Title:

Diversity of carbapenem-resistant *Acinetobacter baumannii* and bacteriophage-mediated spread of the Oxa23 carbapenemase

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The genome sequences of the isolates from this study have been deposited in GenBank under the BioProject accession number PRJNA659545.

Abstract

Carbapenem-resistant *A. baumannii* are prevalent in low- and middle-income countries such as Egypt, but little is known about the molecular epidemiology and mechanisms of resistance in these settings. Here we characterise carbapenem-resistant *A. baumannii* from Alexandria, Egypt, and place it in a regional context. 54 carbapenem-resistant isolates from Alexandria Main University Hospital, Egypt, collected between 2010 and 2015 were genome sequenced using Illumina technology. Genomes were *de novo* assembled and annotated. Genomes for 36 isolates from the Middle East region were downloaded from GenBank. Core gene compliment was determined using Roary, and analyses of recombination were performed in Gubbins. MLST sequence type and antibiotic resistance genes were identified. The majority of Egyptian isolates belonged to one of 3 major clades, corresponding to Pasteur MLST clonal complex (CC^{PAS}) 1, CC^{PAS}2 and sequence type (ST^{PAS}) 158. Strains belonging to ST^{PAS}158 have been reported almost exclusively from North Africa, the Middle East and Pakistan, and may represent a region-specific lineage. All isolates carried an *oxa23* gene, six carried *bla*$_{NDM-1}$, and one carried *bla*$_{NDM-2}$. The *oxa23* gene was located on a variety of different mobile elements, with Tn*2006* predominant in CC^{PAS}2 strains, and Tn*2008* predominant in other lineages. Of particular concern, in 8 of the 13 CC^{PAS}1 strains, the *oxa23* gene was located in a temperate bacteriophage phiOXA, previously identified only once before in a CC^{PAS}1 clone from the US military. The carbapenem-resistant *A. baumannii* population in Alexandria Main University hospital is very diverse, and indicates an endemic circulating population, including a region-specific lineage. A major mechanism for *oxa23* dissemination in CC^{PAS}1 isolates appears to be a bacteriophage, presenting new concerns about the ability of these carbapenemases to spread throughout the bacterial population.

Impact Statement

In this study we have analysed the whole genomes of a group of antibiotic-resistant bacteria – *Acinetobacter baumannii* – from Alexandria, Egypt, to identify why they are antibiotic resistant, and

53 how resistance is being spread between bacteria. This is to help address the current knowledge gap
54 regarding the mechanisms and spread of antibiotic resistance in low- and middle-income countries
55 like Egypt. We found that for the vast majority of bacteria, resistance was due to a specific gene –
56 *oxa23*. However, the bacteria carrying this gene were very varied, showing that they do not
57 represent a specific outbreak, but rather the continuous circulation of multiple different antibiotic-
58 resistant lineages. A significant number of bacteria belonged to a subgroup that have only been
59 sporadically reported from North Africa, the Middle East and Pakistan, providing evidence that there
60 may be a specific subgroup of *A. baumannii* from this geographic region. Of particular significance, in
61 a number of bacteria the *oxa23* gene was found to be carried by a bacteriophage – a virus that
62 infects bacteria. We present evidence that it is likely that this bacteriophage is responsible for
63 spreading the *oxa23* gene between bacteria, which is not currently widely recognised as a major
64 mechanism for antibiotic resistance dissemination.
65

66 **Data Summary**

67 The whole genome shotgun sequences of the isolates from this study have been deposited at
68 DDBJ/ENA/GenBank under the BioProject accession number PRJNA659545. The individual genome
69 accession numbers for each isolate are as follows:
70 A1a, JACSUC000000000; A2, JACSUB000000000; A4, JACSVQ000000000; A5, JACSUA000000000; A6,
71 JACSTZ000000000; A7-T, JACSVP000000000; A8-T, JACSVO000000000; A8a, JACSTY000000000; A9,
72 JACSTX000000000; A10, JACSTW000000000; A10a, JACSTV000000000; A11a, JACSTU000000000;
73 A13a, JACSTT000000000; A14a, JACSTS000000000; A15, JACSTR000000000; A16, JACSTQ000000000;
74 A18, JACSTP000000000; A21, JACSVN000000000; A22, JACSTO000000000; A27, JACSTN000000000;
75 A30, JACSTM000000000; A31, JACSTL000000000; A34, JACSTK000000000; A35, JACSTJ000000000;
76 A36, JACSTI000000000; A39, JACSTH000000000; A40, JACSTG000000000; A41, JACSTF000000000;
77 A42, JACSTE000000000; A43, JACSTD000000000; A44, JACSTC000000000; A45, JACSTB000000000;
78 A46, JACSTA000000000; A64, JACSSZ000000000; A68, JACSSY000000000; A69, JACSSX000000000;

