 kit are listed in Supplementary Table 4.

Table 2. Overview of β -lactam resistance genes with regard to species, sample source and antimicrobial resistance gene family. β -lactam-resistant genes of all isolates obtained from the different human samples of the nephrological hospital in Rawalpindi, Pakistan. Also given are the sample sources, species and isolate IDs. A detailed overview to all detected resistance genes for each isolate is listed in the Supplementary Table 3.

Number	Isolate number	Species	Sample source	Sample ID	AMR gene family	Susceptibility to meropenem/imipenem	Carbapenemase genes												ESBL genes					Other BL genes					AmpC		
							<i>bla</i> NDM (consensus)	<i>bla</i> OXA-48-like	<i>bla</i> OXA-181/232	<i>bla</i> VIM (consensus)	<i>bla</i> VIM-2	<i>bla</i> OXA-23-like	<i>bla</i> OXA-51-like	<i>bla</i> IMP (consensus)	<i>bla</i> IMP25 (<i>bla</i> SIM-1)	ISAba1 to <i>bla</i> OXA-51	<i>bla</i> CTX-M1/15	<i>bla</i> TEM (consensus)	<i>bla</i> SHV (consensus)	<i>bla</i> PER-1	<i>bla</i> VEB (consensus)	<i>bla</i> CTX-M9	<i>bla</i> OXA-1	<i>bla</i> OXA-10	<i>bla</i> OXA-2	<i>bla</i> OXA-9	<i>bla</i> MIR	<i>bla</i> ACT		<i>bla</i> CMY (consensus)	<i>bla</i> ADHA
1	PAK15	KLEP	Foley catheter	FT-899	Carbapenemase	R	■	■	■						■	■	■	■	■	■			■	■	■	■	■				
2	PAK29	KLEP	Foley catheter	FT-750	Carbapenemase	R	■	■	■									■	■	■				■	■	■	■				
3	PAK16	KLEP	Urine	U-861	Carbapenemase	R	■	■	■									■	■	■				■	■	■	■				
4	PAK1	KLEP	Bronchoalveolar lavage	BAL-794	Carbapenemase	R	■	■										■	■	■											
5	PAK52	AXYL	Central venous pressure tube	CVP-2051	Carbapenemase	R	■				■																				
6	PAK51	AXYL	Foley catheter	FT-1935	Carbapenemase	R	■								■			■	■	■											
7	PAK60	KLEP	Pus	PS-558	Carbapenemase	R	■	■										■	■	■									■		
8	PAK2	KLEP	Throat swab	TH-752	Carbapenemase	R	■	■										■	■	■											
9	PAK3	CLOA	Urine	U-680	Carbapenemase	R	■	■											■	■											
10	PAK6-2	KLEP	Urine	U-1113	Carbapenemase	R	■	■										■	■	■											
11	PAK22	KLEP	Urine	U-1139	Carbapenemase	R	■	■										■	■	■											
12	PAK56	ECOL	Bronchoalveolar lavage	BAL-2433	Carbapenemase	R	■												■			■		■	■					■	
13	PAK58	ECOL	Bronchoalveolar lavage	BAL-2248	Carbapenemase	R	■											■	■				■	■							
14	PAK21	CLOA	Endotracheal tube	ETT-1098	Carbapenemase	R	■																								
15	PAK23-1	ECOL	Endotracheal tube	ETT-1122	Carbapenemase	R	■											■	■				■							■	
16	PAK59	CLOA	Foley catheter	FT-2209	Carbapenemase	R	■											■	■												
17	PAK9	ECOL	Foley catheter	FT-2166	Carbapenemase	R	■												■											■	
18	PAK18	KLEP	Foley catheter	FT-811	Carbapenemase	R	■											■		■										■	
19	PAK46	KLEP	Foley catheter	FT-1925	Carbapenemase	R	■											■	■												
20	PAK48-1	KLEP	Foley catheter	FT-1890	Carbapenemase	R	■											■	■	■				■						■	

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Number	Isolate number	Species	Sample source	Sample ID	AMR gene family	Susceptibility to meropenem/imipenem	Carbapenemase genes															ESBL genes					Other BL genes					AmpC	
							<i>blaNDM</i> (consensus)	<i>blaOXA-48</i> -like	<i>blaOXA-181/232</i>	<i>blaVIM</i> (consensus)	<i>blaVIM-2</i>	<i>blaOXA-23</i> -like	<i>blaGES</i>	<i>blaIMP</i> (consensus)	<i>blaIMP25</i> (<i>blaSIM-1</i>)	<i>ISAba1</i> to <i>blaOXA-51</i>	<i>blaCTX-M1/15</i>	<i>blaTEM</i> (consensus)	<i>blaSHV</i> (consensus)	<i>blaPER-1</i>	<i>blaVEB</i> (consensus)	<i>blaCTX-M9</i>	<i>blaOXA-1</i>	<i>blaOXA-10</i>	<i>blaOXA-2</i>	<i>blaOXA-9</i>	<i>blaMIR</i>	<i>blaACT</i>	<i>blaCMY</i> (consensus)	<i>blaDHA</i>			
21	PAK73	CLOA	Pus	P5-65	Carbapenemase	R	■											■															
22	PAK68	KLEP	Pus	P5-613	Carbapenemase	R	■											■															
23	PAK7	ECOL	Throat swab	TH-1151	Carbapenemase	R	■											■															
24	PAK28-2	ECOL	Throat swab	TH-627	Carbapenemase	R	■											■															
25	PAK39-3	ECOL	Throat swab	TH-871	Carbapenemase	R	■											■															■
26	PAK32	ECOL	Urine	U-1173	Carbapenemase	R	■											■															■
27	PAK57	ECOL	Urine	U-2516	Carbapenemase	R	■											■															
28	PAK64	ECOL	Urine	U-76	Carbapenemase	R	■											■															
29	PAK66	ECOL	Urine	U-660	Carbapenemase	R	■											■															
30	PAK26-2	KLEP	Urine	U-1292	Carbapenemase	R	■											■															
31	PAK43-1	KLEP	Urine	U-1891	Carbapenemase	R	■											■															
32	PAK49	KLEP	Double-J stenting tube	DJ-1858	Carbapenemase	R		■										■															
33	PAK54	KLEP	Double-J stenting tube	DJ-168	Carbapenemase	R		■										■															
34	PAK34-1	ECOL	Endotracheal tube	ETT-1197	Carbapenemase	R		■											■														
35	PAK42-2	KLEP	Endotracheal tube	ETT-1693	Carbapenemase	R		■										■															
36	PAK35	KLEP	Foley catheter	FT-1214	Carbapenemase	R		■										■															
37	PAK63-1	KLEP	Pus	P5-38	Carbapenemase	R		■										■															
38	PAK28-1	KLEP	Throat swab	TH-627	Carbapenemase	R		■										■															
39	PAK40	KLEP	Throat swab	TH-1782	Carbapenemase	R		■										■															
40	PAK19	KLEP	Urine	U-980	Carbapenemase	R		■										■															

