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Stepwise evolution of pandrug-resistance in *Klebsiella pneumoniae*

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Carbapenem resistant Enterobacteriaceae (CRE) pose an urgent risk to global human health. CRE that are non-susceptible to all commercially available antibiotics threaten to return us to the pre-antibiotic era. Using Single Molecule Real Time (SMRT) sequencing we determined the complete genome of a pandrug-resistant *Klebsiella pneumoniae* isolate, representing the first complete genome sequence of CRE resistant to all commercially available antibiotics. The precise location of acquired antibiotic resistance elements, including mobile elements carrying genes for the OXA-181 carbapenemase, were defined. Intriguingly, we identified three chromosomal copies of an IS*Ecp1*-$bla_{OXA-181}$ mobile element, one of which has disrupted the *mgrB* regulatory gene, accounting for resistance to colistin. Our findings provide the first description of pandrug-resistant CRE at the genomic level, and reveal the critical role of mobile resistance elements in accelerating the emergence of resistance to other last resort antibiotics.

The "golden era" when modern medicine saved lives through antibiotic treatment is under serious threat[1]. In 2013, the Centers for Disease Control and Prevention (CDC) released a landmark report on "Antibiotic Resistance Threats[2]". Three microorganisms were tagged as posing a threat level of urgent – *Clostridium difficile*, carbapenem-resistant Enterobacteriaceae (CRE) and drug-resistant *Neisseria gonorrhoeae*[2]. CRE, which include organisms such as *Klebsiella pneumoniae* and *Escherichia coli,* are resistant to almost all currently available antibiotics. Almost 50% of patients who develop bloodstream infections with these organisms die from the infection[2]. In healthcare settings, carbapenem resistant Enterobacteriaceae have increased sharply over the past decade[3]. Carbapenem resistance is typically mediated by the production of beta-lactamases[4], and patients with CRE infections are treated with last-resort antibiotics such as colistin[5].

The CDC and the European Centre for Disease Prevention and Control (ECDC) have jointly developed definitions for multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria[6]. Pandrug-resistance implies non-susceptibility to all commercially available antibiotics relevant to the treatment of a particular bacterial infection. Although there has been an anecdotal report of probable pandrug-resistance in *K. pneumoniae*[7], no such isolates have been comprehensively analyzed.

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In this manuscript, we describe the genetic basis of pandrug-resistance in a *K. pneumoniae* isolate using single molecule real-time (SMRT) sequencing. We show that a genetic element conferring resistance to carbapenem antibiotics has been acquired and mobilized, leading to insertional inactivation of a gene that results in resistance to colistin. Overall, our analysis provides a comprehensive description of a pandrug-resistant *K. pneumoniae* isolate at the whole genome level.

Results

Case Record. An 87 year old man, hospitalized in the United Arab Emirates in April 2014, was found to be colonized with multidrug-resistant *Klebsiella pneumoniae*. The isolate grew from urine and a pre-sacral pressure area but blood cultures were sterile. Susceptibility testing by way of a commercial semi-automated method (Vitek, bioMérieux) showed resistance to all antibiotics tested. The urinary isolate (strain MS6671) was therefore sent to a reference laboratory for further testing. Other *K. pneumoniae* isolates with this antibiotic resistance phenotype were not detected at the index patient's hospital.

Pandrug-resistant phenotype of *K. pneumoniae* MS6671. MS6671 was found to be non-susceptible to all antibiotics tested, which includes cephalosporins, penicillins, carbapenems, aztreonam, aminoglycosides, ciprofloxacin, colistin, tetracyclines, tigecycline, chloramphenicol, trimethoprim-sulfamethoxazole and fosfomycin (Table 1). Thus, the isolate can truly be described as pandrug-resistant[6].

***K. pneumoniae* MS6671 general genome features.** The complete genome of *K. pneumoniae* MS6671 consists of a circular chromosome 5,402,900 base-pairs in length with an average G-C content of 57%, five circular plasmids and a linear plasmid prophage (Supplementary Table S1). The sequence type of the isolate was ST147. The chromosome of MS6671 is highly similar to *K. pneumoniae* NTUH-k2044[8]; a hypervirulent strain associated with liver abscess and meningitis, with most variation attributable to differences in their mobile genetic element (MGE) content (Supplementary Figure 1). Further details of the complete genome are provided in the Supplementary Results.

Genetic determinants of pandrug-resistance. In order to determine the genetic basis of pandrug-resistance, we interrogated the genome to identify acquired and intrinsic resistance genes. The majority of acquired antibiotic resistance genes were located on the chromosome, with most beta-lactamase and aminoglycoside resistance genes carried within two copies of a class 1 integron or as part of mobile elements that incorporate the IS*Ecp1* insertion sequence (Table 1, Supplementary Table S2 and Supplementary Results). Mutations in *gyrA* and *parC* that have previously been linked with fluoroquinolone resistance (GyrA Ser83Ile and ParC Ser80Ile) were identified[9,10]. Fosfomycin resistance was mediated by a chromosomally encoded copy of *fosA*[11]. Mutations in chromosomal genes encoding major outer membrane porins (OmpK35 and OmpK36) were also identified. A novel variant of the *ompK36* gene was encoded on the chromosome. The amino acid sequence change is located in loop 3 (L3) of the porin, which constitutes the porin channel eyelet[12]. L3 mutations have previously been associated with increased resistance to carbapenems[13–15]. Additionally, *ompK35* has been disrupted by IS insertion. Inactivation of *ompK35* has been associated with increased resistance to a number of different classes of antibiotics, including quinolones and cephalosporins[16,17]. Genes encoding three beta-lactamases, including an extended-spectrum beta-lactamase (ESBL) and a carbapenemase, were detected at different genomic locations – $bla_{\text{SHV-36}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-181}}$.

Insertional inactivation of *mgrB* by a carbapenem-resistance element and colistin resistance. OXA-181 is an oxacillinase capable of hydrolysing carbapenems[18]. Three copies of an IS*Ecp1*-$bla_{\text{OXA-181}}$ transposon were identified throughout the chromosome (Fig. 1). One of these insertions has resulted in the inactivation of the *mgrB* gene, a negative regulator of *phoPQ*. Insertions in *mgrB* have previously been shown to cause colistin resistance in *K. pneumoniae* clinical isolates[19–21]. Examination of the DNA flanking the IS*Ecp1*-$bla_{\text{OXA-181}}$ transposons shows that the primary insertion site is within MS6671_10430, followed by intra-chromosomal transposition of IS*Ecp1*-$bla_{\text{OXA-181}}$ and a 37 bp fragment of MS6671_10430 to two other locations in the genome (Fig. 2). The three transposons are bracketed by imperfect 14 bp inverted repeats and flanking 5 bp direct repeats (TATCT, TGAAA and TATAA), providing direct evidence for their transposition activity (Supplementary Table S3).

In a similar fashion, a single copy of IS*Ecp1*-$bla_{\text{CTX-M-15}}$ has inserted into *ompK35*, leading to inactivation this gene (Fig. 1). The IS*Ecp1*-mediated mobilisation and transposition of $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-181}}$ and other clinical relevant beta-lactamase resistance genes, including bla_{CMY} and bla_{ACC}, has been reported previously[22–26].

***K. pneumoniae* MS6671 contains two copies of a class 1 integron.** A class 1 integron was identified on the chromosome encoding multiple antibiotic resistance genes (*arr-3, aac(6′)-Ib-cr, rmtF, catB1*) (Fig. 1). These genes result in resistance to rifampin, all aminoglycosides and chloramphenicol. A near-identical copy of this integron was also found on one of the six plasmids (Supplementary Figure S2).

Antimicrobial Category	Antimicrobial Agent	MIC (mg/L)	EUCAST Interpretation	Genes previously associated with resistance†
Aminoglycosides	Gentamicin	>256	R	*rmtF*, *aac(6′)-lb-cr*
	Tobramycin	>256	R	
	Amikacin	>256	R	
	Netilmicin	>256	R	
AntiMRSA Cephalosporins	Ceftaroline	>32	R	$bla_{OXA-181}$,$bla_{CTX-M-15}$
Antipseudomonal Penicillins and Beta-lactamase Inhibitors	Ticarcillin/Clavulanate	>256	R	$bla_{OXA-181}$
	Piperacillin/Tazobactam	>256	R	
Carbapenems	Ertapenem	>32	R	$bla_{OXA-181,}$ *ompK36* (ins aa135–136DT)
	Imipenem	4	NS	
	Meropenem	8	R	
	Doripenem	4	R	
Non-extended Spectrum Cephalosporins	Cefazolin*	>32	R	$bla_{CTX-M-15}$
	Cefuroxime	>256	R	
Extended Spectrum Cephalosporins	Ceftriaxone, Cefotaxime	>32	R	$bla_{CTX-M-15}$
	Ceftazidime	32	R	
	Cefepime	32	R	
Cephamycins	Cefoxitin*	128	R	$bla_{OXA-181}$
	Cefotetan*	32	NS	
Fluoroquinolones	Ciprofloxacin	>32	R	*gyrA* (Ser83Ile), *parC* (Ser80Ile), *qnrB ompK35* inactivation
Folate-pathway Inhibitors	Trimethoprim/Sulfamethoxazole	8	R	*dfrA12, dfrA14*‡
Glycylcyclines	Tigecycline	4	R	*acrAB*#
Monobactams	Aztreonam	32	R	$bla_{OXA-181}$, $bla_{CTX-M-15}$
Penicillins	Ampicillin	>256	R	bla_{SHV-36}
Penicillins and Beta-lactamase Inhibitors	Amoxycillin/Clavulanate	>256	R	$bla_{OXA-181}$
	Ampicillin/Sulbactam	>256	R	
Phenicols	Chlorampenicol	128	R	*catB1*, *ompK35* inactivation,
Phosphonic acids	Fosfomycin	64	R	*fosA*,
Polymyxins	Colistin	128	R	*mgrB* inactivation
Tetracyclines	Tetracycline*	32	R	*acrAB*#, *ompK35* inactivation, *tetC*
	Doxycycline*	32	R	
	Minocycline*	32	R	

Table 1. Antibiotic resistance in *K. pneumoniae* MS6671. *Based on the breakpoints of CLSI criteria; Polymyxin B MIC was 32 mg/L. †Several additional intrinsic factors, such as efflux pumps and porins, which may be involved in multi-drug resistance are also encoded in the genome (Supplementary Table S2). ‡Resistance to trimethoprim. No *sul* genes identified. #Tigecycline resistance has been associated with upregulation of *acrAB*, often resulting from the aberrant expression of *ramA* and/or r*amR*. A description of the potential mechanism of tigecycline resistance in MS6671 is provided in the Supplementary Results.

Discussion

This is the first genomic analysis of a pandrug-resistant CRE isolate, as defined by the rigorous CDC/ECDC assessment criteria[6]. With the advantage of long-reads provided by SMRT sequencing we were able to identify the genomic context of multiple resistance elements. In contrast to short-read technologies, SMRT sequencing allows complex resistance elements to be properly characterized[27]. This technology platform was used to investigate the German *E. coli* O104:H11 outbreak[28] and more recently to identify plasmid-borne resistance in a large-scale study of CRE following an outbreak at the National Institute for Health Clinical Center[29,30]. Critically, elucidation of the complete *K. pneumoniae* MS6671 genome using long-read sequencing enabled the context of multiple, identical carbapenem resistance elements to be determined. Based on this analysis we propose a model for the development of pandrug-resistance in this *K. pneumoniae* isolate, whereby mobile resistance determinants are responsible

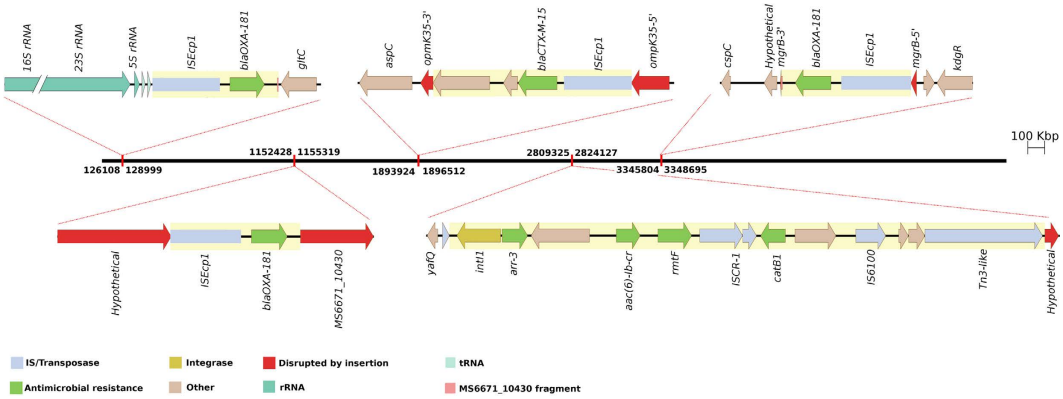

Figure 1. Diagram of the pandrug-resistant *K. pneumoniae* MS6671 chromosome highlighting the position and context of mobile genetic elements that harbor antimicrobial resistance genes. The chromosome of MS6671 is represented to scale by the black bar with IS*Ecp1* and integron insertion points indicated with red rectangles. Pop-outs display schematic representations of the four IS*Ecp1* elements which harbor beta-lactamase genes (three copies of $bla_{\text{OXA-181}}$ and one copy of $bla_{\text{CTX-M-15}}$) and a class 1 integron located on the chromosome of MS6671. Insertion elements are highlighted in yellow. The coordinates of each element are indicated above and below the genome bar.

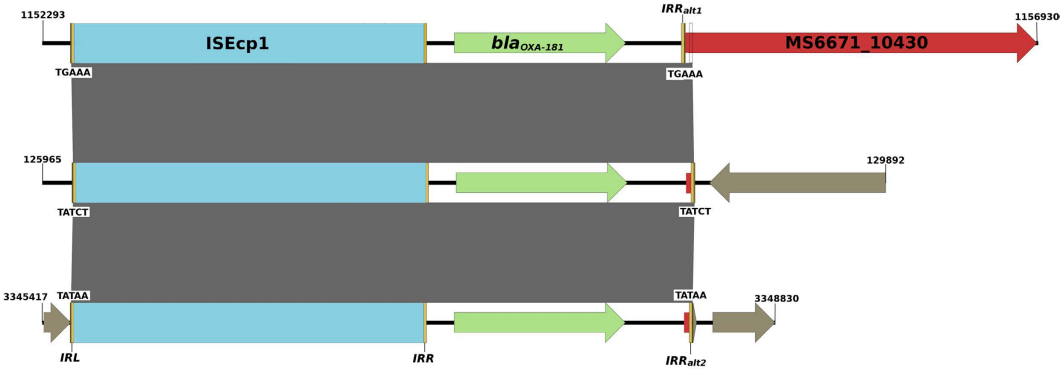

Figure 2. Comparison of IS*Ecp1*-$bla_{\text{OXA-181}}$ transposons from MS6671. Pairwise nucleotide comparison of IS*Ecp1*-$bla_{\text{OXA-181}}$ (OXA-181) transposons and flanking genomic regions from *K. pneumonaie* MS6671. IS*Ecp1* elements are represented by blue rectangles. Protein-coding genes are represented by coloured arrows: $bla_{\text{OXA-181}}$ (green); MS6671_10430 encoding a hypothetical protein (red); other (brown). Left and right flanking inverted repeats (IRL, IRR, IRRalt1, IRRalt2) are represented by yellow bars and 5 bp direct repeat sequences created by duplication of the target sequence during transposition are given (TGAAA, TATCT or TATAA). In the primary insertion site, a single 2,855 bp transposon carrying $bla_{\text{OXA-181}}$ has inserted at TGAAA (position 1152428..1155282) within MS6671_10430. This transposon, similar to Tn2013 previously described in *K. pneumoniae Kp3*[31], is flanked by 14-bp inverted repeat sequences, namely IRL and IRRalt1. IRRalt2 lies 23 bp downstream of IRRalt1 within the MS6671_10430 sequence. Mobilisation of the IS*Ecp1*-$bla_{\text{OXA-181}}$ transposon using IRRalt2 instead of IRRalt1 has resulted in a 37 bp fragment of MS6671_10430 (indicated by a small red rectangle) being packaged at the 3′ end of the other two IS*Ecp1*-$bla_{\text{OXA-181}}$ transposons inserted at TATCT and TATAA (position 126108..128999 and position 3345804..3348695, respectively). Grey shading indicates regions of homology (100% nucleotide sequence identity) between sequences.

for driving additional resistance. In this example, IS*Ecp1* carrying the $bla_{\text{OXA-181}}$ carbapenem resistance gene has inserted three times in the chromosome, with one event causing colistin resistance by insertional inactivation of *mgrB*.

IS*Ecp1*-like insertion sequences are the most common genetic element associated with $bla_{\text{CTX-M}}$, bla_{CMY} and bla_{ACC} genes and have more recently been associated with $bla_{\text{OXA-181}}$[22–26,31]. By recognizing a variety of DNA sequences as right inverted repeats (IRR), IS*Ecp1*s are capable of mobilising adjacent genes and inserting at new location[32,33]. Similar to previous reports on the hydrolytic activities of OXA-181[18,34,35], elevated MICs for ertapenem, imipenem, meropenem and doripenem were observed for

MS6671, indicating hydrolytic activity of OXA-181 towards these carbapenems and a possible $bla_{OXA-181}$ copy number effect (Table 1). Notably, doripenem resistance was higher than previously reported[18]. The *ompK36* variant encoded by MS6671 has previously been associated with increased resistance to doripenem and doripenem-colistin[13], and may contribute to the elevated MIC for doripenem observed in MS6671. Porin deficient *E. coli* expressing OXA-48-like beta-lactamases have also been shown to have elevated MICs towards carbapenems[18].