79 A70, JACSSW000000000; A71, JACSVM000000000; A72, JACSSV000000000; A73, JACSSU000000000;
80 A74, JACSST000000000; A75, JACSSS000000000; A78, JACSSR000000000; A82, JACSSQ000000000;
81 A83, JACSVL000000000; A84, JACSSP000000000; A85, JACSSO000000000; A86, JACSVK000000000;
82 A87, JACSSN000000000; A88, JACSSM000000000; A89, JACSSL000000000; A92, JACSSK000000000;
83 A5910, JACSSJ000000000; A6135, JACSVJ000000000.

84

85 **Introduction**

86 The bacterium *Acinetobacter baumannii* is a major opportunistic hospital-acquired pathogen that is
87 listed by the World Health Organization (WHO) as in critical need of new treatment options due to
88 its multidrug-resistant nature (1). In particular, the frequency of carbapenem-resistant *A. baumannii*
89 has been steadily increasing over the last two decades, leaving very few treatment options available
90 to combat this pathogen (2). However, carbapenem-resistant *A. baumannii* are not uniformly
91 distributed across the globe, with higher rates of resistance found in low- and middle-income
92 countries (3-6), though rates in some southern and eastern European countries have now also
93 reached very high levels (7). In countries in the Middle East and North Africa high levels of
94 carbapenem-resistant *A. baumannii* are reported, with frequencies of 70% of isolates or greater
95 being common (8). Despite these very high rates of resistance, there are relatively few studies
96 investigating the molecular epidemiology of the antibiotic-resistant strains.

97

98 Carbapenem resistance in *A. baumannii* is usually the result of the expression of an OXA-type β-
99 lactamase, or occasionally metallo-β-lactamases such as the IMP, VIM and NDM groups (9). The
100 acquired OXA-type β-lactamases in *A. baumannii* are encoded by genes belonging to five main
101 groups – *oxa23* (or $bla_{\text{OXA-23-like}}$), *oxa40* (or $bla_{\text{OXA-40-like}}$), *oxa58* (or $bla_{\text{OXA-58-like}}$), *oxa134* (or $bla_{\text{OXA-134-}}$
102 $_{\text{like}}$), and *oxa143* (or $bla_{\text{OXA-143-like}}$) (10, 11). In addition, all *A. baumannii* carry an intrinsic OXA β-
103 lactamase gene called *oxaAb* (or $bla_{\text{OXA-51-like}}$), certain alleles of which when highly expressed due to
104 the presence of an IS*Aba1* insertion sequence upstream can confer carbapenem resistance (12-14).

105 The most common of these resistance mechanisms globally is *oxa23* (15). In Egypt, and other
106 countries in the region, *oxa23* is so prevalent it can be found in up to 100% of carbapenem-resistant
107 isolates, with frequencies greater than 70% being the norm (16-19). In *A. baumannii oxa23* is usually
108 located on a transposon mobilised by one or more insertion sequences (IS), which has enabled the
109 resistance gene to be spread to many different plasmids and many different lineages within the
110 species (20). Despite the particularly high prevalence of *oxa*23 in low- and middle-income countries
111 such as Egypt, there are very few studies that have investigated the mobile genetic elements
112 carrying the gene which is crucial to gaining an understanding of the local population genetics of the
113 species.

114

115 The majority of *A. baumannii* isolates belong to one of eight International Clones (ICs), which
116 correspond to specific multi-locus sequence typing (MLST) sequence types (STs) and clonal
117 complexes (CCs) (15, 21). There are two MLST schemes for *A. baumannii* – the Pasteur scheme (22)
118 and the Oxford scheme (23) with the Pasteur scheme containing genes that are less prone to
119 recombination than those in the Oxford scheme (24). Globally, isolates belonging to IC2,
120 corresponding to CC^{PAS}2, are the most common, though there are exceptions such as Latin American
121 countries where isolates belonging to IC4 (CC^{PAS}15), IC5 (CC^{PAS}79) and IC7 (CC^{PAS}25) are predominant
122 (25, 26). In many low- and middle-income countries MLST is too costly to perform on large numbers
123 of isolates, and so at present we often rely on a small number of studies to provide an indication of
124 what the national epidemiology may be. In Egypt, studies have indicated that CC^{PAS}2 is the most
125 common CC, but that a large number of isolates from other CCs or that don’t belong to any of the
126 defined CCs make up a substantial portion of the population (16, 18, 19, 27). The aim of our study
127 was to define the local population structure of *A. baumannii* in Alexandria Main University Hospital,
128 Egypt, and identify the mobile genetic elements responsible for resistance gene dissemination.