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							<i>bla</i> NDM (consensus)	<i>bla</i> OXA-48-like	<i>bla</i> OXA-181/232	<i>bla</i> VIM (consensus)	<i>bla</i> VIM-2	<i>bla</i> OXA-23-like	<i>bla</i> OXA-51-like	<i>bla</i> GES	<i>bla</i> IMP (consensus)	<i>bla</i> IMP25 (<i>bla</i> SIM-1)	<i>ISA</i> Ba1 to <i>bla</i> OXA-51	<i>bla</i> CTX-M1/15	<i>bla</i> TEM (consensus)	<i>bla</i> SHV (consensus)	<i>bla</i> PER-1	<i>bla</i> VEB (consensus)	<i>bla</i> CTX-M9	<i>bla</i> OXA-1	<i>bla</i> OXA-10	<i>bla</i> OXA-2	<i>bla</i> OXA-9	<i>bla</i> MIR	<i>bla</i> ACT		<i>bla</i> CMY (consensus)	<i>bla</i> DDHA
41	PAK43-3	KLEP	Urine	U-1891	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
42	PAK47	KLEP	Urine	U-1940	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
43	PAK14	KLEP	Wound swab	WS 636	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
44	PAK38	OLEO	Foley catheter	FT-1041	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
45	PAK27	PSEU	Foley catheter	FT-644	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
46	PAK33-1	PSEU	Foley catheter	FT-1381	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
47	PAK25	OLEO	Foley catheter	FT-1318	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
48	PAK61	PSEU	Pus	PS-39	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
49	PAK24	PSEU	Throat swab	TH-1102	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
50	PAK4	PSEU	Urine	U-950	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
51	PAK20	STEMA	Urine	n.d.	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
52	PAK71	ACBA	Bronchoalveolar lavage	BAL-72	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
53	PAK11	ACBA	Pus	PS-466	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
54	PAK62	ACBA	Pus	PS-45	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
55	PAK67	ACBA	Pus	PS-613	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
56	PAK69	ACBA	Pus	PS-601	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
57	PAK72	ACBA	Pus	PS-70	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
58	PAK36	ACBA	Throat swab	TH-1098	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
59	PAK65	ACBA	Urine	U-561	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
60	PAK30	PSEU	Blood	B-84	Carbapenemase	R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Table 2. Overview of β -lactam resistance genes with regard to species, sample source and antimicrobial resistance gene family. β -lactam-resistant genes of all isolates obtained from the different human samples of the nephrological hospital in Rawalpindi, Pakistan. Also given are the sample sources, species and isolate IDs. A detailed overview to all detected resistance genes for each isolate is listed in the Supplementary Table 3 (cont.).

Number	Isolate number	Species	Sample source	Sample ID	AMR gene family	Susceptibility to meropenem/imipenem	Carbapenemase genes															ESBL genes					Other BL genes					AmpC																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
							<i>bla</i> NDM (consensus)	<i>bla</i> OXA-48-like	<i>bla</i> OXA-181/232	<i>bla</i> VIM (consensus)	<i>bla</i> VIM-2	<i>bla</i> OXA-23-like	<i>bla</i> OXA-51-like	<i>bla</i> GES	<i>bla</i> IMP (consensus)	<i>bla</i> IMP25 (<i>bla</i> IMP-1)	ISAba1 to <i>bla</i> OXA-51	<i>bla</i> CTX-M1/15	<i>bla</i> TEM (consensus)	<i>bla</i> SHV (consensus)	<i>bla</i> PER-1	<i>bla</i> VEB (consensus)	<i>bla</i> CTX-M9	<i>bla</i> OXA-1	<i>bla</i> OXA-10	<i>bla</i> OXA-2	<i>bla</i> OXA-9	<i>bla</i> MIR	<i>bla</i> ACT	<i>bla</i> CMY (consensus)	<i>bla</i> DHA																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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Table 3. Genotyping vs. phenotyping: in detail comparison between the microarray-based genotype and the phenotype obtained by a VITEK-2 system (antimicrobial resistance)