Inactivation of *mgrB* has recently been associated with resistance to colistin, and appears to be the most common mechanism for polymyxin resistance in *K. pneumoniae*[19,20]. Specifically, disruption of *mgrB* results in over expression of the *phoPQ* signaling system and of the *pmrHFIJKLM* operon which controls modification of LPS, the target of polymyxin antibiotics[36]. Insertional inactivation of *mgrB* with IS*5*-like or IS*1* elements has been previously reported[21,37], however, the present study is the first to show colistin resistance caused by insertion of a carbapenem resistance element itself. While we cannot rule out the possibility that this mechanism may have occurred in other colistin-resistant *K. pneumoniae* carrying IS*Ecp1*-$bla_{OXA-181}$[38], the generation of a complete genome sequence of MS6671 provides unequivocal evidence for this novel insertion event.

We also found a fourth IS*Ecp1* element encoding an ESBL (CTX-M-15), which was inserted within the outer membrane porin gene *ompK35*. Disruption of *ompK35* reduces the permeability of the outer membrane and mutants lacking this porin have increased resistance to quinolones, tetracyclines, beta-lactams and chloramphenicol[39]. Pan-aminoglycoside resistance was mediated by the rRNA methyltransferase RmtF, which was encoded on both chromosomal and plasmid copies of a class 1 integron. We did not have access to investigational antibiotics, such as plazomicin, eravacycline, ceftazidime/avibactam or ceftolozane/tazobactam, to assess their potency against MS6671.

MS6671 was defined by multi-locus sequence typing (MLST) as being ST147. *K. pneumoniae* ST147 was first described in Hungary in 2008[40]. Subsequently, it has been frequently associated with carbapenem resistance, with ST147 producing KPC well described in Greece and Italy[41,42]. For example, epidemics of VIM-producing carbapenem resistant *K. pneumoniae* ST147[43], and KPC-2-producing carbapenem resistant *K. pneumoniae*[44,45] have been reported in Greece. Notably, ST147 isolates carrying both bla_{VIM} and bla_{KPC-2} genes were identified[41,46]. Carbapenem resistant ST147 carrying the bla_{NDM-1} gene have been isolated in Iraq, Switzerland, Canada and the United Kingdom[47–49]. In MS6671, carbapenem resistance was most likely mediated by the beta-lactamase OXA-181, possibly in combination with permeability defects as has been reported previously in other strains[18,31]. ST147 *K. pneumoniae* producing OXA-181 have been previously reported from the Indian sub-continent[18,31,50]. Clearly, in addition to the KPC-producing, carbapenem-resistant *K. pneumoniae* ST258 clone[51], ST147 also represents a clone of *K. pneumoniae* with a potential for global significance.

Fortunately, in six months there have been no further isolates with this resistance phenotype at the index patient's hospital. However, the occurrence of this strain in the Arabian Gulf is of great significance. OXA-48-like-producing *K. pneumoniae* are frequent in this region[52]. It is unknown if this strain originated in the index patient, in another patient at the same hospital or was imported from another hospital, perhaps in another country. There are a large number of expatriates in the Gulf region, and travel to the Indian sub-continent, Europe and the United States is frequent[53]. The potential for international transfer of multidrug-resistant bacteria[54,55] emphasizes the need for global surveillance efforts as one part of a strategy to control antibiotic resistance[3].

In summary, we have provided the first report of a pandrug-resistant isolate of CRE using high-resolution genome data. The CDC has denoted CRE as an urgent threat. The emergence of this highly resistant strain, in a clone that has proven capable of causing outbreaks, raises this threat level even higher.

Methods

Antibiotic Resistance Phenotypic Testing. The *K. pneumoniae* isolate (hereafter referred to as MS6671) was sent to a reference laboratory (University of Queensland, Centre for Clinical Research) where confirmatory susceptibility testing was performed in order to determine the minimal inhibitory concentrations of all antibiotics used by the Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC) in defining a pandrug-resistant isolate[6]. Susceptibility of most tested antibiotics was determined using Etests and following the breakpoints of the European Committee for Antibiotic Susceptibility Testing (EUCAST)[56], except for cefazolin, cefoxitin, cefotetan, tetracycline, doxycycline, and minocycline which were determined using the breakpoints of the Clinical and Laboratory Standards Institute (CLSI)[57]. The minimum inhibitory concentration of colistin (sulfate, Sigma-Aldrich) was determined by broth microdilution in cation-adjusted Mueller-Hinton broth (Oxoid).

Genome sequencing. Pacific Biosciences (PacBio) RS II Single-Molecule Real Time (SMRT) sequencing of *K. pneumoniae* MS6671 was performed using ~4 μg of the genomic DNA sheared using g-TUBE™ (Covaris®) into fragments size targeted at 10 kb. Purification of the sheared DNA was then carried out using 0.45-fold volume of washed Agencourt AMPure XP magnetic beads (Beckman Coulter Inc.). SMRTbell template libraries were subsequently prepared using the commercial Template Preparation Kit from Pacific Biosciences Inc. that involved steps of DNA end repair, adapters ligation

followed by exonuclease digestion of incompletely ligated products. Next, 0.83 nM of the libraries were then annealed with sequencing primers followed by binding to 50 nM of P4 DNA polymerase, as provided in the Template Binding Kit from Pacific Biosciences Inc. For enhanced loading efficiency, 15 pM of the bound complexes were immobilized into Magbeads (Pacific Biosciences Inc.) prior to loading into the sequencing zero-mode waveguides (ZMWs). Duration for the sequence collection was set at 180 minutes with stage start option. Reads with length that were less than 50 bp were filtered off upon acquisition of the sequencing data and minimum polymerase read quality was set at 0.75.

Genome assembly. *De novo* genome assembly of PacBio SMRT reads from the *K. pneumoniae* MS6671 genome was performed using the hierarchical genome assembly process (HGAP)[58] from the PacBio SMRT analysis software suit (version 2.2.0), with default parameters and a seed read length cut-off of 5 kb. Following assembly, all contigs were screened for duplicate sequences at their 3′ and 5′ ends. Overlapping sequences were manually trimmed and joined based on sequence similarity. Individual contigs with duplicate sequences on their 5′ and 3′ ends were manually trimmed and circularised. Following circularisation the chromosome and plasmid sequences were polished using quiver[58] whereby the raw reads were mapped back to the chromosome and plasmid sequences to validate the assembly and resolve any remaining sequence errors. Non-circularised chromosomal and plasmid contigs were closed using primers designed on their 5′ and 3′ ends. The amplified PCR products were sequenced by the Australian Genome Research Facility and their sequences were manually integrated into the assembly.

Genome annotation. Gene calling and automatic functional annotation of the complete MS6671 chromosome and plasmids was performed using Prokka (*Prokka: Prokaryotic Genome Annotation System* - http://vicbioinformatics.com/) identifying 5,054 putative coding regions on the chromosome with an additional 644 putative coding regions distributed amongst the 5 plasmids and linear plasmid prophage. The complete annotated genome sequence has been deposited at the European Nucleotide Archive (Bioproject: PRJEB7538, Accessions: LN824133-LN824139).

Identification of antibiotic resistance genes. Initial identification of antimicrobial resistance genes from the complete PacBio assembly was performed using ResFinder (version 2.0)[59]. Additional screening for antimicrobial resistance genes was performed by comparison (BLASTp; sequence identity $>=40\%$; E-value $<=0.0001$) of all predicted coding regions against the Antibiotic Resistance Genes Database (ARDB)[60] and the Comprehensive Antimicrobial Resistance Database (CARD)[61]. Antimicrobial resistance genes were then subject to manual inspection to improve their functional annotation, correct start sites and identify point mutations, which may contribute to a resistant phenotype. Finally, resistance gene loci were screened for known insertion sequences and integrons by comparison against the ISFinder database[62] and Integrall[63], respectively.

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Author Contributions

H.M.Z., B.M.F., M.A.S., S.A.B. and D.L.P. designed the study, interpreted the results and wrote the manuscript. H.M.Z., B.M.F., M.A., A.A., Y.F. and J.L. collected the data. H.M.Z., B.M.F., M.A.S., S.A.B., D.L.P., T-M.C., W-F.Y. and K-G.C. performed the analysis.

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Original Paper

The Potential Role of Social Media Platforms in Community Awareness of Antibiotic Use in the Gulf Cooperation Council States: Luxury or Necessity?

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Abstract

The increasing emergence and spread of antimicrobial resistance (AMR) is a serious public health issue. Increasing the awareness of the general public about appropriate antibiotic use is a key factor for combating this issue. Several public media campaigns worldwide have been launched; however, such campaigns can be costly and the outcomes are variable and difficult to assess. Social media platforms, including Twitter, Facebook, and YouTube, are now frequently utilized to address health-related issues. In many geographical locations, such as the countries of the Gulf Cooperation Council (GCC) States (Saudi Arabia, United Arab Emirates, Kuwait, Oman, Qatar, and Bahrain), these platforms are becoming increasingly popular. The socioeconomic status of the GCC states and their reliable communication and networking infrastructure has allowed the penetration and scalability of these platforms in the region. This might explain why the Saudi Ministry of Health is using social media platforms alongside various other media platforms in a large-scale public awareness campaign to educate at-risk communities about the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV). This paper discusses the potential for using social media tools as cost-efficient and mass education platforms to raise awareness of appropriate antibiotic use in the general public and in the medical communities of the Arabian Peninsula.

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KEYWORDS

social media; antibiotics; awareness; health campaigns; Gulf States

Introduction

Antibiotic resistance has become a severe public health threat worldwide, including in the Gulf Cooperation Council (GCC) states [1]. Hence, various initiatives across the globe have been launched to combat this issue. In 2011, the World Health Organization (WHO) themed its annual day to address this issue with the slogan "No action today, no cure tomorrow" and listed the actions to be undertaken. These actions included providing education to achieve effective antibiotic use [2]. Antibiotic misuse, such as demanding unneeded antibiotics, purchasing antibiotics over-the-counter without a prescription, and not completing a course of antibiotics, is associated with the emergence and selection of antibiotic-resistant bacteria [3]. Nosocomial infections caused by antibiotic-resistant pathogens are significantly associated with an increased length of hospital stay and increased cost [4].

European surveys have shown that the general public still misunderstands the function and correct use of antibiotics [5,6]. On many occasions, the WHO has highlighted the importance of involving the general public alongside health care professionals for combating the emergence of antimicrobial resistance (AMR) [7,8]. To alleviate the public's lack of cognizance about AMR, various countries in Europe [7,9], as well as the United States [10] and other countries, have initiated public campaigns to raise awareness about the appropriate use of antibiotics.

Considering Saudi Arabia and other Gulf countries, various studies have addressed the issue of antibiotic misuse in hospital settings and the easy over-the-counter access to prescription antibiotics. For example, antibiotic use in intensive care units (ICUs) in Saudi Arabia has been found to be 10 times greater than that in the United States and some European countries [1,11-13]. This issue also extends to the wider GCC community. A Saudi study found that 77.6% of pharmacies dispensed antibiotics without a prescription primarily to treat scenarios consistent with viral infections [14], whereas 68.4% of antibiotics from Abu Dhabi pharmacies were sold over-the-counter [15], suggesting lack of antibiotics knowledge [16].

These examples of antibiotics misuse and others suggest the urgent need for a public campaign in Saudi Arabia and beyond to provide greater education on the proper use of antibiotics along with the concept of reserving antibiotics for use only when they are truly needed. In April 2015, the GCC Center for Infection Control released the multilevel GCC Strategic Plan for Combating Antimicrobial Resistance, which sets the framework for the regional and national plans [17]. One of the 5 strategic roadmaps addressed is the importance of preserving and restricting the available antimicrobial agents for human use. Interventional methods include educating antibiotic prescribers, patients, and the general public on the importance of appropriate antimicrobials use and basic infection prevention and control (eg, immunization and hygiene) [17].

Social media platforms are being widely used for health promotion advocates and to endorse traditional awareness campaigns. They have unique characteristics for sharing open access information, providing a platform for dynamic conversations with communities and social groups, and keeping users connected with their topics of interest [18]. They have been used to raise awareness for obesity, diabetes [19], and adolescent dating violence [20]. As an outreach effort, the European Antibiotic Awareness Day released a toolkit to advise on how to engage in social media activities promoting prudent antibiotic use [18]. Information provided in the toolkit relates to European countries and may not fully apply to the GCC states.

In this paper, we discuss the planning, setup, and potential effectiveness of developing a mass education campaign via social media platforms to raise general public and medical awareness of appropriate antibiotic usage in the GCC states.

The Influence of Educational Campaigns on Antibiotic Use and Antibiotic Resistance

Before initiating an educational media campaign, it is important to review the effectiveness of previous initiatives that were developed and launched in other parts of the world to raise awareness and provide guidance on responsible antibiotic use (Table 1).

Table 1. Summary of selected antibiotic awareness campaigns worldwide.

Name	Site (country)	Duration	Method used	Target audience	Reduction rate
Belgian Antibiotic Policy Coordinating Committee (BAPCOC) establishment [21]	Belgium	Launched in 1999-present	Multimedia campaigns, national campaigns, publication of clinical practice guidelines, support for the establishment of antibiotic management teams (AMTs)	Belgian community	36% reduction in outpatient antibiotic use per 1000 inhabitants per day during winter season
"Les antibiotiques c'est pas automatique" ("Antibiotics are not automatic"); part of the national campaign "Keep antibiotics working" [22]	All 22 regions across France	2001-2007	Mass media campaigns, one-on-one physician education sessions	General public and health care professionals	26.5% reduction of antibiotic prescriptions per 100 inhabitants during winter season over a 5-year period
Antibiotics Awareness Week [23]	Australia	2012-present	Facebook, Twitter, online pledging	All Australians	Unknown as yet
Local low-cost information campaign [9]	Emilia-Romagna region (Northern Italy)	November 2011-Februrary 2012	Brochures, posters, local media advertisements, and visual aids	General public	4.3% reduction in defined daily doses of prescribed antibiotics in intervention group
English public antibiotic campaigns [7]	England and Scotland	2008	Posters displayed in magazines and newspapers	General public	No improvement observed in postsurvey of public's understanding
e-Bug [24]	European countries and Saudi Arabia	Launched in 2006-present	Website-based games	Junior and senior school students	Not assessed

Data from Belgium and France have also revealed a reduction in the misuse of antibiotics after educational interventions. A decrease of 26.5% in antibiotic prescriptions was observed in France between 2002 and 2007 compared with the preintervention period (2000-2002), with the largest reduction observed in children [22]. A 36% reduction in antibiotic prescriptions was also observed in Belgium from 1999-2000 to 2006-2007 [21]. Both countries also reported a decrease in the incidence of infection with invasive penicillin- and macrolide-resistant *Streptococcus pneumoniae*. It was noted that the decrease in Belgium occurred before the wider use of the pneumococcal conjugate vaccine (PCV7), indicating that the vaccine did not contribute to this initial reduction in the incidence of infection with invasive penicillin-resistant pneumococci [5,6]. These data suggest the effectiveness of antibiotic awareness media campaigns in decreasing the use of antibiotics and hence in reducing the impact of antibiotic resistance.

Recently, Formoso et al [9] reported the effectiveness of a low-cost media campaign on antibiotic use in an Italian province that lasted for 5 months during the cold season. The intervention materials included visual aids, such as posters, brochures, and advertisements, which were used in the local media. The key messages of the campaign were codesigned by a physician practicing in the intervention area. Antibiotic prescriptions were significantly reduced by 4.3% in the intervention area compared with the control area. However, the general population's knowledge and attitudes about antibiotic resistance were not changed by the campaign [9].

In 2008, antibiotic awareness campaigns were carried out in England and Scotland by broadcasting key messages in advertising published in magazines and newspapers. Unfortunately, the campaign did not show any positive effects in either England or Scotland. No improvement was observed in the general public's understanding of antibiotic misuse to treat coughs and colds despite the fact that in 2009 more public respondents agreed that "resistance to antibiotics is a problem in British hospitals" than in 2008 [7]. In fact, this is not the only documented failure of antibiotic awareness campaigns. Huttner et al [5] reviewed 22 campaigns launched in high-income countries between 1990 and 2007. At least 3 of these campaigns failed and the effect of 3 others is unknown because of a lack of follow-up assessment on antibiotic use [5].

The e-Bug project is an example of an innovative approach to raise awareness about microbes and infection prevention. The aim of the e-Bug project is to disseminate educational materials about microbes (both beneficial and pathogenic) to junior and senior school students across Europe. The project relies on website-based gaming and entertainment-based lessons [24]. The e-Bug project now has partners in 26 different European countries, as well as Saudi Arabia, providing educational materials in different European languages and in Arabic [25]. On May 2015, the e-Bug website had a total of 17,391 visitors and the Saudi Arabian site had 76 visitors in total [26]. The impact of the e-Bug project is not clear because no evaluation of its implementation and impact on behavioral changes in targeted groups was ever performed [24].