Detected AMR genotype	AMR gene family	Expected AMR phenotype	Antibiotics tested	Genotype detected by microarray	Resistant detected by VITEK	Susceptibility detected by VITEK	Concordance (%)	Mean of concordance within AMR gene family
<i>blaNDM</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	20	20	0	100.0	
<i>blaOXA-181/232</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	12	12	0	100.0	
<i>blaOXA-23-like, blaOXA-51-like</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	8	8	0	100.0	
<i>blaVIM</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	7	7	0	100.0	
<i>blaNDM, blaOXA-48-like</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	6	6	0	100.0	
<i>blaNDM, blaOXA-181/232</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	3	3	0	100.0	
<i>blaGES, blaNDM</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	2	2	0	100.0	
<i>blaGES, blaVIM</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	1	1	0	100.0	
<i>blaIMP</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	1	1	0	100.0	
<i>blaIMP25 (blaSIM-1)</i>	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β -lactams	MEM, IMP, FEP, CTX, CAZ, PIP, TZP, AZA	1	1	0	100.0	
no genes detected		Susceptible to carbapenems	MEM, IMP	21	11	10	47.6	95.2
<i>blaCTX-M1/15</i>	ESBL	Resistant to 4G/3G cephalosporins, other β -lactams	FEP, CTX, CAZ, PIP, TZP, AZA	10	10	0	100.0	
<i>blaVEB</i>	ESBL	Resistant to 4G/3G cephalosporins, other β -lactams	FEP, CTX, CAZ, PIP, TZP, AZA	2	2	0	100.0	
<i>blaSHV, blaTEM (consensus)</i>	ESBL	Resistant to 4G/3G cephalosporins, other β -lactams	FEP, CTX, CAZ, PIP, TZP, AZA	1	1	0	100.0	
no genes detected		Susceptible to 3G/4G cephalosporins	FEP, CTX, CAZ	8	4	4	50.0	87.5
<i>blaOXA-1, blaOXA-10</i>	NSBL	Resistant to other β -lactams	PIP, TZP, AZA	1	1	0	100.0	
<i>blaOXA-2</i>	NSBL	Resistant to other β -lactams	PIP, TZP, AZA	1	1	0	100.0	
no genes detected		Susceptible to all β -lactams	PIP, TZP, AZA	6	4	2	33.3	77.8
<i>aac(6)-Ib</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	5	5	0	100.0	
<i>aac(6)-Ib, aadA1, aadA2, armA, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	5	5	0	100.0	
<i>aac(6)-Ib, aadA2</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	5	5	0	100.0	

AK: Amikacin; AZA: Aztreonam; CAZ: Ceftazidime; CN: Gentamicin; CTX: Cefotaxime; FEP: Cefepime; IMP: Imipenem; MEM: Meropenem; MXF: Moxifloxacin; PIP: Piperacillin; SXT: Co-trimoxazole; TOB: Tobramycin; TZP: Piperacillin-tazobactam; ESBL: Extended-spectrum β -lactamase; NSBL: Narrow-spectrum β -lactamase; AMR: Antimicrobial resistance.

Table 3. Genotyping vs. phenotyping: in detail comparison between the microarray-based genotype and the phenotype obtained by a VITEK-2 system (antimicrobial resistance) (cont.).

Detected AMR genotype	AMR gene family	Expected AMR phenotype	Antibiotics tested	Genotype detected by microarray	Resistant detected by VITEK	Susceptibility detected by VITEK	Concordance (%)	Mean of concordance within AMR gene family
<i>aac(6')-Ib, aadA2, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	5	5	0	100.0	
<i>aac(6')-Ib, rmtC, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	5	5	0	100.0	
<i>aac(6')-Ib, aadA4, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	4	4	0	100.0	
<i>strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	4	2	2	50.0	
<i>aac(6')-Ib, aadA1</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	2	1	66.7	
<i>aac(6')-Ib, aadA2, armA</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	3	0	100.0	
<i>aac(6')-Ib, aadA4, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	3	0	100.0	
<i>aac(6')-Ib, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	2	1	66.7	
<i>aadA1</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	2	1	33.3	
<i>aadA2</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	3	0	3	100.0	
<i>aac(3')-Ia, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	2	2	0	100.0	
<i>aac(6')-Ib, aadA4</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	2	2	0	100.0	
<i>aac(6')-Ib, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	2	2	0	100.0	
<i>aadB, ant2, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	2	2	0	100.0	
<i>aac(3')-Ia, aadA1, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	0	1	0.0	
<i>aac(6')-Ib, aadA1, aadA2, armA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aadA1, aphA, armA, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aadA1, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aadA2, aadA4, strA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aadB, ant2, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aphA</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aac(6')-Ib, aphA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadA1, aadA2, aadB, ant2, aphA, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadA1, aadB, ant2</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadA2, rmtB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadA4, strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	0	1	0.0	

AK: Amikacin; AZA: Aztreonam; CAZ: Ceftazidime; CIP: Ciprofloxacin; CN: Gentamicin; CTX: Cefotaxime; FEP: Cefepime; IMP: Imipenem; MEM: Meropenem; MXF: Moxifloxacin; PIP: Piperacillin; SXT: Co-trimoxazole; TOB: Tobramycin; T2P: Piperacillin-tazobactam, ESB: Extended-spectrum β -lactamase, NSBL: Narrow-spectrum β -lactamase, AMR: Antimicrobial resistance.

Table 3. Genotyping vs. phenotyping: in detail comparison between the microarray-based genotype and the phenotype obtained by a VITEK-2 system (antimicrobial resistance) (cont.).