Various factors have been suggested to be necessary to achieve success in antibiotic awareness campaigns. These include carefully designing key messages that are clear and simple, targeting both general public individuals and clinicians, and using television and radio [6,7]. Moreover, motivating

physicians to be involved in communicating with patients about the appropriate use of antibiotics and antibiotic resistance is also important [7]. Physicians' participation in developing campaign messages and communicating with the general public might significantly improve the chances of success. Early engagement can also have an impact on the sense of ownership of the campaign and facilitate physicians' consistent support [27]. This may indirectly influence physicians, focusing their attention on antibiotic prescribing and providing greater patient education.

Variable Sociological Factors

Awareness messages that display local surveillance data or amount of antibiotics locally misused may be important. Sharing with the general public the real-life experiences of individuals who have been infected with "superbugs" could be useful and may help the audience identify with those affected. Sharing real-life medical experience has been shown to be a useful communication platform from which to clarify public health-related stories, such as acne and cancer [28,29]. Considering these factors when designing awareness campaigns about antibiotics may help make an impact in GCC communities. Based on the Health Belief Model, perceived susceptibility (ie, you are at risk of getting infected) can be used to raise awareness [30,31]. However, delivering known messages to the target audience might result in a loss of interest and later disengagement. Educational interventions would be more successful if local contexts and barriers are adequately analyzed and addressed.

Replicating campaign strategies that have been initiated in different global regions outside the GCC region might not result in an effective outcome. It is crucially important to study the cultural factors and antibiotic distribution infrastructure in the GCC before thinking about the awareness messages. For example, the United States' Get Smart campaign [32] highly recommends that parents do not demand antibiotics for their children from the treating physician. We do not believe that this message line will be as effective as it might be in the United States because antibiotics can be purchased without a prescription from community pharmacies; therefore, a doctor's refusal might not make a difference. Approximately 37% of the total population of the GCC states consists of nonnational expatriates [33]; hence, it is important to consider cultural differences and not to neglect this segment when setting up a public awareness campaign. For example, the Saudi Ministry of Health has generated educational materials in multiple languages to fulfill this requirement [34].

Community pharmacies have a significant role in dispensing antibiotics in GCC communities. For example, 24.4% of 1645 recently surveyed antibiotic transactions in community pharmacies in Abu Dhabi were carried out without a prescription, including amoxicillin-clavulanic acid for sore throats and ceftriaxone for sexually transmitted infections [35]. The illegal practice of selling antibiotics over-the-counter, without a prescription, did not favor expatriates over citizens in the surveyed pharmacies in Abu Dhabi [15].

The self-prescription of antibiotics is another sociological factor that must be considered when designing antibiotic awareness campaigns in the GCC states [36,37]. This factor is strongly associated with the availability of antibiotics over-the-counter.

Before creating content to be used for awareness campaigns, it is necessary to conduct formative research to assess the public's existing knowledge of antibiotics resistance, understand the motivations for inappropriate antibiotics use, and learn about the social and cultural backgrounds for the targeted population. That will subsequently help to develop tailored key messages that can potentially encourage behavioral change [30]. Knowing these critical elements has lead to the success of many awareness campaigns, such as The Magic Glasses video to prevent soil-transmitted helminthes in China [31,38]. On the other hand, content produced for social media-based campaigns can be unrelated to the campaign's target. For example, it was found that the majority of Movember campaign-related tweets did not associate with prostatic and testicular cancer awareness [39], and the majority of tweets produced during breast cancer awareness week did not promote any specific preventive behaviors [40]. Despite the importance of developing related content, research into the correlation between social media-based awareness campaigns and behavior change is minimal because it is a new avenue in public health awareness [41].

Social media platforms can also contain contradictory health messages with potentially negative impact. Because social media platforms give users the freedom to publish their content, some of that content can contain medically misleading information, as found in YouTube videos promoting anorexia [42].

Funding

Funding is an important factor that may significantly affect a campaign's functionality and outcome. A systematic review of more than 20 international campaigns aimed at raising awareness of antibiotic use showed that these campaigns sourced their funding from different sectors, including the pharmaceutical industry [6]. The funding spent by pharmaceutical companies on promoting and marketing antibiotics is massive. For example, in 1998, it was estimated that pharmaceutical companies in the United States spent approximately US $1.6 billion to promote antibiotics [43]. On the other hand, media campaigns that encourage the prudent use of antibiotics are not widely supported [6]. Government funding is important. Because antibiotic awareness campaigns might translate into wiser use of antibiotics and potentially lead to a reduced selection of resistant bacteria, public funding should be offered to support awareness campaigns.

The cost of running a traditional mass media campaign to promote prudent antibiotic use in the community can be very expensive. For example, developing and conducting the French antibiotic awareness campaign carried out from 2002 to 2007 cost approximately €500 million over a 6-year period [44], whereas the Belgian campaign cost considerably less at approximately €400,000 per year [21]. The "Get Smart Colorado" campaign, which took place for 4 months in 2002, reported a cost of US $88,500 to purchase advertising space that included bus tails, bus stop posters, interior bus signs, and national public radio spots [10]. Similarly, the recent Italian campaign in 2013 cost approximately US $60,800 for purchasing media spots on television and radio, and in newspapers.

Approximately the same cost was spent to develop and print written visual aid materials, such as posters [9].

Considering social media platforms are free, establishing a social media-based campaign may be far cheaper than traditional media-based campaigns. However, in order to maintain continuous cyber presence and followers scalability, social media managers are usually hired [45], which can be an additional cost burden on social media-based campaigns. The key advantage of social media is the possibility to measure and track impressions and responses to online posts. These data can be used to guide social media campaigns to improve marketing strategy. However, platforms available to analyze big data generated from social media can be costly and may require technical expertise.

Time

Repeating the educational intervention over a long period of time is essential for the awareness success of mass media campaigns. Repetition over a long period of time has been demonstrated for causes such as smoking cessation and has helped achieve effectiveness [46]. The vast majority of antibiotic awareness campaigns launched in high-income countries between 1990 and 2007 were conducted over a period of more than 1 year [6]. Other campaigns, such as European Antibiotic Awareness Day (on November 18 each year) [47], are seasonal and have a long-term sustainable plan. However, the "Get Smart Colorado" campaign, which lasted for only 4 months, successfully showed a 3.8% net reduction in antibiotic dispensing at retail pharmacies as well as an 8.8% net decrease in managed care-associated antibiotic dispensing [10].

The ease of using social media, along with the indirect community contribution via "share" and "retweet" features, might provide long-term exposure and awareness messages to the wider general public. However, it is important to consider the temporal effect of social media feeds due to their short lifespan. It was found that the half-life of a tweet is approximately 24 minutes, whereas the half-life of Facebook posts is approximately 90 minutes [48]. A hashtag is a keyword preceded by a hash sign (#) that is used to identify and categorize messages on a specific topic, which can give the topic a longer lifespan in social media [49,50]. Keeping the audience engaged and interested in the topic is another important consideration. This might be achieved by ensuring that the key messages and materials are not overrepeated throughout the campaign's duration. Updating campaign materials with new and relevant data might keep the audience more engaged and keen to receive updated educational materials. Lastly, the time chosen to post the social media message can be critical for the lifespan of social media posts [51].

The Value of Social Media Platforms to Communities in the Gulf Cooperation Council States

The total number of users of the social networking website Facebook in the Arab world (22 countries) had grown to 54,552,875 by the end of May 2013; 33.4% of users are female and 68% are younger than 30 years [52]. Facebook users in the GCC states represent approximately 22% (12 million) of the total Facebook users in the Arab world [52] (Table 2).

Table 2. The use of Internet and social media platforms in the GCC states.

Country	Population (million)[a]	Internet users (million), n (%)[b]	Facebook users (million),[c] n (%)	Twitter users (million),[d] n (%)
Saudi Arabia	28.4	13.0 (45.8)	6.4 (22.5)	1.9 (6.7)
United Arab Emirates	8.3	5.7 (71.0)	3.4 (41.7)	0.4 (4.8)
Kuwait	3.1	2.0 (63.2)	0.8 (26.8)	0.2 (7.3)
Bahrain	1.2	1.0 (80.0)	0.3 (25.1)	0.1 (5.6)
Qatar	1.7	1.7 (99.9)	0.6 (34.4)	0.1 (4.4)
Oman	3.3	2.1 (63.6)	0.5 (16.4)	0.04 (1.2)

[a] Population figures obtained from [53].

[b] Internet user figures obtained from [54].

[c] Facebook user figures obtained from [52].

[d] Twitter user figures obtained from [55].

For the microblogging website Twitter, the number of active users in the Arab world reached 3,766,160 individuals as of March 2013, with an estimated 10,832,000 tweets per day. Saudi Arabia has the highest number of active Twitter users in the Arab world, with 1.9 million individuals, which is approximately 50% of the total Twitter users in the Arab region. Approximately 47% and 11% of the total tweets in the Arab world are generated from Saudi Arabia and United Arab Emirates, respectively.

The video-sharing website YouTube is also a popular media platform in the GCC, particularly in Saudi Arabia. As an update to research conducted by Forbes Middle East, we present data from selected local GCC talk shows on YouTube (Table 3). It is clear that these shows attract many viewers, although some shows from Saudi Arabia receive the most attention. This audience would make these shows an excellent platform for delivering awareness messages to a larger number of viewers.

Table 3. The popularity of selected YouTube-based shows in the GCC states.

Name of show	Origin	Launch date	Episodes[a]	Subscribers[a]	Total views[a]	Average views per episode[a]
EyshElly	Saudi Arabia	Feb 2011	63	1,714,699	197,686,128	3,137,875
3al6ayer	Saudi Arabia	Sep 2010	45	810,119	61,462,338	1,365,029
	Saudi Arabia	Sep 2010	33	649,465	72,887,865	2,208,723
Endam Cinema	Oman	Jul 2013	6	215	50,864	8477
Balalee6	United Arab Emirates	Jun 2012	13	6686	972,165	74,781
	United Arab Emirates	May 2012	5	10,133	954,815	190,963
shenoya3nitv	Kuwait	Jan 2012	54	67,477	8,076,193	149,559
How to Prevent from Corona	Saudi Arabia	May 2014	1	17,759	2,973,376	NA

[a] The figures were obtained from YouTube channels on November 15, 2013.

Examples of Saudi Public Health Awareness Messages Delivered Through Social Media Platforms

Owing to its high profile and popularity among Internet users in the GCC region, YouTube has often been used in Saudi Arabia as a platform to deliver public health–related awareness messages and campaigns (Table 4). We have noticed 2 different models for delivering health-related topics on YouTube in Saudi Arabia. One model was noticed in many campaigns that used comedy talk shows with large audience as a platform to deliver the awareness messages. With the help of other social media platforms, such as Facebook and Twitter, these messages have traveled far and wide, attracting a large number of viewers. For example, a Saudi-based comedy show named “Fe2aFala” released an episode about acquired immune deficiency syndrome (AIDS) and this episode attracted over 1 million viewers.

Table 4. Health messages delivered through Saudi YouTube-based shows.[a]

Show/channel name	Type	Awareness about	Number of viewers	Channel subscribers
Phosphine	Special episode	Phosphine gas	3,288,241[b]	14,821
Lumink	Special show	Health promotion	2,583,534	807,561
Telfaz11	Special episode	Breast cancer	1,489,802	296,563
Fe2aFala	Special episode	AIDS	1,139,948	351,907
Sen_tube	Entire show	Dental care	1,292,959	29,373
3almezan	Entire show	Obesity and well-being	1,097,713	51,746
Hotcoldshow	Special episode	Diabetes	455,485	81,725
MedScoope	Entire show	Health promotion	88,703	937

[a] The figures were obtained by accessing the YouTube channels on the March 4, 2014.

[b] This number of views was achieved within only 3 days of uploading the video on YouTube.

Another model is to use YouTube as a channel similar to traditional mass media to distribute health awareness messages. For example, in June 2014, the Saudi Arabia Ministry of Health launched an engaging public awareness campaign using YouTube, Twitter, Facebook, educational posters, and health guideline updates to educate the general public and medical communities on the emergence of, and health precautions needed for, MERS-CoV [34]. In this example, social media platforms may have been used to create online presence to endorse awareness messages delivered locally on traditional media. It must be noted that the incidence rate of MERS-CoV has declined [56] with the multi-faceted intervention, which social media has been a part of.

Publishing awareness content on social media might allow international distribution and an indefinite exposure period. However, in order to potentiate the effectiveness of a YouTube video, additional advertising strategies must take place. Table 4 summarizes some of the campaigns that have used YouTube shows to deliver public health awareness in Saudi Arabia and have attracted a high number of views.

Our Pilot Experience

Many health care facilities and organizations have started using Twitter as a teaching tool; for example, “tweeting” about specific health problems has been used by major organizations such as the Centers for Disease Control and Prevention (CDC), WHO, the National Cancer Institute (NCI), and the National Institutes of Health (NIH). “Tweets” go out from a sender and

are simultaneously received by all members of a group of "followers," providing a fast, open, and easy way to deliver a particular focused message.

We used Twitter in our online Arabic-language pilot campaign focusing on superbugs using a hashtag. We delivered short tweets and links to various articles and videos related to superbugs. We also translated multiple non-Arabic research articles and news for our more than 34,000 followers, who were primarily from Saudi Arabia and the surrounding areas. This medium gave us the ability to have a real-time conversation by answering their questions and concerns about the topic. To track our efforts, we regularly reviewed the number of followers, updates, retweets, and "mentions" in Twitter. Many evaluation metrics for Twitter can be collected. We evaluated our Twitter pilot study by analyzing the influence of some of our tweets. We kept track of how many of our "followers" published updates including "retweets" or "at replies" over time. A simple analysis of 147 selected tweets with the Arabic superbug hashtag resulted in approximately 4100 retweets between July and November 2013. This information does not tell us which of the tweets encouraged followers to go on to read the full article or watch the video, but it does give us useful information on general interest levels.

We created a whiteboard animation with a voiceover video [57] that discussed the importance of antibiotics, how superbugs are created, and the spread of superbugs in the GCC. We also discussed possible factors that could contribute to the emergence and spread of superbugs in addition to advice on how to control superbugs as reviewed previously [1].

Interestingly, the number of retweets that we received from our superbugs hashtag correlated with the number of YouTube views that we recorded for our video. The video viewing pattern as determined by YouTube was as expected: there were a large number of views soon after the video was posted and released on Twitter, and there were fewer views from 3 weeks later until the present day. We observed 2 large peaks in viewing rates within the first 2 weeks of the video being released. We believe the first one was due to our successful Twitter activities. We are unclear about what caused the second and largest jump in YouTube views. It is possible that the video was picked up by another group and spread on Twitter again (Figure 1).

We also observed sporadic smaller peaks in YouTube views from 3 weeks onward. These numbers suggest that the video was still drawing attention and being shared, most likely on social media, even months after being posted.

Our viewer retention rate was consistent with what YouTube estimates to be the average retention rate for videos of a similar length—approximately half of the video. This rate suggests that the length of our video—5.5 minutes—is adequate to capture the viewers' attention.

Subsequently, we created an infographic video uploaded on YouTube [58] that combined a selection of our tweets with visual and audio enhancement. The tweet that featured the video received 152 retweets and 130 favorites, and the video was viewed more than 3000 times in only 2 weeks (Figure 2).

The content generated for our pilot campaign was primarily dependent on our existing knowledge of antibiotic misuse in Saudi Arabia and neighboring states as previously reviewed [1,14-16,35-37]. We acknowledge the limitations of our pilot campaign resulting from the lack of formative research, its Arabic content, and reliance on social media. In order to achieve prospective behavioral change, it is important to understand the motivation for antibiotic misuse in the general public. Future research should conduct a thorough survey, interviews, and observations to cover the diverse population of the GCC states. This will help us design relevant key messages to be used in future awareness campaigns. The diverse ethnic groups and socioeconomic status of people in the Gulf States should also be considered in future campaigns because more languages and media platforms might need to be used. Relying solely on a social media platform in an awareness campaign might overlook the large population of migrant workers in the GCC who might not have access to social media platforms.

Figure 1. YouTube views over a period of 84 days for the whiteboard animated and voiceover video about superbugs and proper antibiotics use.