Detected AMR genotype	AMR gene family	Expected AMR phenotype	Antibiotics tested	Genotype detected by microarray	Resistant detected by VITEK	Susceptibility detected by VITEK	Concordance (%)	Mean of concordance within AMR gene family
<i>aadB</i> , <i>ant2</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadB</i> , <i>ant2</i> , <i>aphA</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>aadB</i> , <i>ant2</i> , <i>strA</i> , <i>strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
<i>armA</i> , <i>strA</i> , <i>strB</i>	Aminoglycosides	Resistant to aminoglycosides	AK, CN, TOB	1	1	0	100.0	
no genes detected		Susceptible to aminoglycosides	AK, CN, TOB	7	2	5	71.4	87.9
<i>qepA</i>	Quinolones	Resistant to fluoroquinolones	CIP, MXF	3	3	0	100.0	
<i>qnrA1</i>	Quinolones	Resistant to fluoroquinolones	CIP, MXF	1	1	0	100.0	
<i>qnrB</i>	Quinolones	Resistant to fluoroquinolones	CIP, MXF	22	22	0	100.0	
<i>qnrB</i> , <i>qnrS</i>	Quinolones	Resistant to fluoroquinolones	CIP, MXF	1	1	0	100.0	
<i>qnrS</i>	Quinolones	Resistant to fluoroquinolones	CIP, MXF	5	5	0	100.0	
no genes detected		Susceptible to fluoroquinolones	CIP, MXF	50	44	6	12.00	85.3
<i>sul1</i> , <i>dfrA1</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	3	3	0	100.0	
<i>sul1</i> , <i>dfrA1</i> , <i>dfrA12</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	1	0	100.0	
<i>sul1</i> , <i>dfrA1</i> , <i>dfrA12</i> , <i>dfrA14</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	3	3	0	100.0	
<i>sul1</i> , <i>dfrA1</i> , <i>dfrA15</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	1	0	100.0	
<i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	1	0	100.0	
<i>sul1</i> , <i>dfrA12</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	8	7	1	87.5	
<i>sul1</i> , <i>dfrA12</i> , <i>dfrA5</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	0	1	0.0	
<i>sul1</i> , <i>dfrA17</i> , <i>dfrA19</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	2	2	0	100.0	
<i>sul1</i> , <i>dfrA5</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	4	4	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA1</i> , <i>dfrA12</i> , <i>dfrA14</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	6	6	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA12</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	0	1	0.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA12</i> , <i>dfrA14</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	4	4	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA12</i> , <i>dfrA17</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	2	2	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA14</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	4	4	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA14</i> , <i>dfrA5</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	1	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA17</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	2	2	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA17</i> , <i>dfrA19</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	2	2	0	100.0	
<i>sul1</i> , <i>sul2</i> , <i>dfrA5</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	1	1	0	100.0	

AK: Amikacin; AZA: Aztreonam; CAZ: Ceftazidime; CIP: Ciprofloxacin; CN: Gentamicin; CTX: Cefotaxime; FEP: Cefepime; IMP: Imipenem; MEM: Meropenem; MXF: Moxifloxacin; PIP: Piperacillin; SXT: Co-trimoxazole; TOB: Tobramycin; T2P: Piperacillin-tazobactam; ESB: Extended-spectrum β -lactamase, NSBL: Narrow-spectrum β -lactamase, AMR: Antimicrobial resistance.

Table 3. Genotyping vs. phenotyping: in detail comparison between the microarray-based genotype and the phenotype obtained by a VITEK-2 system (antimicrobial resistance) (cont.).									
Detected AMR genotype	AMR gene family	Expected AMR phenotype	Antibiotics tested	Genotype detected by microarray	Resistant detected by VITEK	Susceptibility detected by VITEK	Concordance (%)	Mean of concordance within AMR gene family	
<i>sul2, dfrA14</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	3	3	0	100.0		
<i>sul2, dfrA17</i>	Co-trimoxazole	Resistant to co-trimoxazole	SXT	3	3	0	100.0		
<i>sul1</i>	Co-trimoxazole	Susceptible to co-trimoxazole	SXT	12	11	1	8.3		
<i>sul1, sul2</i>	Co-trimoxazole	Susceptible to co-trimoxazole	SXT	2	2	0	0.0		
<i>sul2</i>	Co-trimoxazole	Susceptible to co-trimoxazole	SXT	7	6	1	14.3		
no genes detected		Susceptible to co-trimoxazole	SXT	8	5	3	37.5	77.0	
Overall concordance								85.1	
AK: Amikacin; AZA: Aztreonam; CAZ: Ceftazidime; CIP: Ciprofloxacin; CN: Gentamicin; CTX: Cefotaxime; FEP: Cefepime; IMP: Imipenem; MEM: Meropenem; MXF: Moxifloxacin; PIP: Piperacillin; SXT: Co-trimoxazole; TOB: Tobramycin; TZP: Piperacillin-tazobactam, ESBL: Extended-spectrum β -lactamase, NSBL: Narrow-spectrum β -lactamase, AMR: Antimicrobial resistance.									

The probes and primers for *bla*TEM and *bla*SHV were designed to aim on consensus areas (Supplementary Table 5). Therefore, *bla*TEM and *bla*SHV ESBLs cannot be distinguished from non-ESBL *bla*TEM and *bla*SHV alleles.

Multiplex labeling by primer extension amplification and hybridization was done according to the manufacturer's instructions. The procedure for multiplex labeling, hybridization and data analysis is described in detail by Braun *et al.* (2014) [27]. Starting from a clonal overnight culture, the complete turnaround time of the described kit is approximately 7 h, with a hands-on time of approximately 3 h.

Ethics statement

All isolates were collected from Kidney Center, Al-Sayed Hospital, Rawalpindi, Pakistan. Isolates were obtained during routine diagnostics and were analyzed anonymously. None of the authors were involved in sample collection, or interaction with the patients. Isolates were provided anonymously and no patient data were forwarded to co-authors or contributors not affiliated to the Al-Sayed Hospital, who also had no access to any patient-identifying information. Ethics approval and informed consent were therefore not required by EU regulations on epidemiological surveillance studies.