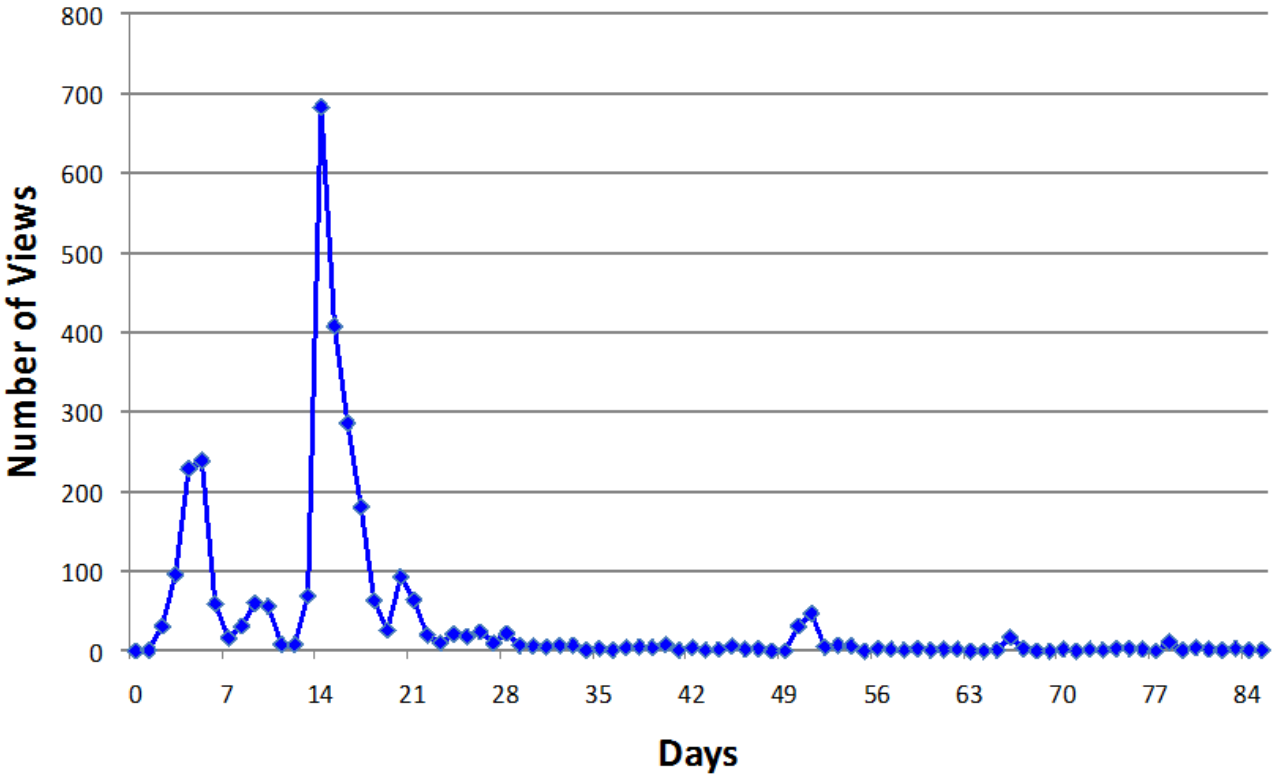
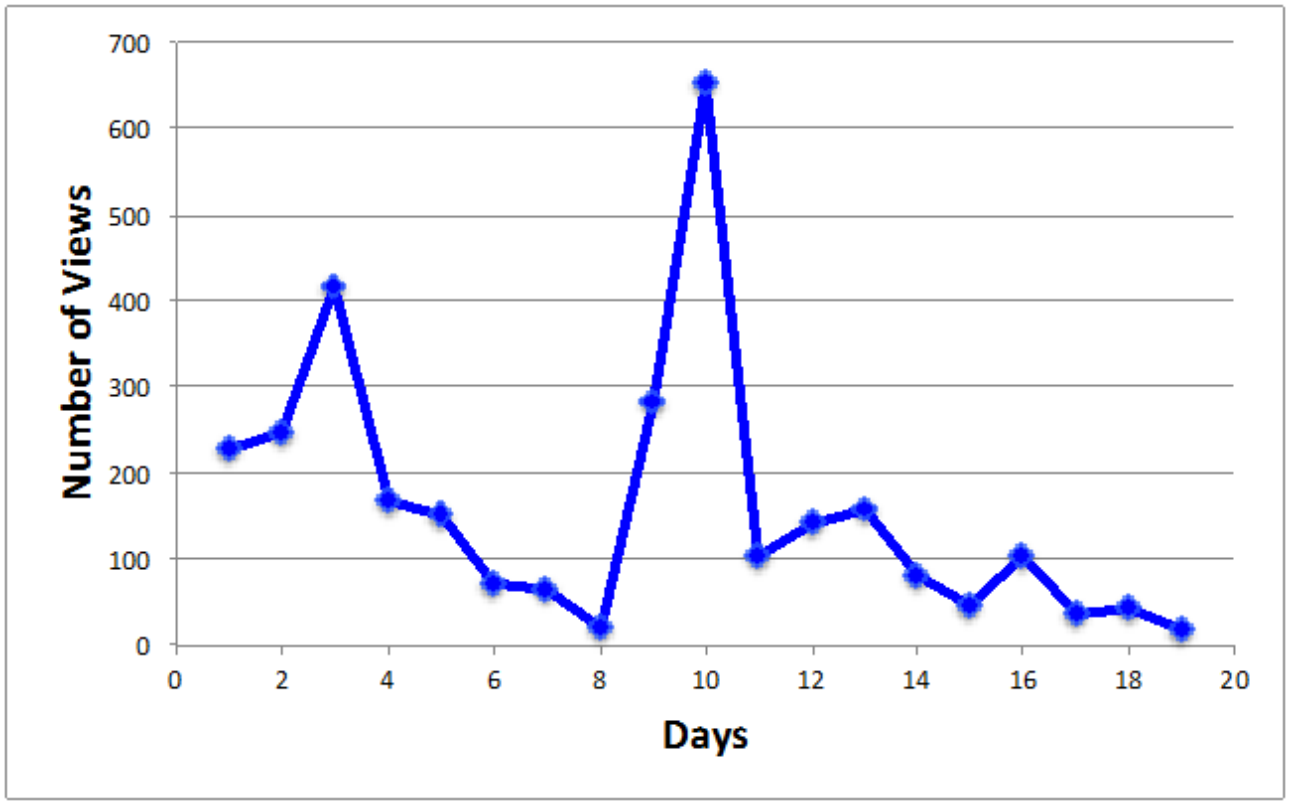


Figure 2. YouTube views over a period of 20 days for the infographic video.



Conclusion

Reducing the suboptimal use of antibiotics among the general public and medical community through awareness activities is an important element in national plans to combat rising AMR. However, it is important to create awareness content that is related to the target audience and based on formative research. Social media platforms seem to be a valuable platform for delivering awareness messages. Owing to social media popularity, awareness messages could reach a large number of

users and the reach can be tracked. Through our pilot experience we have successfully distributed antibiotics awareness messages through Twitter and YouTube to our target audience in the Gulf counties and Saudi Arabia. The use of social media can also enhance awareness campaigns delivered in traditional media channels. However, it is important to consider the cultural demographic diversity, which could limit the reach of awareness massages, such as the high population of immigrant workers in the GCC who might not have access to these emerging social media platforms. Social media–based messages can also have short life span, which might limit the effect and reach of key awareness messages. Measuring the impact of the social media–based awareness campaigns on the general public's understanding and behavioral change is a challenge and needs further research.

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Conflicts of Interest

None declared.

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Abbreviations

AIDS: acquired immune deficiency syndrome
AMR: antimicrobial resistance
GCC: Gulf Cooperation Council
ICU: intensive care unit
NCI: National Cancer Institute
NIH: National Institutes of Health

WHO: World Health Organization

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Genetic Contexts of bla_{NDM-1} in Patients Carrying Multiple NDM-Producing Strains

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The carbapenem resistance determinant bla_{NDM-1} has been found in various Gram-negative bacteria and upon different plasmid replicon types (Inc). Here, we present four patients within two hospitals in Pakistan harboring between two and four NDM-1-producing Gram-negative bacilli of different species coresident in their stool samples. We characterize the bla_{NDM-1} genetic contexts of these 11 NDM-1-producing Gram-negative bacilli in addition to other antimicrobial resistance mechanisms, plasmid replicon profiles, and sequence types (STs) in order to understand the underlying acquisition mechanisms of carbapenem resistance within these bacteria. Two common plasmid types (IncN2 and IncA/C) were identified to carry bla_{NDM-1} among the six different bacterial species isolated from the four patients. Two of these strains were novel *Citrobacter freundii* ST 20 and ST 21. The same IncN2-type bla_{NDM-1} genetic context was found in all four patients and within four different species. The IncA/C-type bla_{NDM-1} genetic context was found in two different species and in two of the four patients. Combining genetic context characterization with other molecular epidemiology methods, we were able to establish the molecular epidemiological links between genetically unrelated bacterial species by linking their acquisition of an IncN2 or IncA/C plasmid carrying bla_{NDM-1} for carbapenem resistance. By combining plasmid characterization and in-depth genetic context assessment, this analysis highlights the importance of plasmids in antimicrobial resistance. It also provides a novel approach for investigating the underlying mechanisms of bla_{NDM-1}-related spread between bacterial species and genera via plasmids.

Infectious pathogens have the ability to be transmitted from one person to another. Antimicrobial resistance in certain instances has been observed to follow this paradigm of transmission; specifically, bacterial species have been described as transmitting antimicrobial resistance determinants. A well-known example of a successful international clone is the *Escherichia coli* sequence type (ST) 131 transmitting $bla_{CTX-M-15}$ (1, 2). However, numerous reports in the last few years provide evidence that plasmids are a major factor in the transmission of antimicrobial resistance (3, 4).

Since the first report (5), bla_{NDM} has been reported to be harbored by a diverse range of bacterial species, among which the most frequent are within the *Acinetobacter* genus and *Enterobacteriaceae* family (6, 7). Furthermore, bla_{NDM} has also been identified to reside upon different plasmid replicon types (Inc) among the *Enterobacteriaceae* family, including IncA/C (8), IncF types (9), IncL/M (10), IncN (11), IncX (12), and IncH (13). In addition, the genetic structure or context in which bla_{NDM} resides varies between different plasmid types and even within the same plasmid type (14). The bla_{NDM-1} genetic contexts observed with *Enterobacteriaceae* plasmids frequently involve various mechanisms of gene acquisition, including different IS*CR* elements (15), class one integrons (16), flanking insertion (IS) elements (15), and singleton IS elements present in close proximity (8, 10). In contrast, bla_{NDM} genetic contexts within *Acinetobacter* spp. have been reported with less variation since bla_{NDM} can be commonly found within the 10,099-bp transposon known as Tn*125*, composed of two flanking IS*Aba125* sequences (7, 17–19).

The Indian subcontinent is recognized as a major reservoir for bla_{NDM} acquisition and has been suggested as the geographical origin of bla_{NDM} (6, 20). In a previous study from our group, 66 NDM-1-producing Gram-negative isolates from stool samples of patients in two Pakistani hospitals were reported (20). Among patients harboring these isolates, four patients were found to carry multiple NDM-1 producers of different species. Sartor and colleagues also determined that the species within each patient were characterized by different plasmid replicon type profiles (20). This warranted further investigation in order to elucidate the underlying nature of bla_{NDM-1} acquisition by different species when the bacterial hosts have different plasmid replicon profiles. The aim of this study was to further characterize the different NDM-producing bacterial species coexisting in multiple patients through whole-genome sequencing, noting their resistance mechanisms and sequence types (STs) with a further focus on characterizing the bla_{NDM-1} genetic contexts.

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TABLE 1 Strains and their respective sequence types, antimicrobial resistance profiles, and plasmid replicon typing results[d]

Patient	Strain characteristics			Plasmid replicon types via Plasmidfinder (Inc)						PBRT (Inc)		Antimicrobial resistance determinants via Resfinder																														
												Beta-lactamases (*bla*)						Aminoglycoside resistance determinants									Quinolone resistance determinants			Other resistance determinants														
	Strain	Bacterial Species	MLST (ST)	A/C_2	N2	HI1	HI2	F types	Others	Plasmid carrying bla_{NDM}	Other plasmids present strain	NDM-1	CTX-M-15	OXA-1	OXA-10	TEM-1B	Other	*aac(6')-Ib-cr*[b]	*rmtC*	*armA*	*strA & strB*	*aac(3)-IIa*	*aadA1*	*aadA16*	*aac(3)-IId*	*aph(3')VIa*	Other	*qnrB1*	*qnrS1*	Other	*catB3*	*catA1*	*sul1*	*sul2*	*dfrA14*	*dfrA27*	*tet(A)*	*ARR-3*	Additional resistance determinants
1	Pn2	*Enterobacter cloacae*	171			FIA (HI1)	HI2A			A/C_2							ACT-7 CMY-6									*aadA4*													
	Pn3	*Aceinteobacter baumannii*	113	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A						OXA-64																					
	Pn4	*Klebsiella pneumoniae*	101					FIB_K FII	R	N2							SHV-1									*aadA5*			*qnrB66* *oqxA* *oqxB*										*mph(A)* *dfrA17*
	Pn5	*Citrobacter freundii*	20[c]				HI2A	FIB		-																			*qnrB34*										*mph(E)* *msr(E)* *floR* *tet(C)* *dfrA5*
2	Pn13	*Pseudo-citrobacter faecalis*	N/A				HI2A	FIB_K		N2																													
	Pn14	*Escherichia coli*	2598				HI2A	FIB_K FIB FIA	I1 Col_{156}	N2	I1 FIA FIB																														
3	Pn27	*Pseudo-citrobacter faecalis*	N/A				HI2A	FIB_K		N2																														
	Pn28	*Escherichia coli*	1431			HI1A HI1B	HI2A	FII	Col (BS512)	-	A/C HI1						CMY-4 OXA-9																							*msr(E)* *dfrA12*
4	Pn66	*Escherichia coli*	10					FIB_K	Y	A/C_2	Y						CMY-6									*aadA4*													
	Pn67	*Citrobacter freundii*	21[c]					FII(pMET) FII_{Yp}		FII_Y							CMY-73												*qnrB54*										*mph(A)*
	Pn68	*Enterobacter cloacae*	171			FIA (HI1)	HI2A			A/C_2							ACT-7 CMY-6									*aadA2* *aacA4*													

[a] PBRT was performed on transconjugants carrying the plasmid harboring bla_{NDM}.
[b] This enzyme is responsible for aminoglycoside and quinolone resistance.
[c] Novel *Citrobacter freundii* sequence types determined by MLST.
[d] Shaded boxes indicate the replicon types and genes present in each strain as determined by PlasmidFinder and ResFinder. N/A, not applicable (no positive plasmid replicon typing results for *Acinetobacter* species and no MLST scheme for *P. faecalis*).

MATERIALS AND METHODS

Bacterial isolates. Eleven clinical isolates were acquired in 2010 from stool samples from four different patients in two hospitals in Rawalpindi, Pakistan (21). In the study conducted by Perry and colleagues (21), one patient carried four species, three patients had three species, and five patients had two species of NDM-1 producers (data not shown). Isolates were selected based on the number of isolates present in the patients and the diversity of their plasmid type profiles, which was determined by PCR-based replicon typing (PBRT) of *Enterobacteriaceae* isolates as previously described (20, 22). The strains identified from each patient are as follows (Table 1): patient 1, *Enterobacter cloacae* Pn2, *Acinetobacter baumannii* Pn3, *Klebsiella pneumoniae* Pn4, and *Citrobacter freundii* Pn5; patient 2, *Pseudocitrobacter faecalis* Pn13 and *Escherichia coli* Pn14; patient 3, *P. faecalis* Pn27 and *E. coli* Pn28; patient 4, *E. coli* Pn66, *C. freundii* Pn67, and *E. cloacae* Pn68.

Plasmid transfer experiments through conjugation. Plasmids carrying bla_{NDM-1} from clinical strains were transferred by conjugation to confirm their transferability and plasmid replicon type. For conjugation experiments, the 11 clinical isolates (donor strains) were verified to be susceptible to sodium azide via agar dilution. Donor strains and sodium azide-resistant *E. coli* J53 recipients were grown in LB broth at 200 rpm to logarithmic phase (23). The conjugation mixture consisted of donor and recipient strains in a 1:1 ratio that were plated onto MacConkey agar and then incubated at 37°C overnight (24). The conjugation mixture of approximately 20 μl of confluent growth on the plate was then harvested into 1 ml of saline and serially diluted in saline to 10^{-8}. This serial dilution method was performed to obtain single colonies of both donors and transconjugants between the serial dilutions of 10^{-4} and 10^{-6}. One hundred microliters of each dilution was then inoculated onto MacConkey agar supplemented with meropenem (0.1 μg/ml) and sodium azide (100 μg/ml) or with sodium azide only (100 μg/ml) and onto a control plate without additives; plates were incubated at 37°C for 24 h. Successful transconjugants were confirmed phenotypically and through PCR for bla_{NDM-1}. PBRT was performed on transconjugants to identify the plasmid replicon type carrying bla_{NDM-1} (22).

Sequencing and bioinformatics. Paired-end libraries of whole genomic DNA of all 11 clinical isolates were prepared and sequenced by the Illumina MiSeq platform (Illumina, San Diego, CA, USA). All sequences were *de novo* assembled using the CLC Genomic Workbench, version 7.5 (CLC Bio, Aarhus, Denmark). pNDM-BJ01 (GenBank accession number JQ001791) (17) was manually annotated and used as a reference for Tn*125* structure annotation. Sequences from the GenBank database and IS Finder (https://www-is.biotoul.fr/) were used to identify and characterize genes flanking the Tn*125* region. The CLC Genomic Workbench was used for a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and to analyze and manually annotate the genetic context of bla_{NDM-1} according to the aforementioned reference sequences. Contigs containing bla_{NDM-1} were named as follows: patient 1, pPN2-ECL-NDM-1 (*E. cloacae* Pn2), PN3-AB-NDM-1 (*A. baumannii* Pn3), pPN4-KP-NDM-1 (*K. pneumoniae* Pn4), and PN5-CF-NDM-1 (*C. freundii* Pn5); patient 2, pPN13-PCF-NDM-1 (*P. faecalis* Pn13) and pPN14-EC-NDM-1 (*E. coli* Pn14); patient 3, pPN27-PCF-NDM-1 (*P. faecalis* Pn27) and PN28-EC-NDM-1 (*E. coli* Pn28); patient 4, pPN66-EC-NDM-1 (*E. coli* Pn66), pPN67-CF-NDM-1 (*C. freundii* Pn67), and pPN68-ECL-NDM-1 (*E. cloacae* Pn68).