Results

Evaluation of the advanced carbapenemase assay

The functionality of the CarbDetect AS-2 kit was tested using 97 different reference strains (see above) with confirmed AMR-resistance genes. A comparison between all characterized reference strains and the detected AMR-genes is listed in the Table 1. The gene *aac*(6')-Ib-cr (strain 278798)-mediating quinolone resistance was false detected as *aac*(6')-Ib. However, in this strain the confirmed AMR-gene *dfpA7* was detected correctly; therefore, the concordance here was 50% (Table 1). The overall concordance between the detected AMR-genes and the AMR-genes confirmed to be present in the reference strains was 98.8%.

No reference strains were available for *bla*KHM, *bla*PAM-1, *bla*SFH-1, *bla*SMB-1, *bla*OXA-279, *bla*OXA-292, *bla*FOX, *bla*MOX, *aac*(6')-II, *aac*(6')-IIa, *aac*-aph, *npmA* and *bla*MOX-CMY9. The detailed, complete experimental data for all 97 reference strains are given in the Supplementary Table 1.

Samples & clinical isolates

Carbapenem-resistant isolates were found in blood, bronchoalveolar lavage, central venous pressure tube, double-J stent, endotracheal tube, Foley catheter, nasogastric tube, pus, throat swab, urine and wound swab. *Escherichia coli* was the most prevalent pathogen (Table 2). However, in urine samples *K. pneumoniae* was as often isolated as *E. coli* and on Foley catheters *K. pneumoniae* was the most common species (n = 7). *Acinetobacter baumannii* was the most frequently found pathogen in pus.

The carbapenemase gene *bla*NDM was found in nearly all types of samples, except in blood, on the double-J stents and wound swab (Table 2), but this might be attributed to low sample numbers. The gene *bla*OXA-23 was exclusively detected in *A. baumannii*.

Susceptibility testing & species identification of clinical isolates

Of 425 isolates collected during the period of our study, 82 (19.3%) were preliminarily identified as carbapenem-resistant using disk diffusion assays. According to confirmatory tests of these 82 isolates, 72 were resistant to meropenem and imipenem indeed. Out of the ten carbapenem susceptible isolates, nine were still resistant to cefepime and one against ceftazidim (Supplementary Table 2).

Carbapenem-resistant isolates were identified as *K. pneumoniae* (n = 26), *E. coli* (n = 22), *P. aeruginosa* (n = 15), *A. baumannii* (n = 8), *E. cloacae* (n = 4), *Stenotrophomonas maltophilia* (n = 2), *Achromobacter xylosoxidans* (n = 2), *Pseudomonas oleovorans* (n = 2) and *P. putida* (n = 1).

Eight isolates (*K. pneumoniae*, n = 6; *S. maltophilia*, n = 2) (9.8%) were resistant to colistin.

Antimicrobial resistance genotype & phenotype

Carbapenemase genes were detected in 61 out of the 72 carbapenem-resistant isolates. The most prevalent carbapenemase gene was *bla*NDM (n = 31) followed by *bla*OXA-181/232 (n = 15), *bla*VIM (n = 8), *bla*OXA-23 (n = 8), *bla*OXA-51-like (n = 8), *bla*OXA-48-like (n = 6), *bla*GES (n = 3), *bla*IMP (n = 1) and *bla*SIM-1 (n = 1) (Table 2). In 12 isolates, multiple carbapenemase genes were detected. The following combinations were

found, *bla*NDM/*bla*OXA-48-like (n = 6), *bla*NDM/*bla*OXA-181/232 (n = 3), *bla*NDM/*bla*GES (n = 2) and *bla*VIM/*bla*GES (n = 1) (Table 2). In the remaining 11 carbapenem-resistant isolates, no carbapenemase genes were detected (Table 2). Modified Hodge tests also yielded negative results suggesting another mechanism of resistance such as overexpression of AmpC accompanied by a porin loss or efflux pumps. Due to these 11 isolates (Supplementary Table 3), the concordance between genotype and phenotype for carbapenem-resistant isolates was 95.2% (Table 3). The insertion sequence *IS*Ab*A1* was not detected upstream to the intrinsic carbapenemase gene *bla*OXA-51 in *A. baumannii*. The underlying mechanism of detected carbapenem-resistant in *A. baumannii* was *bla*OXA-23 (Table 2).

Out of 21 isolates with no carbapenem-resistance genes, 17 were still resistant to third and fourth generation cephalosporins (Table 3). In 13 of these 17 isolates, ESBL genes were detected (Table 2). The gene *bla*CTX-M-1/15 was found in ten of these isolates, two of which harbored *bla*VEB while one isolate was only positive for consensus marker *bla*TEM and *bla*SHV (both including also ESBL variants); thus, the pheno- and genotypes correlated perfectly (Table 3).

In the remaining eight isolates, no ESBL gene was detected, albeit four of them were still resistant to all cephalosporins. These included two *S. maltophilia* and two *P. aeruginosa* isolates that were resistant to all other β -lactams. For details see Supplementary Table 3.