Contigs of each clinical strain were submitted to the ResFinder, version 2.1 (25) (https://cge.cbs.dtu.dk/services/ResFinder/), and PlasmidFinder, version 1.1 (26), databases (available at the Center of Genomic Epidemiology website [http://www.genomicepidemiology.org]) to characterize their resistance mechanism genes and plasmid Inc types.

Sequence typing via an MLST scheme. Each clinical isolate was submitted to the MLST, version 1.7, database (27) (https://cge.cbs.dtu.dk/services/MLST/) for sequence typing via respective multilocus sequence

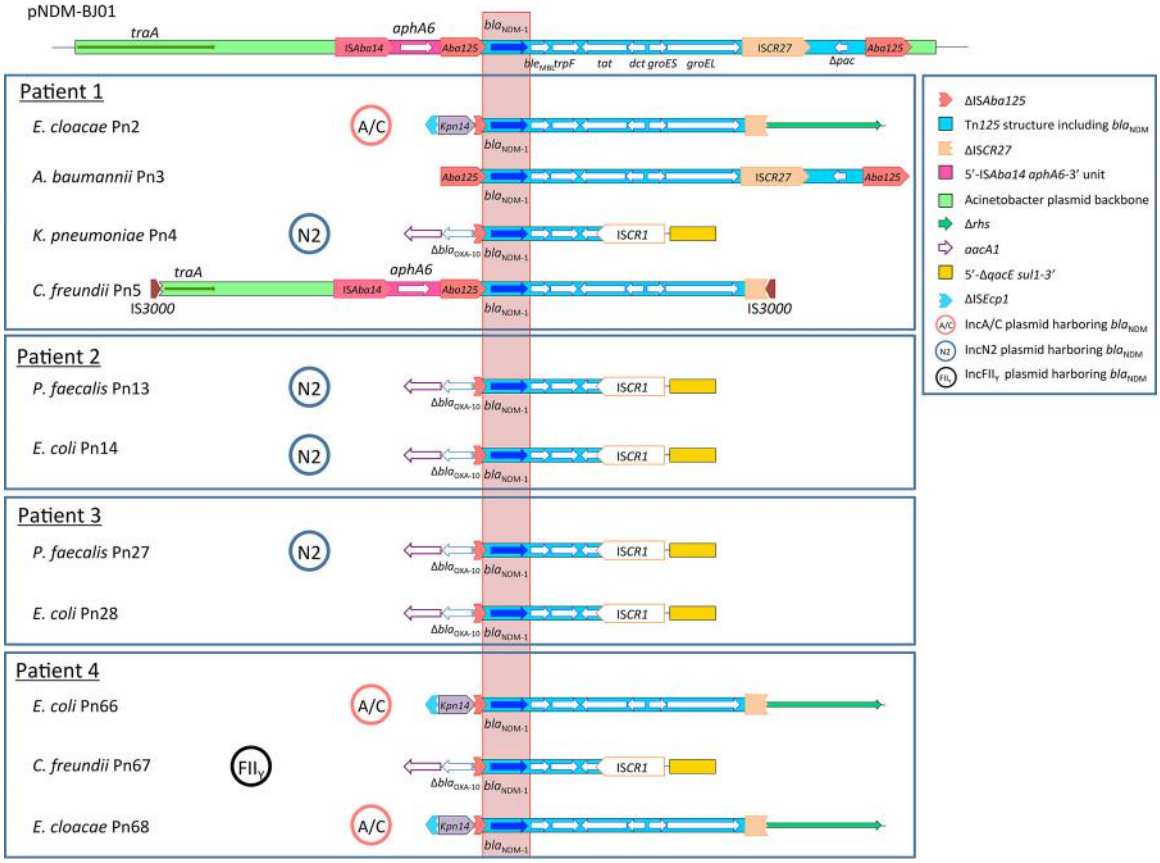

FIG 1 Schematic representation of all bla_{NDM} genetic contexts described within this study and the reference sequence pNDM-BJ01 (GenBank accession number JQ001791). bla_{NDM} genetic contexts and their GenBank accession numbers are as follows: Pn2, pPN2-ECL-NDM-1 (KP770024); Pn3, PN3-AB-NDM-1 (KP770025); Pn4, pPN4-KP-NDM-1 (KP770033); Pn5, PN5-CF-NDM-1 (KP770032); Pn13, pPN13-PCF-NDM-1 (KP770031); Pn14, pPN14-EC-NDM-1 (KP770030); Pn27, pPN27-PCF-NDM-1 (KP770029); Pn28, PN28-EC-NDM-1 (KP770023); Pn66, pPN66-EC-NDM-1 (KP770028); Pn67, pPN67-CF-NDM-1 (KP770027); and Pn68, pPN68-ECL-NDM-1 (KP770026). Δ, truncated gene. Insertion sequence (IS) elements are represented as block arrows.

typing (MLST) schemes, except for *P. faecalis* strains Pn13 and Pn27 as no MLST schemes were available (28). Both *C. freundii* strain Pn5 and strain Pn65 were identified as novel sequence types and subsequently submitted to the *C. freundii* MLST website (http://pubmlst.org/cfreundii/) (29) for assignment of new sequence types.

Nucleotide sequence accession numbers. Each nucleotide sequence was deposited in the GenBank database under the following accession number: pPN2-ECL-NDM-1, KP770024; PN3-AB-NDM-1, KP770025; pPN4-KP-NDM-1, KP770033; PN5-CF-NDM-1, KP770032; pPN13-PCF-NDM-1, KP770031; pPN14-EC-NDM-1, KP770030; pPN27-PCF-NDM-1, KP770029; PN28-EC-NDM-1, KP770023; pPN66-EC-NDM-1, KP770028; pPN67-CF-NDM-1, KP770027; and pPN68-ECL-NDM-1, KP770026.

RESULTS AND DISCUSSION

The molecular and *in silico* analysis results of each NDM-1-producing strain are summarized in Table 1. These results also include for each strain the clinically significant antimicrobial resistance determinants found within each strain, ST type, and replicon type (Inc) of the plasmid carrying bla_{NDM-1} (determined via PBRT of successful transconjugants). *In silico* analysis of each contig with bla_{NDM-1} initially identified a truncated Tn*125* structure harboring bla_{NDM-1} (ΔTn*125*). The sizes of ΔTn*125* structures were variable (Fig. 1). Different genes and IS elements were identified flanking the ΔTn*125* structures (Fig. 1). The combination of the different ΔTn*125* structure sizes and flanking contexts identified four distinct bla_{NDM-1} genetic contexts.

The most common bla_{NDM-1} genetic context, 5′-*aacA1* Δbla_{OXA-10} ΔTn*125*-3′ (identified in 5 of 11 strains and in all four patients), contained a 2,341-bp ΔTn*125* structure with *aacA1* conferring aminoglycoside resistance and a truncated narrow-spectrum β-lactamase, bla_{OXA-10}, upstream and was determined to be on IncN2-type plasmids (expect in Pn28 and Pn67). This bla_{NDM-1} genetic context was found within three different bacterial species, *K. pneumoniae* Pn4 (pPN4-KP-NDM-1), *P. faecalis* Pn13 and Pn27 (pPN13-PCF-NDM-1 and pPN27-PCF-NDM-1, respectively), *E. coli* Pn14 and Pn28 (pPN14-EC-NDM-1 and PN28-EC-NDM-1, respectively), and *C. freundii* Pn67 (pPN67-CF-NDM-1), and is similar to an *E. coli* isolate from Japan (direct submission under GenBank accession number AB769140) and to two IncN3 plasmids, pLK75 (GenBank accession number KJ440076) of *E. coli* and pLK78 (GenBank accession number KJ440075) of *K. pneumoniae* isolated from Taiwan (11). Transmission of bla_{NDM-1} may have occurred via conjugation of an IncN2 plasmid with this genetic context, as suggested by our successful conjugation experiments involving strains carrying IncN2 plasmids. These conjugation events would subsequently result in the different bacterial

species producing NDM-1 within three of the four patients. The IncN2 bla_{NDM-1} genetic context was also found in *C. freundii* Pn67 in the fourth patient; however, it was identified on an $IncFII_Y$-type plasmid. This genetic context was also identified in *E. coli* Pn28 and believed to be located on the chromosome or on a nonconjugative plasmid as conjugation experiments were unsuccessful after multiple attempts. The bla_{NDM-1} genetic contexts in Pn28 and Pn67 are highly similar to those of the IncN2 plasmids of our study as well as to those in the literature; however, they were not located on IncN2 plasmids. It may be speculated the "IncN-like" bla_{NDM-1} genetic contexts of Pn28 and Pn67 may have diverged from the IncN2 plasmids of our study through such events as homologous recombination (30). Alternatively, Pn28 and Pn67 could have been the source from which the IncN2 plasmids acquired the bla_{NDM-1} genetic context prior to horizontal gene transfer among the different species. Further investigation, including full plasmid construction, will be required to clarify the nature of these two isolates and the potential for bla_{NDM-1} cassette transfer between the plasmid backbones IncN2 and $IncFII_Y$.

Another bla_{NDM-1} genetic context observed, found in 3 of the 11 isolates, involved a longer 6,332-bp ΔTn*125* structure. In contrast to the aforementioned IncN2 ΔTn*125* structure, this longer ΔTn*125* structure was flanked by an IS*Kpn14* element and truncated IS*Ecp1* upstream and by a truncated type IV secretion protein, *rhs*, downstream (Fig. 1). This IncA/C bla_{NDM-1} genetic context was determined to be on IncA/C-type plasmids and was found in two patients, patient 1, carrying *E. cloacae* strain Pn2 (pPN2-ECL-NDM-1), and patient 4, carrying *E. coli* strain Pn66 (pPN66-EC-NDM-1) and *E. cloacae* strain Pn68 (pPN68-ECL-NDM-1). Similar to the aforementioned IncN2 bla_{NDM-1} genetic contexts, the IncA/C bla_{NDM-1} genetic context was also observed within two different species within the same patient (patient 4). Further, it is very similar to the most frequently reported IncA/C bla_{NDM-1} genetic context of the *Enterobacteriaceae* family (8, 14, 31). The combination of IncA/C bla_{NDM-1} genetic context identification (Fig. 1), the previously reported clonal spread of *E. cloacae* and *E. coli* within these hospitals, and the identification of IncA/C plasmids as the most prevalent plasmid types (20) might suggest the contribution of this specific IncA/C plasmid to the carriage and transmission of carbapenem resistance among *Enterobacteriaceae* within this clinical setting.

Out of the 11 isolates, two unique genetic contexts for bla_{NDM-1} were found in PN3-AB-NDM-1 (*A. baumannii* Pn3) and PN5-CF-NDM-1 (*C. freundii* Pn5) in patient 1. PN3-AB-NDM-1 carried a context composed of the longest Tn*125* structure (7,962 bp), which is very similar to the Tn*125* lengths frequently described within the *Acinetobacter* genus; i.e., it was composed of a full Tn*125* structure extending from the left-hand IS*Aba125* element to the right-hand IS*Aba125* (7, 18, 32, 33). PN5-CF-NDM-1 contained a 7,288-bp ΔTn*125* structure flanked by two IS*3000* sequences (truncated left-hand IS*Aba125* to truncated IS*CR27*) with plasmid backbone elements in close proximity, such as *traA*. This context has not been previously reported. Both bla_{NDM-1} genetic contexts, PN3-AB-NDM-1 and PN5-CF-NDM-1, may potentially be located on a nonconjugative plasmid or on the chromosome, as suggested by unsuccessful transfers in conjugation experiments. Of note, both PN3-AB-NDM-1 and PN5-CF-NDM-1 were found in patient 1, who carried four different NDM-1-producing species, i.e., *E. cloacae*, *A. baumannii*, *K. pneumoniae*, and *C. freundii*, each with a different bla_{NDM-1} genetic context (Fig. 1).

The characterized bla_{NDM-1} genetic contexts of 11 clinical isolates retrospectively may suggest interspecies transmission of antimicrobial resistance at an *in vivo* level via plasmids. This has been previously described with bla_{KPC} (34). Initial characterization of our isolates identified different plasmid replicon profiles with multiple species within the same patient. Genetic characterization in combination with other molecular typing methods has described, clarified, and provided an insight into the spread and acquisition of bla_{NDM-1}. Apart from the nonconjugative bla_{NDM-1} found in Pn3, Pn5, and Pn28 and the single $IncFII_Y$ plasmid in Pn67, the remaining strains have become carbapenem resistant by acquiring IncN2- and IncA/C-type plasmids with the specific aforementioned bla_{NDM-1} genetic contexts.

The prevalence of highly similar bla_{NDM-1} genetic contexts within different species and among different patients highlights the possible role plasmids are able to provide in interspecies transmission of carbapenem resistance. We suggest that genetic characterization of plasmids with respect to bla_{NDM-1} could be considered a tool similar to the multilocus sequencing typing (MLST) approach, which utilizes the typing of conserved regions within the genome for comparison, to investigate the clonal epidemiology of antibiotic-resistant bacteria (1, 35, 36). By considering genetic context characterization in combination with standard molecular methods (bacterial species identification, resistance mechanism profiling, and clonality via MLST) during epidemiological studies, sophisticated epidemiological links between patients and genetically unrelated bacterial species can be clarified in the case of bla_{NDM-1} and potentially other plasmid-mediated antimicrobial resistance determinants that have genetic context variation, such as $bla_{CTX-M-type}$ (37, 38) or $bla_{CMY-type}$ (39). Such investigation will assist in clarifying whether within a specific geographical region and facility antimicrobial resistance acquisition by species has occurred via a particular plasmid with a specific genetic context.

Here, we have characterized the genetic nature in which bla_{NDM-1} resides in different NDM-1-producing bacterial species coexisting in multiple patients and identified IncA/C and IncN2 plasmids as the platform providing carbapenem resistance to otherwise diverse and unrelated species of *Enterobacteriaceae* within the clinical setting of two hospitals in Pakistan. The combination of in-depth genetic plasmid characterization and epidemiological molecular investigation methods presented here not only highlights the ability of plasmids to transmit and provide antimicrobial resistance determinants to various bacterial species and genera but also provides a novel approach for investigating the underlying mechanisms of bla_{NDM-1}-related spread associated with hospitalized patients.

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Species identification within *Acinetobacter calcoaceticus–baumannii* complex using MALDI-TOF MS

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ABSTRACT

Acinetobacter baumannii, one of the more clinically relevant species in the *Acinetobacter* genus is well known to be multi-drug resistant and associated with bacteremia, urinary tract infection, pneumonia, wound infection and meningitis. However, it cannot be differentiated from closely related species such as *Acinetobacter calcoaceticus*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* by most phenotypic tests and can only be differentiated by specific, time consuming genotypic tests with very limited use in clinical microbiological laboratories. As a result, these species are grouped into the *A. calcoaceticus–A. baumannii* (*Acb*) complex. Herein we investigated the mass spectra of 73 *Acinetobacter* spp., representing ten different species, using an AB SCIEX 5800 MALDI-TOF MS to differentiate members of the *Acinetobacter* genus, including the species of the *Acb* complex. *RpoB* gene sequencing, 16S rRNA sequencing, and *gyrB* multiplex PCR were also evaluated as orthogonal methods to identify the organisms used in this study. We found that whilst 16S rRNA and *rpoB* gene sequencing could not differentiate *A. pittii* or *A. calcoaceticus*, they can be differentiated using *gyrB* multiplex PCR and MALDI-TOF MS. All ten *Acinetobacter* species investigated could be differentiated by their MALDI-TOF mass spectra.

1. Introduction

Acinetobacter baumannii is an opportunistic, aerobic, Gram-negative nosocomial pathogen that has become one of the six most important multi-drug resistant microorganisms worldwide (Antunes et al., 2014). *A. baumannii* is commonly known to cause difficult to treat pneumonia, bacteremia, urinary tract infection, wound infection and meningitis (Maragakis and Perl, 2008).

Accurate identification of *A. baumannii* and its closely related species *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *Acinetobacter calcoaceticus* is important as each of these species may display different characteristics in regard to antimicrobial susceptibilities, pathogenicity and clinical outcomes (Chuang et al., 2011; Sedo et al., 2013). The environmental strain *A. calcoaceticus* has not been reported to cause infection in humans whilst the other organisms are all of clinical significance (Peleg et al., 2008, 2012). A recent paper has described increased severity and mortality in bacteraemia patients infected with *A. baumannii* compared with those infected with *A. pittii* and *A. nosocomialis*, emphasising the need for accurate differentiation (Fitzpatrick et al., 2015).