All isolates were screened for 28 different aminoglycoside resistance genes. Sixty-nine out of 82 (84.1%) isolates were resistant to at least one tested aminoglycoside (amikacin, tobramycin and/or gentamicin). Thirty-three different genotypes (i.e., combinations of these genes) were identified (Table 3). The most prevalent genotypes were *aac*(6')-Ib (n = 5) and *aac*(6')-Ib in combination with *aadA1*, *aadA2*, *armA*, *strA*, *strB* (n = 5) or *aac*(6')-Ib with *aadA2* (n = 5) or *aac*(6')-Ib with *aadA2*, *strA*, *strB* (n = 5) or *aac*(6')-Ib with *rmtC*, *strA*, *strB* (n = 5). All isolates harboring these genotypes were resistant to all tested aminoglycosides (Table 3). The genotype *aac*(6')-Ib in combination with *aadA4*, *strA*, *strB* (n = 4) showed a 100% concordance to the detected resistance to all aminoglycosides tested.

Two out of four isolates that harbored only *strA/strB* (mediating resistance to streptomycin only, which was not tested) were also resistant to amikacin, tobramycin and gentamicin. These isolates were found to be resistant to more compounds than expected; therefore the concordance was set to 50%. For isolates carrying only *aadA2* (n = 3), which confers resistance only to streptomycin and spectinomycin, the concordance was 100%, because the isolates were susceptible to all tested aminoglycosides. For isolates carrying just *aadA1* (n = 3), which only confers resistance to streptomycin and spectinomycin, two were still resistant to all tested aminoglycosides, and therefore the concordance was 33.3%. One isolate harbored the combination of *aadA4* and *strB*, and it was susceptible to all aminoglycosides (concordance 0.0%). Although streptomycin and spectinomycin were not tested in the recent study, previous studies using the same combination of probes and primers for *aadA1* and *aadA2* showed that isolates encoding these genes were only resistant to streptomycin and spectinomycin [32].

In eight isolates, no aminoglycoside resistance genes were detected, although two of them were resistant to all aminoglycosides tested. The mean concordance between aminoglycoside resistance genotype and phenotype was 87.9% (Table 3).

Seventy-six out of 82 (92.7%) isolates were resistant to fluoroquinolones. However, in only 32 isolates corresponding fluoroquinolones resistance genes were detected (*qepA*, *qnrA1*, *qnrB*, *qnrS*). All isolates that harbored at least one of these genes were resistant to both tested fluoroquinolones (ciprofloxacin, moxifloxacin). For these isolates, geno- and phenotype correlated perfectly. In 44 fluoroquinolone-resistant isolates and in six susceptible isolates, no genes could be found that are associated with fluoroquinolone resistance. Therefore, the mean concordance between fluoroquinolone-resistant phenotype and genotype was 85.3% (Table 3).

Seventy-four out of 82 (90.2%) isolates were resistant to co-trimoxazole. Resistance to co-trimoxazole suggests a genotype of *sul* in combination with *dhfrA*. Most tested isolates (50 of 53) with this gene combination were resistant to co-trimoxazole indeed. Isolates with the following genotypes were exceptions: *sul1/dhfrA12* (seven of eight isolates were resistant, 87.5% concordance); *sul1/dhfrA5/dhfrA12* (isolate was susceptible, 0.0% concordance), *sul1/sul2/dhfrA12* (isolate was susceptible, 0.0% concordance). Of 12 isolates that harbored only the gene *sul1*, 11 were still resistant (concordance 8.3%) and all isolates with the genotype *sul1/sul2* were resistant (concordance 0.0%). Six of seven isolates where only the gene *sul2* was detected were resistant (concordance 14.3%). Five of eight isolates harbored no gene for co-trimoxazole resistance but were still found to be resistant (concordance 37.5%). Therefore, the mean concordance of genotype and phenotype was 77% (Table 3).

Eight out of 82 (9.8%) isolates were colistin resistant, but none of these isolates harbored the genes *mcr-1* or *mcr-2*.

The overall concordance among all genotypes to expected phenotypes was 85.1% (Table 3).

Discussion

We report the prevalence of carbapenemase-producing Gram-negative pathogens at the Kidney Center, Rawalpindi, Pakistan. All presented genotyping data were determined using the CarbDetect AS-2 kit, which is an updated version of the CarbDetect AS-1 kit [27]. This update includes not only additional markers for carbapenemase genes, but also markers for resistance mechanisms toward other classes of antibiotics (i.e., aminoglycosides, fluoroquinolones and others). Evaluation with 97 defined reference strains showed a 98.8% concordance between expected and detected genotypes. No reference strains were available for some rare resistance genes (see above); in these cases an evaluation was not possible.

Since *bla*NDM-1 was first reported from India in 2009, it is believed that CPOs are highly prevalent in this region and are disseminating globally from there [11,33–37]. The current worldwide emergence of resistance to carbapenems in Gram-negative pathogens constitutes an important and growing public health threat. Sporadic outbreaks of carbapenem-resistant organisms have been reported not only in hospitals, but also in the community [26]. In this study, the prevalence of carbapenem-resistance isolates was about 1% of all clinical samples collected during the period of this study ($n = 7857$), but 19.3% of all samples from which clinically relevant Gram-negatives were cultured ($n = 425$). Our findings were in accordance to a previous study at a military hospital in Rawalpindi, Pakistan. Carbapenemase-producing Enterobacteriaceae were detected in 32 out of 175 isolates (18.3%) from stool samples and all produced *bla*NDM carbapenemase [13].

In the present study, 82 of 425 isolates were initially identified as carbapenem resistant using disc diffusion tests. However, confirmatory tests using VITEK-2 system revealed that in fact 72 samples were resistant. Carbapenemase genes were detected in 61 isolates. For the remaining 11 isolates in which no carbapenemase genes were detected, negative modified Hodge tests (MHT) indicated that the resistance mechanisms were an AmpC overexpression in combination with porin loss [28,30] or a different mechanism. For *P. aeruginosa*, the carbapenem-resistance is usually mediated by OprD loss, which primarily confers a resistance to imipenem but also confers a low-grade resistance to meropenem [38]. *Stenotrophomonas maltophilia* is resistant to all β -lactams because of two native and ubiquitous broad-spectrum metallo- β -lactamases (L1/L2) [31] that are not covered by the assay used herein. It is well known that MHT lacks specificity and may produce false-positive results for bacteria with complex ESBL or AmpC backgrounds [39]. In the recent study all carbapenem-resistant clinical isolates with at least one detected carbapenemase gene were positive using MHT. All carbapenem-resistant isolates lacking carbapenemase genes were negative. All used methods (VITEK, MHT and microarray) correlated perfectly. Furthermore, to the best of our knowledge, up-to-date false-negative MHTs were not described.

While *bla*KPC [40] is the most prevalent carbapenemase gene globally, *bla*NDM proved to be the most common carbapenemase gene in the study region, and it was detected in almost 50% of all carbapenemase-producing isolates belonging to different species in this study. Previous studies from Pakistan have reported variable prevalence for *bla*NDM. One study at a military hospital reported that *bla*NDM was the only carbapenemase resistance gene detected in their samples [13]. In 2010, Kumarasamy *et al.* also reported *bla*NDM-1 in Pakistan [11]. In their study, the gene encoding NDM was the most frequently found carbapenemase and it was detected in nearly all sample types. Another study reported 18.5% prevalence of *bla*NDM-1-positive Enterobacteriaceae isolated from 200 stool samples [14]. Additionally, Nahid *et al.* indicated 23% prevalence of *bla*NDM-1 gene, 25% *bla*VIM gene and 1.5% *bla*IMP gene [4]. Sattar *et al.* documented a prevalence of *bla*NDM of 28.8% in *P. aeruginosa*, 14.6% in *E. coli* and 5.0% in *K. pneumonia* [41]. *bla*NDM-1 β -lactamase-producing bacteria are detected not only in hospital settings, but also in environmental samples (drinking water and seepage water). In New Delhi, *bla*NDM-1 was detected in two out of 50 drinking water samples and in 51 out of 171 seepage samples [42].

The second most prevalent enzyme in the current study was *bla*OXA-181/232 followed by *bla*VIM. However, *bla*OXA-23 was detected in only *A. baumannii* isolates. Irfan *et al.* collected carbapenem-resistant *A. baumannii* (CRAB) samples from a tertiary care hospital in Karachi and found *bla*OXA-23-like acquired-oxacillinase genes in 47 out of 50 isolates [43]. In another study, *bla*OXA-23 was most commonly associated with resistance among 125 CRAB isolates [44]. Similar results were also found by Atrouni *et al.* [45]. Another study reported *bla*OXA-23 in 14 out of 59 CRAB isolates [35]. In the current study, *bla*OXA-23 was the only carbapenemase gene identified in all eight CRAB isolates.

The treatment of carbapenem-resistant pathogens is very challenging and colistin is commonly used to treat such patients. Colistin is an old antibiotic that has largely been avoided during the last decades due to its toxicity.

Nonetheless, it is one of the few agents that remain effective against multidrug-resistant Gram-negative bacteria, resulting in an increasing clinical usefulness for colistin [46]. Colistin resistance is normally mediated due to mutations affecting chromosomal genes and not associated with horizontal gene transfer. However, in PR China, a plasmid-encoded colistin-resistance gene, *mcr-1*, was recently identified in colistin-resistant *E. coli*, isolated particularly from swine [19]. Recently, a second variant of a colistin resistance gene named *mcr-2* was detected in *E. coli* also from swine [47]. In our study, 10% of all isolates were colistin resistant, but none of the isolates harbor the gene *mcr-1* and *mcr-2* genes, so we assume that all detected resistance to polymyxin was chromosomally mediated, that is, due to mutations and possibly favored by previous episodes of colistin treatment of these individual patients. Unfortunately, data on prior antibiotic prescriptions were not accessible.

Combined resistance to various antibiotics was also evaluated in the present study and an overall 89% resistance to fluoroquinolones was observed in carbapenem-resistant isolates. Zhang *et al.* conducted a study in PR China and they reported 93% fluoroquinolone resistance in carbapenem-resistant *E. coli* strains [48]. Sekyere *et al.* (2017), recently reported of a higher fluoroquinolone, tigecycline and colistin resistance among carbapenemase-producing Enterobacteriaceae isolates from South Africa [49,50].

Interestingly, 12 isolates were shown to harbor multiple carbapenemase genes (Table 2). These isolates carried *bla*NDM in combination with *bla*OXA-48-like genes ($n = 9$) or *bla*GES ($n = 2$); or *bla*VIM in combination with *bla*GES ($n = 1$). There has been a recent report from south India on the occurrence of oxacillinase production that also demonstrated predominant presence of *bla*VIM along with *bla*OXA-23 among clinical isolates [51]. Although a report from south India has investigated co-presence of *bla*OXA-23 with *bla*NDM-1 [52], this is the first study from Pakistan which has detected multiple genetic mechanisms, *bla*NDM, *bla*VIM, different *bla*OXA-48-like genes and *bla*GES in different species (*K. pneumoniae*, *A. xylosoxidans*, *P. oleovorans*). We hypothesize that this co-existence of multiple resistance genes is due to the presence of multiple resistance plasmids in the isolates. This hypothesis will be supported by the assumption that the acquisition of plasmids carrying resistance genes may not impose a substantial cost to the recipient bacteria [53–56]. We assume that antibiotic selective pressure is the driving force to include more than one carbapenemase genes. Maybe the overuse of different carbapenems and other antibiotics in hospitals selected these pathogens which are resistant to multiple antibiotics.