However, these four species which together make up the *A. calcoaceticus–A. baumannii* (*Acb*) complex are indistinguishable by phenotypic based tests (Peleg et al., 2008; Wang et al., 2013; Lee et al., 2014).

Molecular methods such as 16S rRNA and *rpoB* gene sequencing and have shown to be useful in differentiating members of the *Acinetobacter* genus (La Scola et al., 2006; Zarrilli et al., 2009; Wang et al., 2014). However, neither method is sufficient to differentiate species such as *A. pittii* and *A. calcoaceticus* (Higgins et al., 2010), and other molecular methods such as PCR amplification of intrinsic resistance genes or *gyrB* are used. However, these methods are mainly applied in research settings and have very limited use in clinical laboratories (La Scola et al., 2006; Higgins et al., 2010; Kamolvit et al., 2014).

Matrix assisted laser desorption ionisation — time of flight mass spectrometry (MALDI-TOF MS) has been shown to be a rapid and highly discriminatory method for the identification of bacteria (Kliem, 2010; Welker et al., 2011; Sedo et al., 2013). MALDI-TOF instruments that are commonly used in clinical settings for bacterial identification rely on the use of proprietary identification software and spectral databases (Martiny et al., 2012).

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In this study, we evaluated the use of a research-use-only (RUO) 5800 MALDI-TOF MS (AB SCIEX, Concord, ON, Canada) to differentiate and identify various species in the *Acb* complex, as well as other *Acinetobacter* spp. using only the standard instrument software and freely available open-source software for the acquisition, processing and interpretation of spectra.

2. Materials and methods

2.1. Bacterial isolates and reference strains

The strains investigated in our study included thirty two multilocus sequence typed (MLST typed) *A. baumannii* isolates, six *Acinetobacter* reference strains and thirty five additional *Acinetobacter* isolates as described below:

The MLST typed *A. baumannii* isolates included sixteen isolates of sequence type (ST)110, four isolates of ST92 and three isolates of ST109 (Huber et al., 2014). In addition to this, four isolates of from the Arabian peninsula were added to the study, including one isolate from Saudi Arabia of the ST195 and one of ST436, respectively, plus one isolate of ST208 from Kuwait and one of ST229 from Qatar (Zowawi et al., 2015).

Two additional isolates of ST208 were of Japanese origin, and one isolate of ST208 was from Thailand, and two Singaporean strains were of ST491 (Kamolvit et al., unpublished data). The isolates from Japan, Thailand and Singapore were collected between 2008 and 2010, and the MLST typing was performed in silico. The Kenyan strains and the strains from the Arabian peninsula were collected and MLST typed as previously described (Huber et al., 2014; Zowawi et al., 2015). MLST typing has been performed according to the Oxford scheme for all isolates (http://pubmlst.org/abaumannii/).

The following reference strains and previously published isolates and were added to the study; *A. baumannii* ATCC 19606, *A. calcoaceticus* ATCC 14987, *Acinetobacter lwoffii* ATCC 15309 and ATCC 17986, *Acinetobacter johnsonii* ATCC 17909, *Acinetobacter junii* ATCC 17908, *Acinetobacter baylyi* ($n = 1$), *A. calcoaceticus* ($n = 1$), *A. pittii* ($n = 1$) (Peleg et al., 2012) and *A. nosocomialis* ($n = 2$) (Peleg et al., 2012; Carruthers et al., 2013).

Various additional *Acinetobacter* spp. from Kenya ($n = 4$, provided by the AGA KHAN University hospital in Nairobi, Kenya and collected between 2010–2011), Japan ($n = 12$, provided by the Toho University in Tokyo, Japan and collected in 2010), Australia ($n = 2$, collected at the Royal Brisbane and Women's hospital in Brisbane, Australia in 2004 and 2006 respectively), Thailand ($n = 7$, provided by the Siriaj Hospital in Bangkok), Singapore ($n = 5$, collected in 2008 and provided by the National University of Singapore). All isolates were grown on Mueller Hinton agar and incubated for 24 h in a 37 °C incubator, and identification and confirmation of species was performed as described in chapter 2.2.

2.2. 16S rRNA identification

All *Acinetobacter* spp. isolates were initially identified by the sequencing of the 16S rRNA gene as previously described (Misbah et al., 2005). Sequencing was performed by Macrogen Inc., Seoul, Korea, and sequences were blasted on NCBI using the megablast function against the 16S ribosomal RNA sequences database with maximum target sequences being set at 100. If 16S rRNA sequencing was unable to identify an isolate using the highest percentage identity, score and an E-value of 0 resulting in a sequence that matches two species with identical lengths, *rpoB* gene sequencing of zones 1 and 2 was performed as previously described (La Scola et al., 2006). A previously described *gyrB* multiplex PCR (Higgins et al., 2010) was used to differentiate *A. calcoaceticus* and *A. pittii*.

2.3. MALDI-TOF MS

MALDI-TOF MS analyses were conducted on a 5800 TOF/TOF set in linear positive mode running the TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) at a laser frequency of 100 Hz with a set mass range of 3000 to 20,000 Da. A continuous stage motion set in a random pattern at 600 μm/s was used for sampling.

An in-house sinapinic acid matrix consisting of 10 mg of sinapinic acid (>99.0% for MALDI-MS, Fluka 85,429) in 500 μL acetonitrile, 475 μL distilled water and 25 μL 80% trifluoroacetic acid (TFA, LC-MS grade, Fluka 40967) was adapted from a previously published protocol (Freiwald and Sauer, 2009). Calibration was performed using calibration mixture 2 (AB SCIEX, Framingham, Massachusetts) which contained Angiotensin I, ACTH (1–17 clip), ACTH (18–39 clip), ACTH (7–38 clip) and insulin (bovine) to ensure mass accuracy within 5 ppm.

2.4. Acquisition of mass spectra

A small amount of bacteria (approximately 10^6–10^8 cfu) was transferred from a 24 h culture by spreading a thin layer onto a sample spot on an Opti-TOF 384 MALDI plate insert (AB SCIEX, Framingham, Massachusetts) and overlaid with 1 μL of sinapinic acid matrix. Each isolate was spotted in quadruplicate and each replicate scanned once. Laser intensity was set at 4322 units and at a pulse rate of 100 Hz with a total of 1000 spectra accumulated for each sample. A mass range of m/z 3000 to m/z 20,000 and a continuous stage motion set in a random pattern at 600 μm/s was used for sampling. The TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) was used to acquire mass spectra.

2.5. Processing of spectra

Mass spectra files were non-manipulatively converted from t2d files to mzXML files using a t2d converter (http://www.pepchem.org) and processed using mMass version 5.50 (Strohalm et al., 2008) (http://www.mmass.org/). Processing of raw spectra was conducted in mMass 5.5 (Martin Strolham) with a peak picking algorithm that used baseline correction, Savitzky-Golay smoothing and a signal to noise ratio of 3. Replicates of the same isolates were averaged to form a consensus spectrum.

3. Results

3.1. Bacterial identification

Using 16S rRNA identification, we confirmed the following *Acinetobacter* spp. in our collection; *A. baumannii* ($n = 37$), *A. nosocomialis* ($n = 15$), *A. junii* ($n = 3$), *A. lwoffii* ($n = 2$), *A. johnsonii* ($n = 1$), *A. baylyi* ($n = 1$), *Acinetobacter soli* ($n = 1$) and *Acinetobacter bereziniae* ($n = 1$).

Using *rpoB* gene sequencing and *gyrB* multiplex PCR we determined the remaining fourteen strains to be *A. pittii* ($n = 12$) and *A. calcoaceticus* ($n = 2$). The results are as summarised in Table 1.

3.2. MALDI-TOF MS based characterisation

All ten *Acinetobacter* species investigated in our study had sufficient differences in their mass spectra to be characterised and differentiated using MALDI-TOF MS. In all of the *A. baumannii* strains investigated ($n = 35$) we observed the presence of a characteristic, high intensity mass of m/z 5743.05, as well as two other specific masses of m/z 8583.00 and m/z 8715.00 that could be used to distinguish *A. baumannii* from other *Acinetobacter* spp. (Fig. 1). Additionally, all the species that were not part of the *Acb* complex could also be characterised and differentiated (Summarised in Table 1).

Table 1
Acinetobacter spp. investigated in this study with the methods that were used to identify each species and their indicative MALDI peak masses.

Bacterial species	Methods of species confirmation	Indicative MALDI peak masses (*m/z*)
A. baumannii ($n = 35$)	16S rRNA gene sequencing	5743.05, 8583.00 and 8715.00
A. nosocomialis ($n = 15$)	16S rRNA gene sequencing	8134.00, 8542.00 and 8315.00 or 8356.00 or 8481.00
A. pittii ($n = 12$)	16S rRNA gene sequencing, *rpoB* gene sequencing and *gyrB* multiplex PCR	5743.05 or 5780.03, 8620.00, 8822.00
A. junii ($n = 3$)	16S rRNA gene sequencing	10,849.00
A. calcoaceticus ($n = 2$)	16S rRNA gene sequencing, *rpoB* gene sequencing and *gyrB* multiplex PCR	5829.00
A. lwoffii ($n = 2$)	16S rRNA gene sequencing	7613.00, 7662.00, 9101.00 and 9219.00
A. johnsonii ($n = 1$)	16S rRNA gene sequencing	4309.00 and 6720.00
A. baylyi ($n = 1$)	16S rRNA gene sequencing	5669.00
A. soli ($n = 1$)	16S rRNA gene sequencing	5519.00
A. bereziniae ($n = 1$)	16S rRNA gene sequencing	8018.00 and 9247.00

Nine of the twelve *A. pittii* isolates studied had a representative high intensity mass of *m/z* 5780.03 and two other specific masses of *m/z* 8620.00 and *m/z* 8822.00. In addition, three of the twelve isolates were also characterised by a mass at *m/z* 5743.05, with similar intensity to that of the one in *A. baumannii*. However, the mass at *m/z* 5780.03 was absent (Fig. 1).

In *A. nosocomialis* we observed a representative mass at *m/z* 8134.00 in all isolates, and another mass at *m/z* 8542.00 in fourteen of fifteen isolates (Fig. 2). In *A. calcoaceticus*, we observed a representative prominent peak at *m/z* 5829.00 that occurred in both isolates of this species (Fig. 2).

4. Discussion

Retrospective studies found that *A. baumannii* was associated with higher mortality rates, greater antimicrobial resistance and different clinical characteristics than the ones of *A. nosocomialis* and *A. pittii* (Chuang et al., 2011; Lee et al., 2011; Yang et al., 2013). This raises the importance of being able to differentiate the organisms within the *Acb* complex to allow for treatment to be optimised for specific organisms.

A. calcoaceticus–A. baumannii complex organisms are hard to differentiate because of high phenotypic similarities (Gerner-Smidt et al., 1991). Molecular methods such as 16S rDNA and *rpoB* sequencing as well as *gyrB* PCR amplification are possible methods to discriminate between these strains. We observed that 16S rRNA sequencing or *rpoB* gene sequencing were not sufficient to differentiate *A. pittii* and *A. calcoaceticus*, and that the identification of these two organisms required *gyrB* multiplex PCR. However, in our investigation these species showed MALDI-TOF mass spectra that are clearly distinct from each other.

MALDI is a soft desorption ionisation method which may result in the formation of ions without significantly breaking chemical bonds, which is particularly useful in protein analysis. MALDI instruments commonly used for bacterial identification use licensed software packages that are incompatible with instruments of other brands. Such instruments are usually single TOF mass analysers that do not allow for efficient structure and sequence investigation of analytes. The ABSciex

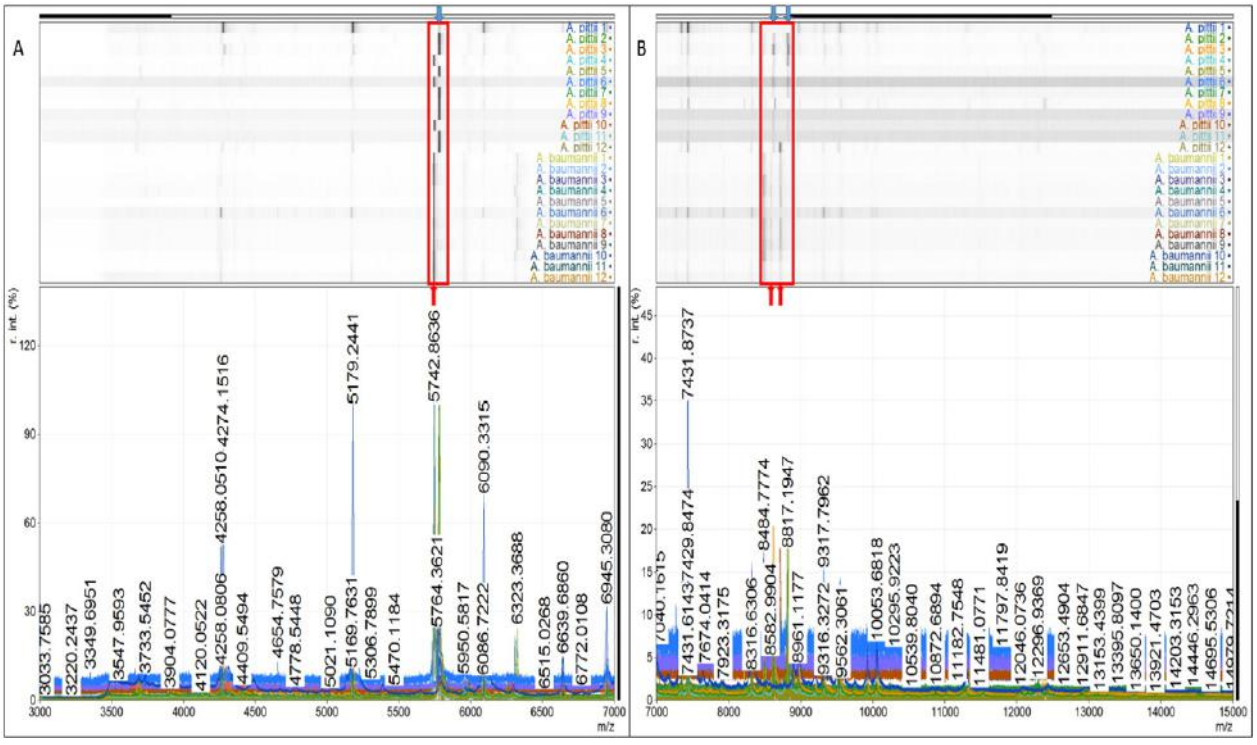

Fig. 1. (A) Mass range of *m/z* 3000–7000 of *A. baumannii* and *A. pittii*. The red arrow indicates the mass of *m/z* 5743.05 and the blue arrow indicates the mass of *m/z* 5780.03. Three *A. pittii* isolates have the mass of *m/z* 5743.05, normally indicative of *A. baumannii*. However, none of the *A. pittii* isolates have both masses. (B) Mass Range of *m/z* 7000–15,000 of *A. baumannii* and *A. pittii*. The red arrows indicate the masses of *m/z* 8583.00 and *m/z* 8715.00 that are representative of *A. baumannii*. The blue arrows indicate the masses of *m/z* 8620.00 and *m/z* 8822.00 which are representative of *A. pittii*.

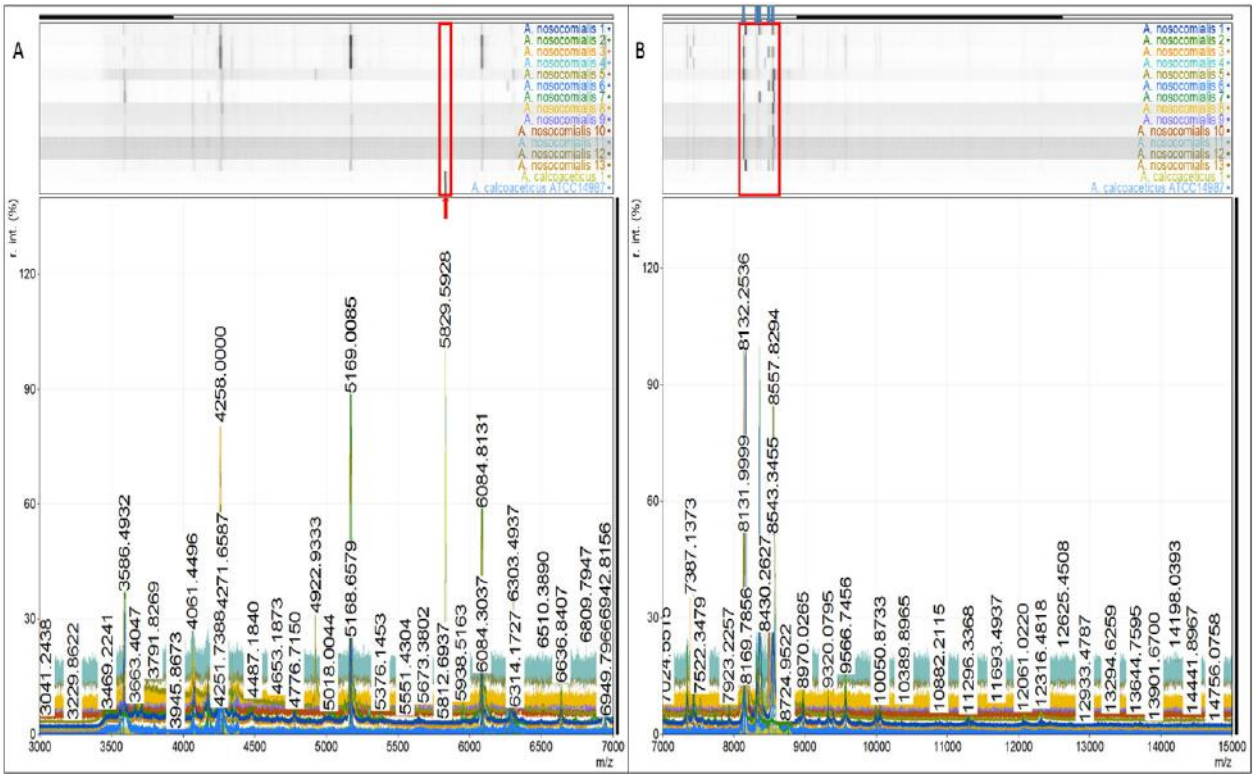

Fig. 2. (A) Mass range of *m/z* 3000–7000 of *A. nosocomialis* and *A. calcoaceticus*. The red arrow indicates the mass of *m/z* 5829.00 which is indicative of *A. nosocomialis*. (B) Mass Range of *m/z* 7000–15,000 of *A. nosocomialis* and *A. calcoaceticus*. The blue arrows indicate the masses *m/z* 8134.00, *m/z* 8542.00, *m/z* 8315.00 or *m/z* 8356.00 or *m/z* 8481.00 that are indicative of *A. nosocomialis*.