In this context, the multiplex microarray method used in this study is well suited for analyzing bacteria which are multidrug resistant. Carbapenemase and other genes encoding for resistance to aminoglycosides, fluoroquinolones and co-trimoxazole can also be detected with a single reaction. Genes coding for narrow-spectrum β -lactamases, AmpC and ESBLs will be detected simultaneously.

We are aware that next-generation sequencing (NGS) is also suitable for multiparameter testing, but microarray platforms have a proven track record spanning nearly two decades in the lab. Researchers worldwide are familiar with both operating the technology and analyzing the results. Microarrays are easier to use with less complicated and less labor-intensive sample preparation than NGS [57]. The same goes for data analysis. Despite the rapid drop in the cost associated with NGS arrays are still more economical and yield higher throughput, providing significant advantages when working with a large number of samples.

Conclusion

Out of 425 Gram-negative isolates, 72 (17%) were resistant to imipenem and meropenem, and in 61 (14%) cases carbapenemase genes were detected. All isolates with at least one carbapenemase gene were resistant against tested carbapenems. The comparison of the genotyping to phenotyping profiles for each target should rather be used to determine to what extent or not the detection of a gene leads to the expected functionality. This because all molecular rapid tests that detect DNA directly from a real sample without previous culture work on the assumption that a gene is always translated into the corresponding function. In the end, the phenotypic resistance is crucial for the clinician and diagnostician when he has to make an appropriate therapy decision. Therefore, these data are of considerable relevance for developers of molecular point of care tests.

Due to the high concordance between the genotypic and phenotypic profiles, we assume that the detection of the genotype gives a very good indication of the distinct phenotype. Therefore, a fast genetic analysis that could detect different resistance genes simultaneously, like the array-based method described in this paper, could be used to detect patients harboring these multiresistance bacteria. Genotyping results may not be used for treatment decisions, but to make a decision whether to isolate a positive patient, and decide the next logical diagnostic procedures for the bacteria.

Furthermore, antimicrobial stewardship has been suggested as a significant way forward to control MDR pathogens especially for CPO pathogens. Therefore, fast detection of responsible genes for ESBL and/or carbapenem resistance could be useful to control the overuse of antibiotics.

Future perspective

One of the main challenges of our time is the dramatic spread of pathogenic microorganisms that are resistant toward antibiotics, such as carbapenem-resistant Enterobacteriaceae (CRE) and MRSA. Recent global trends like constant economic growth, globalization, industrial agriculture, farming and food production, outsourcing and centralization of pharmaceutical production of antimicrobial substances, mass transport of livestock, worldwide travel and migration, aging societies combined with modern medicine, false and nondirected use of antibiotics, lack of hygiene, insufficient availability of safe food and clean drinking water, anti-vaxxer movements, insufficient epidemiological monitoring, poor patient to staff ratios in rehabilitation centers, hospitals, and care facilities, as well as delayed diagnosis combined with the economization of medical procedures are main drivers to antibiotic resistance, and this trend will accelerate in the future. This also affects Pakistan. To stop and to reverse these developments, global and trans-sectoral action is urgently needed. One out of many important challenges is the global availability of decentralized (point of care) diagnostics that can guide therapy in order to avoid an unnecessary use of antimicrobial substances.

Summary points

The medical need

- Misidentification of carbapenem resistance could lead to inappropriate antibiotic treatment and dissemination of resistance.
- An urgent need exists for diagnostic laboratories to introduce methods for a rapid molecular identification of carbapenemase and extended-spectrum β -lactamases genes.
- Carbapenem resistance appears to be common in Pakistan. Therefore, we characterized carbapenemase-producing organisms from a tertiary care center in Rawalpindi, Pakistan using a DNA microarray.

The microarray technique

- The microarray-based assay presented herein provides a new option to simultaneously identify 43 globally important carbapenemase genes and their allelic variants starting from a clonal culture fast and economically.
- Comparing genotypic to phenotypic data, their correlation indicates whether a genotype can be used to develop rapid tests or can be used directly in hospital settings and epidemiological investigations.

Evaluation of the microarray-based assay

- The herein described DNA-based antimicrobial resistance (AMR) assay was evaluated using defined reference strains.
- Clinical isolates from the Kidney Center of Rawalpindi, Pakistan were used to compare the DNA microarray-detected genotype with the corresponding phenotype (analyzed by VITEK-2).

Outcome

- The overall concordance between expected and detected genotypes using 97 defined reference strains was 98.8%.
- The overall concordance between genotyping and phenotyping profile was over 85%.
- The detection of the genotype gives a very good indication of the distinct phenotype.
- During routine diagnostics of 7857 human samples 425 Gram-negative isolates were tested for resistance to carbapenems.
- Seventy-two of four hundred and twenty-five (17%) were resistant to imipenem and meropenem, and in 61 (14%) cases carbapenemase genes were detected.
- The most prevalent carbapenemase gene was *bla*NDM (n = 31).

Conclusion

- The current DNA-based AMR assay could be used to control multidrug-resistant pathogens especially carbapenemase-producing organism.
- Our study demonstrates that the detection of the AMR genotype gives a very good indication of the distinct AMR phenotype.
- The data presented in this study is of considerable relevance for developers of molecular point of care tests.

Acknowledgments

The authors are thankful to the kidney center at the Al-Sayed Hospital, Rawalpindi, Pakistan for providing microbial samples for this research.

Financial & competing interests disclosure

This study was supported by the Office of the Vice Chancellor and the Office of Research, Innovation and Commercialization of the University of Haripur, Haripur, Pakistan. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2018-0082

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