5800 instrument in our laboratory is a TOF/TOF instrument which can overcome the limitations of a single TOF mass analyser by linking two TOF mass analysers in series, making it a much more powerful tool in protein research (Ng et al., 2014). However, due to the lack of a database, bacterial identification is not readily performed with this instrument.

The use of freeware to augment the abilities of RUO instruments such as the 5800 TOF/TOF greatly increase the functionality and availability of these instruments for bacterial identification. The ability to differentiate species without using automated systems may be a step back from the automated systems that the VITEK-MS and the Biotyper offer, but it validates the ability of MALDI-TOF instruments to visualise spectral differences for greater discrimination between closely related species.

As we have seen in our study, MALDI-TOF was able to differentiate closely related species such as *A. pittii* and *A. calcoaceticus*. It appears that MALDI-TOF may also differentiate various *Acinetobacter* spp. from each other, as well as organisms that fall within the *Acb* complex. Automated processes would be required if this method is to be used as part of a diagnostic procedure.

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Leading Article

Antimicrobial resistance in Saudi Arabia

An urgent call for an immediate action

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ABSTRACT

تَزايد تسليط الضوء على الميكروبات المقاومة للمضادات كقضية مُلحة لصحة الحيوان ولصحة البشر في جميع أنحاء العالم. يتجلى هذا الموضوع بشكل واضح في البكتيريا التي تقاوم المضادات الحيوية عند استخدامها كخيار أخير. مما ينبيء بعدوى غير قابلة للعلاج في المستقبل. أقترحت الوكالات الدولية استراتيجيات لمكافحة الميكروبات المقاومة للمضادات. هناك العديد من التحديات في المملكة العربية السعودية التي يمكن أن تحفز ظهور وانتشار البكتيريا المقاومة للأدوية المتعددة. لمواجهة هذه التحديات لابد من بذل مجهود من قطاعات متعددة للسيطرة بنجاح على انتشار وظهور الميكروبات المقاومة للمضادات. لابد أن تتضمن الإجراءات المراقبة الفعّالة لتتبع ظهور هذه الميكروبات وانتشارها. ولابد أيضاً من إعطاء الأولوية في تحسين احتياطات الوقاية من العدوى ومكافحتها للحد من انتشارها. من الضروري رفع مستوى الوعي للحد من استخدام المضادات الحيوية غير المناسبة، وينبغي على برامج إدارة المضادات الحيوية في المستشفيات، والعيادات الخارجية، والصيدليات تنظيم الاستخدام المستمر للمضادات الحيوية.

Antimicrobial resistance (AMR) is increasingly being highlighted as an urgent public and animal health issue worldwide. This issue is well demonstrated in bacteria that are resistant to last-line antibiotics, suggesting a future with untreatable infections. International agencies have suggested combating strategies against AMR. Saudi Arabia has several challenges that can stimulate the emergence and spread of multidrug-resistant bacteria. Tackling these challenges need efforts from multiple sectors to successfully control the spread and emergence of AMR in the country. Actions should include active surveillance to monitor the emergence and spread of AMR. Infection prevention and control precautions should also be optimized to limit further spread. Raising awareness is essential to limit inappropriate antibiotics use, and the antibiotic stewardship programs in hospital settings, outpatients, and community pharmacies, should regulate the ongoing use of antimicrobials.

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Antimicrobial resistance (AMR) in bacteria is emerging and spreading rapidly worldwide. This phenomenon is nowadays affecting public and animal health dramatically on a global level. The discovery of penicillin in the last century provided significant advancements in modern medicine, as well as our ability to treat commonly encountered infectious diseases, one of the biggest killers during the pre-antibiotics era. Many of life-saving interventions, such as cancer chemotherapy and major surgeries depend on effective antimicrobials to succeed.[1] Unfortunately however, the current dependence on antibiotics - whether to treat, prevent, or stimulate food animal growths - have exponentially increased this resistance. When antibiotics are used, selective pressure is created, and possibly forcing the exposed bacteria to mutate or acquire pieces of DNA to become antibiotic resistant.[2] The selection pressure resulting from the overwhelming use and misuse of antibiotics is exponentially supporting the AMR phenomenon.[3] Despite this known fact, approximately 10 million tons of antibiotics are globally used every 10 minutes, which mostly are not related to

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justified medical use.[4] As a result to the global march of AMR, common infections, such as urinary-tract infections are becoming difficult to treat. This is mainly due to bacteria that are resistant to last-line antibiotics,[2] or even pan-drug resistant that are not responding to any commercially available antibiotics.[5] The multidrug-resistant (MDR) pathogens are spreading rapidly in many parts of the world causing severe medical and economic consequences. It is estimated that at least every 10 minutes a patient dies in the USA or Europe because of fatal infections caused by antibiotic resistant bacteria.[6] This article gives a brief overview on the current situation of AMR in Saudi Arabia and the immediate actions needed to tackle this issue.

The emergence and spread of AMR bacteria in Saudi Arabia. ***Current and future challenges.*** A systematic literature review of MDR in Gram-negative bacilli (GNB) showed a substantial increase in the rate of carbapenem-resistant GNB in Saudi Arabia over the last decade in comparison with the rates of the 1990s. It also documented the increasing prevalence of extended spectrum beta-lactamase (ESBL) producing isolates from Saudi Arabia, where some institutes had 29% ESBL rates among *Escherichia coli* (*E. coli*) and 65% ESBL rates among *Klebsiella pneumoniae (K. pneumoniae)*. As a result, these increasing rates have been associated with many reported outbreaks and mortality that ranged between 11-40%.[7]

Recent region-wide surveillance studies reported that most of carbapenem resistant Enterobacteriaceae (CRE) from the Gulf Cooperation Council States (GCC) have been found to harbor the carbapenemase encoding genes $bla_{\text{OXA-48-type}}$, and $bla_{\text{NDM-1}}$.[8] Carbapenem resistant *Acinetobacter baumannii (A. baumannii)* (CRAB) from Saudi Arabia have also increased dramatically over the years. A recent study from Riyadh[9] showed that the susceptibilities of *A. baumannii* to meropenem and imipenem in 2006 ranged between 64-81.2%, while the susceptibility in 2012 ranged between 8.3-11%. Molecular investigation on different CRAB isolates obtained from all of the GCC states revealed that large number of isolates, from different states, have clustered together, suggesting clonality.[10]

The last-line resource antibiotic currently available to tackle many of carbapenem resistant GNB is colistin. However, colistin resistant and even pan-drug resistant GNB have already been reported. Colistin resistance is typically chromosomally mediated and generally not transmissible between bacteria. Nevertheless, the recent description of plasmid-mediated colistin resistance mechanism *mcr-1* has enormous implications on the lifespan of colistin.[11] Plasmid carrying *mcr-1* was found in many parts of the world among Enterobacteriaceae and non-fermentative GNB.[11] A recent report highlighted the presence of the *mcr-1* gene in 4 *E. coli* that were isolated between 2012-2015 from Saudi Arabia, Bahrain, and the United Arab Emirates.[12] It is likely though that *mcr-1* carrying GNB are widely disseminated in more isolates and between other species in the Arabian Peninsula.

Other studies from the GCC region[5,13] affirmed that the region harbours other rare and novel antibiotic resistance mechanisms. For example, PME-1 ESBL producing *Pseudomonas aeruginosa (P. aeruginosa)* from Qatar,[13] and the pandrug-resistant *K. pneumoniae* from neighboring UAE.[5] The latter report provides a very significant finding, as this breakthrough should raise the global attention to avoid the possible catastrophic future of AMR, which can be caused by such an untreatable pathogen.

A Saudi national surveillance on Gram-positive cocci demonstrated that 32% of *Staphylococcus aureus* (*S. aureus*) are methicillin-resistant (MRSA), and 33% of *Streptococcus pneumoniae* are resistant penicillin G and 26% are resistant to erythromycin.[14] A study from Riyadh[15] demonstrated that *S. aureus* was colonizing the nasal cavity of 40% of the 200 tested healthcare workers. Among those *S. aureus*, 45% were methicillin-resistant (MRSA), resulting in total prevalence of 18% health workers carrying MRSA. A national survey[16] on anti-tuberculosis drug resistance found that only 1.6% of total TB demonstrated MDR phenotype. These figures of high prevalence among different bacterial species in Saudi Arabia are unfortunately likely to be sustained, if not increased to due to several factors.

Local risk factors contributing to the emergence and spread of AMR. Several factors are associated to the increasing emergence and spread of MDR bacteria in Saudi Arabia. It is evident that the unoptimized use of antibiotics is a major factor for AMR development. A hospital in Riyadh has well demonstrated the overuse of antimicrobial agents from 4 adult ICUs in 2010, where the highest use was meropenem (33.2 defined daily doses [DDD] per 100 bed-days), followed by piperacillin-tazobactam (16.0 DDD/100 bed-days). On the other hand, the DDD/100 bed-days in 37 ICUs

in the United States was 3.75 for carbapenems and 7.08 for antipseudomonal penicillins.[7] Over-the-counter antibiotics without prescription in Saudi community pharmacies is another issue that is driving the improper use of antibiotics. Only one out of 88 pharmacists in Eastern province refused to sell antibiotics without a prescription, and 77.6% of the pharmacies in Riyadh dispensed antibiotics without a prescription.[7] To my knowledge these are no published evidences from Saudi Arabia demonstrating the use of antibiotics as a growth promoter. However, several reports, in fact, have described the isolation of various MDR GNB from food animal specimens.[7]

Heavy international travel activities are occurring due to the large population of expatriates and to pilgrimage the holy cities. Travel is a known risk factor for acquiring and transmitting infectious diseases, including those caused by antibiotic resistant bacteria. A recent study[17] showed that returned travellers from Hajj have acquired MDR *A. baumannii* and NDM producing *E. coli* during the Hajj event. Previous data from 2 major hospitals in Makkah showed that ceftazidime resistance is evident in 24.6% of *E. coli*, 34.4% of *K. pneumoniae*, and 52.7% of *P. aeruginosa*. Another report showed that septicemia episodes in Makkah are increased by 16.5% during Hajj time due to the influx of international patients.[7]

Another issue that can contribute to the spread of AMR is the challenges related to the adherence of infection control practices. The hand hygiene compliance rate in a hospital in Makkah in 2011 was 50.3%. The effectiveness of hand hygiene compliance was well demonstrated in controlling a nosocomial outbreak caused by carbapenem-resistant *K. pneumoniae* in Riyadh.[7] **Figure 1** illustrates the several factors that may contribute to the emergence and spread of AMR.

Immediate actions needed. To limit this alarming threat on human health, the World Health Organization launched the Global Action Plan on Antimicrobial Resistance.[18] This plan was signed off by most of member states, including Saudi Arabia during the

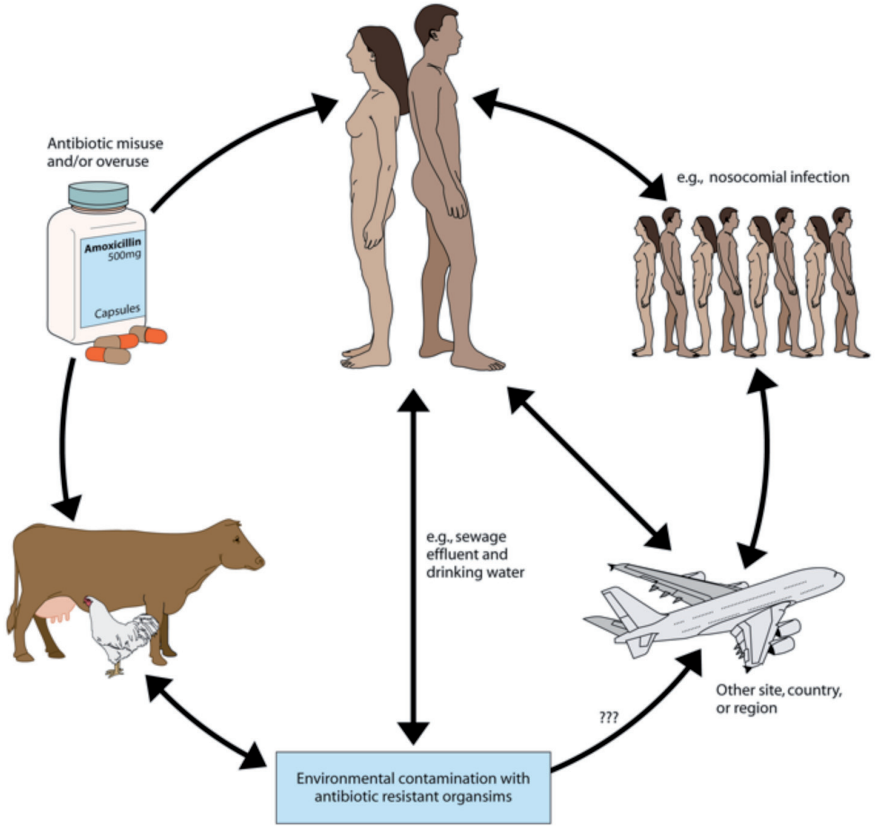

Figure 1 - Different factors can stimulate the emergence and spread of multidrug-resistant bacteria.

World Health Assembly in 2015. The plan consists of 5 pillars; to improve awareness, stringing knowledge through surveillance, reduce the incidence of infection, optimize the use of antimicrobial agents, and develop the economic case for sustainable investment to support the need in all countries in regards to new medicines, diagnostic tools, vaccines, and other interventions. To have tailored strategies, different countries initiated their own national AMR action plans, and the most notable similarity between all action plans is the willingness to tackle the antimicrobial misuse in both human and animal/agricultural sectors.

Raising public, medical, and veterinary awareness of AMR is an important element that is urgently needed to tackle AMR in Saudi Arabia. Previous research[7] has shown that antibiotics are widely used in the community, without prescription, to treat unrelated scenarios. Multilevel and nationwide awareness campaigns in Saudi Arabia are needed to align with the World Antibiotic Awareness Week. The success of these campaigns can be dependent on the tailored and relevant content and key messages that are designed after formative research. The increasing use of social media platforms in the country can also help to disseminate awareness messages. It is very essential however, to consider all segments and socioeconomic groups in the Saudi society, as some members might not speak the local language, or actively use social media platform.[19]

Infection prevention and control plays a very important role to limit the spread of AMR. Conducting educational programs for healthcare workers regarding the importance of hand hygiene compliance can limit the spread of outbreak stains within hospitals, and potentially to the community.[7] Hospitals might apply screening scheme for high-risk patients prior to admission to identify carriers of MDR pathogens and to apply isolation or contact precautions in order to limit spread.

Active ongoing surveillance on AMR is essential to aid the development of tailored treatment guidelines for empirical antibiotic therapy, particularly for community-acquired infections. Developing local surveillance of AMR bacteria that are related to nosocomial infections can also helps to track emerging resistance to antibiotics and to identify outbreaks.[7] Hence, microbiology laboratories should be well equipped to provide valid and reliable identification for pathogen, as well as antibiotic susceptibility tastings. As part of their routine services, microbiology laboratories should also be able to provide up-to-date antibiograms at a hospital level and contribute to local surveillance programs. With the widely accessible molecular-based techniques and

Table 1 - Recommendations for actions to stakeholders in the Saudi Arabia to combat antimicrobial resistance.

Recommendations
Antimicrobial stewardship programs
Active guidelines should be implemented to restrict unnecessary use of antibiotics in Saudi hospitals.
Abolition of over-the-counter sales of antimicrobials without prescriptions.
Regulations and strategies should be used to enforce the ban of antibiotics as growth-promoters in Saudi animal farms and restrict the use of important antimicrobials as listed by the World Health Organization - Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGSIR).
Initiating mass educational campaign about antibiotic use
To raise awareness about antibiotic resistance and limit the improper use of antibiotics.
Cultural differences should be considered when implementing educational campaigns to be delivered to the population of Saudi Arabia.
The highly utilized social media platforms should be used to disseminate messages, but not solely.
Basic infection control precautions (for example, hand hygiene)
Should be emphasized among healthcare workers.
Patients who have been hospitalized internationally need to be screened by the receiving institute for multidrug-resistant organisms.
Infection prevention policy defining at-risk patients and management strategies (for example, empirical treatment, and isolation) should be developed.
Microbiology laboratories
Microbiologists should be updated with local, national, regional and international surveillance to prepare screening and confirmatory testing for emerging resistance mechanisms.
Molecular and protein-based identification tools should also be considered to improve diagnosis and to reduce turn-around time.
Active surveillance
Developing local surveillance of antimicrobial resistant organisms in hospitals, regional and national levels to help suggesting empirical treatments and in outbreak tracking.
Molecular-based techniques and whole genome sequencing should be utilized to give greater insight about the antibiotic resistance mechanisms and clone disseminations.

whole genome sequencing, the use of these technologies on AMR can provide a richer insight on the clonal disseminations of certain species, and the genotypic characterization of antibiotic resistance mechanisms.[7] Surveillance in Saudi Arabia can help contribute to the global understanding of the burden of the AMR, which is still poorly quantified.[2] It was suggested that countries should introduce death register records "deaths caused by antimicrobial-resistant infection".[6]

Last but not least, active antibiotic stewardship guidelines should be implemented to restrict the irrational use of antibiotics in Saudi Arabia. These guidelines should aim to reduce over prescription and misprescription of antibiotics in outpatient settings, community pharmacies, hospital, and agricultural sectors. The WHO and Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGSIR) have published an updated list of antimicrobials according to their importance in human medicine.[20] This list can immediately be used to control the use of medically important antimicrobials from food animal and agricultural settings in Saudi Arabia. **Table 1** summarizes actions needed to tackle AMR in Saudi Arabia.

In conclusion, AMR is a global issue that requires tremendous attention. International agencies have suggested recommendations and plans to combat AMR. It is now up to countries to take the lead and implement local actions plans to limit AMR. Saudi Arabia faces several challenges that can stimulate the emergence and spread of MDR bacteria. These challenges require cultivated efforts from different sectors to successfully achieve a significant control of AMR in the country. Actions should include active surveillance of AMR on hospital-based, regional, and nationwide levels. Infection prevention and control precautions should also be optimized to limit the spread. Raising awareness of AMR is an essential mandate to limit inappropriate use of antibiotics, and the use of antimicrobial should be maintained by antibiotic stewardship programs not only in hospital settings, but also in outpatients and community pharmacies.

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PME-1-Producing *Pseudomonas aeruginosa* in Qatar

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The novel extended-spectrum β-lactamase (ESBL) PME-1 was first described in 2010 in an isolate from a *Pseudomonas aeruginosa* strain obtained from multiple clinical specimens from a single patient admitted to the University of Pittsburgh Medical Center in 2008. The patient had had a prolonged period of hospitalization in United Arab Emirates immediately before being transferred to the United States (1). We describe here the first case of *P. aeruginosa* carrying bla_{PME-1} isolated from Qatar and present the second report to date of this enzyme.

The *P. aeruginosa* HZ-QTR-51 isolate was sent to the reference laboratory at The University of Queensland Centre for Clinical Research (UQCCR) as part of a region-wide collaborative study on multidrug-resistant Gram-negative bacilli (2, 3). The Etest was used to measure the MIC of several antimicrobial compounds as listed in Table 1.

The bacterial genomic DNA was extracted using an UltraClean Microbial DNA isolation kit (Mo Bio Laboratories). Species identification was performed using the PAduplex assay as previously described (4). Paired-end libraries of whole genomic DNA of HZ-QTR-51 were prepared via the use of a Nextera XT DNA sample preparation kit and sequenced by the use of an Illumina HiSeq platform (Illumina, San Diego, CA, USA). The 100-bp-paired-end reads were *de novo* assembled using CLC Genomic Workbench with a minimum contig length of 200 bp. A total of 167 contigs were assembled with depth coverage of ca. 100×.

The sequence type (ST) of *P. aeruginosa* HZ-QTR-51 was confirmed as ST 654 by *in silico* multilocus sequence typing (MLST) (https://cge.cbs.dtu.dk/services/MLST/) (5). The ResFinder 2.1 platform (http://cge.cbs.dtu.dk/services/ResFinder/) (6) was also used to characterize acquired antimicrobial resistance mechanism genes among those in the draft genome. The isolate carried bla_{PME-1} as well as bla_{OXA-50}, bla_{GES-5}, and bla_{PAO} contributing to β-lactam resistance and *strA*, *aph(3′)-VIa*, *aph(3′)-IIb*, and *strB* for aminoglycoside resistance. The isolate also carried *fosA* for fosfomycin resistance, *catB7* for chloramphenicol resistance, *sul1* for sulfonamide resistance, and *tet*(A) and *tet*(G) for tetracycline resistance. For further confirmation, bla_{PME}-specific primers were used as previously described (1).

P. aeruginosa HZ-QTR-51 was phenotypically resistant to all tested antibiotics and was on the breakpoint border for amikacin (Table 1). The carbapenem resistance in this isolate might be due to the production of GES-5, which is a carbapenemase (7).

P. aeruginosa ST 654 is noteworthy for several reasons. VIM-2-producing *P. aeruginosa* ST 654 was isolated from a patient in Sweden following hospitalization in Tunisia (8). ST 654 was also associated with KPC-producing *P. aeruginosa* from Argentina (9). More recently, VIM-2-producing *P. aeruginosa* ST 654 was identified among the international "high-risk clones" in the United Kingdom (10). These reports highlight that *P. aeruginosa* ST 654 is an internationally disseminated clone with a multidrug-resistant phenotype, which might facilitate the rapid international spread of PME-1 (and GES-5)-producing *P. aeruginosa*.

TABLE 1 Antimicrobial MICs for PME-1-producing *P. aeruginosa* HZ-QTR-51

Antimicrobial category	Antimicrobial agent	MIC (mg/liter)	EUCAST interpretation[a]
Aminoglycosides	Gentamicin	>256	R
	Amikacin	16	NS
	Netilmicin	>8	R
Antipseudomonal penicillins and β-lactamase inhibitors	Ticarcillin/clavulanate	>256	R
	Piperacillin/tazobactam	>32	R
Carbapenems	Imipenem	>32	R
	Meropenem	>32	R
	Doripenem	>32	R
Extended-spectrum cephalosporins	Ceftazidime	32	R
	Cefepime	16	R
Fluoroquinolones	Ciprofloxacin	>32	R
Monobactams	Aztreonam	128	R

[a] R, resistant; NS, nonsusceptible.

In conclusion, this report presents the first description of PME-1-producing *P. aeruginosa* in Qatar and the second in the world (11). The currently described *P. aeruginosa* isolate belongs to successful international clone ST 654, which might contribute to the global spread of multiple antibiotic resistance mechanisms, including bla_{PME-1} and bla_{GES-5}. We suggest active surveillance for multidrug-resistant *P. aeruginosa* to assess the dissemination and prevalence of beta-lactamase-mediated antibiotic resistance in the Gulf region.

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Differentiation of *Acinetobacter* Genomic Species 13BJ/14TU from *Acinetobacter haemolyticus* by Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

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Acinetobacter genomic species 13BJ and genomic species 14TU were delineated independently in 1989 based on DNA-DNA hybridization among proteolytic strains of human origin (1, 2). Later studies suggested that these two taxa were congruent with each other at the species level and represented a distinct phylogroup within the hemolytic-proteolytic clade of the genus *Acinetobacter* (3). Reports from different countries showed that strains of *Acinetobacter* genomic species 13BJ/14TU were found in clinical specimens at a frequency similar to or even higher than that of another hemolytic and proteolytic species, *Acinetobacter haemolyticus* (4–6). It has also been noted that strains of *Acinetobacter* genomic species 13BJ/14TU are intrinsically resistant to polymyxins (4, 7), which further underpins the clinical relevance of this taxon.

Previously, *Acinetobacter* genomic species 13BJ/14TU had not been correctly identified using some phenotypic identification systems and the VitekMS matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) microbial identification system, although correct identification was achieved using partial *rpoB* gene sequencing (4, 8). In the present study, we showed that *Acinetobacter* genomic species 13BJ/14TU can be reliably differentiated from other *Acinetobacter* species using MALDI-TOF mass spectrometry (MS).

The strains of *Acinetobacter* genomic species 13BJ/14TU and *A. haemolyticus* for this study were selected mostly from the collection of the Laboratory of Bacterial Genetics (National Institute of Public Health, Prague, Czech Republic) and also included reference strains analyzed in the original DNA hybridization studies (Table 1). The whole-genome sequences for seven of these reference strains were published recently (3, 9). The species identity of the seven strains was confirmed by whole-genome sequence comparison using average nucleotide identity based on BLAST (ANIb), which was calculated using the JSpecies web program (http://imedea.uib-csic.es/jspecies/) with the default setting (3). The identification of the 13 remaining strains was based on a combination of validated methods, including comparative analysis of the partial *rpoB* gene, amplified rRNA gene restriction analysis (ARDRA), and/or phenotypic analysis (5, 7). All strains were epidemiologically unrelated as confirmed by their unique ApaI macrorestriction profiles of their genomic DNA and/or differences in their *rpoB* gene sequences (data not shown).

A subculture of each of the strains investigated was streaked onto sheep blood agar (Difco Laboratories, Detroit, MI; 5% sheep blood from Oxoid, Hampshire, United Kingdom) and incubated at 37°C for 24 h. Cells from representative single bacterial colonies were directly smeared onto a target spot of a steel target plate by using a disposable loop, overlaid with 1 μl of matrix consisting of a saturated solution of sinapinic acid (catalog no. 49508; Sigma-Aldrich, Buchs, Switzerland) in 60% acetonitrile (catalog no. 154601; Sigma-Aldrich)-3% trifluoroacetic acid (catalog no. T6508; Sigma-Aldrich) or α-cyano-4-hydroxycinnamic acid (catalog no. 145505; Sigma-Aldrich, Buchs, Switzerland) in 33% ethanol-33% acetonitrile (catalog no. 154601; Sigma-Aldrich, Buchs, Switzerland)–3% trifluoroacetic acid (catalog no. T6508; Sigma-Aldrich), and air dried within minutes at room temperature. Matrices were tested in parallel to assess the impact of matrices on the ability to identify the strains.

Protein mass fingerprints were obtained using a MALDI-TOF Axima Confidence mass spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range of 3,000 to 30,000 Da. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. For each sample spot, a total of 100 spectral profiles, each consisting of 10 single spectra, were averaged and processed using Launchpad v.2.8 software (Shimadzu-Biotech Corp., Kyoto, Japan). This software was also used for peak processing of all raw spectra with the following settings: the advanced scenario was chosen from the Parent Peak Cleanup menu, peak width was set at 80 channels, the smoothing filter width was set at 50 channels, the baseline filter width was set at 500 channels, and for peak detection

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TABLE 1 Strains used in this study

Strain	Species	Country, yr of isolation	Specimen (human)	WGS[a] accession no.	Reference(s)
CIP 64.3^T	*A. haemolyticus*	Unknown	Sputum	APQQ00000000.1	3, 13
NIPH 1878 (=Tjernberg 197)	*A. haemolyticus*	Sweden, 1980s	Wound		2
NIPH 58	*A. haemolyticus*	Czech Republic, 1992	Blood		5
NIPH 261	*A. haemolyticus*	Czech Republic, 1993	Tissue	APQR00000000.1	3, 5
NIPH 939	*A. haemolyticus*	Czech Republic, 1998	Cervix		5
ANC 3857	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 3923	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 3927	*A. haemolyticus*	Czech Republic, 2010	Wound		
ANC 4581 (=CIP 70.27)	*A. haemolyticus*	Unknown	Nose	ADMT00000000.1	9, 13
ANC 4583 (=CIP 70.26)	*A. haemolyticus*	Unknown	Ocular pus	AMJB00000000.1	9, 13
NIPH 1860 (=CIP 64.2)	*Acinetobacter* genomic species 13BJ/14TU	Germany	Conjunctiva	APRT00000000.1	1, 2, 3
NIPH 1859 (=Tjernberg 71)	*Acinetobacter* genomic species 13BJ/14TU	Sweden, 1980s	Conjunctiva	APRZ00000000.1	2, 3
NIPH 1861 (=Bouvet 496)	*Acinetobacter* genomic species 13BJ/14TU	France	Skin		1
NIPH 2036 (=Bouvet 1191)	*Acinetobacter* genomic species 13BJ/14TU	Belgium	Catheter	ATGK00000000.1	1, 3
ANC 3881	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 2010	Lungs (autopsy)		
NIPH 378	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1995	Eye		5
NIPH 637	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1995	Blood		5
NIPH 669	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1997	Blood		5
NIPH 1035	*Acinetobacter* genomic species 13BJ/14TU	Czech Republic, 1998	Blood		5
ANC 4056	*Acinetobacter* genomic species 13BJ/14TU	Germany, 2004	Wound		
J80	*Acinetobacter* genomic species 13BJ/14TU	Japan	Unknown		

[a] WGS, whole-genome sequence.

method, the threshold apex was chosen. For the threshold apex peak detection, the threshold type was set as dynamic and the threshold offset was set to 0.025 mV with a threshold response factor of 1.25. The processed spectra were exported as peak lists with *m/z* values and signal intensities for each peak in the ASCII format. Calibration was conducted for each target plate using spectra of the reference strain *Escherichia coli* DH5α.

All 21 strains were analyzed in duplicate using two sample spots from the same colony. Generated protein mass fingerprints were analyzed with SARAMIS software (AnagnosTec, Potsdam-Golm, Germany) and the Putative Assigned Protein Masses for Identification Database (PAPMID) (Mabritec, Switzerland).

In the first step, a biomarker mass pattern, called a superspectrum, was calculated for the *Acinetobacter* genomic species 13BJ/14TU, using the SARAMIS SuperSpectrum tool. As ribosomes are known to be among the most abundant proteins (10), 23 ribosomal subunit protein masses, namely, L36 (4,265.1 Da), L34 (5,175.1 Da), L33 (6,104.3 Da), L30 (6,641.7 Da), L32 (6,930.8 Da), L35 (7,185.8 Da), L29 (7,448.6 Da), L31 (8,343.6 Da), S21 (8,349.9 Da), L27 (8,769.1 Da), L28 (8,986.7 Da), S17 (9,407.0 Da), S20 (9,570.2 Da), S15 (10,007.5 Da), S19 (10,063.9 Da), S14 (11,253.2 Da), L18 (12,390.1 Da), L17 (13,540.7 Da), S12 (13,649.0 Da), L13 (15,859.3 Da), S5 (17,008.7 Da), S7 (17,554.5 Da), and L6 (18,968.0 Da), were retrieved from the genome of *Acinetobacter* genomic species 13BJ/14TU strain CIP 64.2, making use of the PAPMID software. The spectra of the *Acinetobacter* genomic species 13BJ/14TU strains investigated in this study were matched against these reference masses for automatic identification, allowing for an error of 500 ppm.

The superspectrum for automated identification of *Acinetobacter* genomic species 13BJ/14TU was generated according to the user guidelines and validated against the entire in-house extended SARAMIS database (version 3.3.2), consisting of 2,087 bacterial species, including 18 *Acinetobacter* spp. with validly published names. These 18 species encompass all *Acinetobacter* spp. which are known to be associated with human clinical specimens, except for *Acinetobacter nosocomialis*, *Acinetobacter seifertii*, *Acinetobacter soli*, and *Acinetobacter variabilis*.

All 21 strains were identified using SARAMIS software, including the new *Acinetobacter* genomic species 13BJ/14TU superspectrum. Using only the SARAMIS database without the new *Acinetobacter* genomic species 13BJ/14TU superspectrum, all 10 *A. haemolyticus* strains were identified correctly as *A. haemolyticus* while the 11 *Acinetobacter* genomic species 13BJ/14TU strains had been identified only as *Acinetobacter* spp. However, with the implementation of the *Acinetobacter* genomic species 13BJ/14TU superspectrum, all strains were correctly identified as *A. haemolyticus* or *Acinetobacter* genomic species 13BJ/14TU, respectively. The choice of matrix used for spectral acquisition made no impact on the identification of the strains.

MALDI-TOF MS, as we have demonstrated, is a useful tool in the differentiation and identification of related species of bacteria such as *A. haemolyticus* and *Acinetobacter* genomic species 13BJ/14TU. Other studies detailing the use of a commercial MALDI mass spectrometer in comparison with other instruments such as the Vitek 2 and MicroScan reported that *Acinetobacter* genomic species 13BJ/14TU could not be accurately identified with these instruments (8). However, the method used here uses the same principle as commercial instruments used in routine identification procedures but with a more in-depth approach in the analysis of the spectra.

The use of calculated theoretical masses from sample genomes as identifying masses contributed to the improved discriminatory power of our method as observed in this study. Use of a sinapinic acid matrix may further increase the discriminatory power of the method due to its ability to better ionize proteins of higher molecular weights that can be used for identification compared to α-cyano-4-hydroxycinnamic acid (11, 12).

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