129

130 **<u>Methods</u>**

131 *Bacterial isolates and antimicrobial susceptibility testing*

132 A total of 54 carbapenem resistant *A. baumannii* clinical isolates obtained from patients presenting
133 at Alexandria Main University Hospital (AMUH) between 2010 and 2015 were included in the study.
134 This is the largest hospital in the northern sector of Alexandria and a major referral hospital. The
135 isolates were identified by conventional methods, including colony morphology, aerobic growth at
136 44°C on MacConkey agar, and species designations obtained using the Vitek system (bioMérieux,
137 UK). The identity of the isolates was further confirmed by PCR amplification of the intrinsic *oxaAb*
138 (*bla*$_{\text{OXA-51-like}}$) gene as well as Matrix Assisted Laser Desorption/Ionization - Time of Flight Mass
139 Spectrometry (MALDI-TOF MS) (Bruker Daltonik, USA). The identified isolates were stored at -80°C
140 prior to subsequent characterisation (28). The susceptibility of the isolates to imipenem and
141 meropenem was determined using agar dilution and the results were interpreted according to the
142 Clinical and Laboratory Standards Institute guidelines (2018)(29).

143

144 *Whole genome sequencing and analyses*

145 Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega) according
146 to the manufacturer’s instructions. A Qubit fluorometer (Life Technologies) was used to quantify the
147 extracted DNA. Dual indexing library preparation was carried out using the Nextera XT DNA
148 Preparation Kit (Illumina Inc., San Diego, CA, USA). Whole genome sequencing of the library was
149 performed on an Illumina MiSeq using the 2 x 250 bp paired-end protocol. Following quality filtering
150 of the reads using Trimmomatic v0.36 (30) and FastQC v0.11.5 (31), genomes were *de novo*
151 assembled with Spades v3.11.1 (32), and annotated using Prokka v1.11 (33). The assemblies were
152 quality checked using QUAST (34). The Sequence Read Archive was searched using keywords of
153 Middle Eastern countries, and the genomes of an additional 36 strains were downloaded and
154 included in subsequent analyses. The genomes of a further 17 geographically and genomically
155 diverse strains (35) were also downloaded and included in subsequent analyses. The core genome
156 content of the strain collection was determined using Roary v3.12.0 (36), and the core gene

157 phylogeny estimated using FastTree v2.1.10 (37). Isolates A8-T and A74 were chosen to be the
158 reference genomes for $CC^{PAS}1$ and $CC^{PAS}2$ respectively for subsequent variant calling. Sequences
159 belonging to $CC^{PAS}1$ and $CC^{PAS}2$ were mapped to the reference genomes and variant called using
160 PHEnix v1.3 (38). A SnapperDB v1.0.6 for each CC was created, allowing inclusion of SNPs with a
161 minimum average read depth of 10 (39). Whole genome alignments were generated including
162 isolates 10,000 SNPs from the CC1 reference and 20,000 SNPs from the CC2 reference, which were
163 used as input for Gubbins. Estimates of recombination within clades identified in the phylogeny
164 were conducted with Gubbins v2.3.1 using default settings (40). The Pasteur MLST sequence type
165 (ST) of each isolate was determined from the whole genome sequence using the online Center for
166 Genomic Epidemiology's MLST software (41), and antibiotic resistance genes were determined using
167 ARIBA (42) with the CARD (43) and SRST2 (44) databases. All *oxaAb* alleles were confirmed using the
168 BLAST function on the Beta-lactamase database (11)(http://www.bldb.eu/). Analyses of the
169 accessory genome were conducted using PANINI (45).
170
171 *Annotation of phiOXA-A35 from the A35 genome assembly*
172 The phiOXA-A35 sequence was constructed by alignment of three contigs and manual resolution of
173 overlaps from assembly data obtained for *A. baumannii* A35. phiOXA open reading frames were
174 initially annotated using PROKKA v1.12 and then refined using BLASTp, InterProScan (46) and
175 HHpred (47). Prediction of tRNAs was performed using tRNAscan-SE 2.0 (48). Alignments of the
176 portal vertex and major capsid proteins were performed using Clustal Omega (49) and phylogenetic
177 trees constructed using IQTree v1.6.12 with ModelFinder, SH-aLRT test and ultrafast bootstrap with
178 1,000 replicates (50-52). Read coverage of phiOXA-A35 was calculated using QualiMap v.2.2.2 (53).
179
180 *Genetic environment of* oxa23
181 In the program Geneious R10 (https://www.geneious.com/), for each *oxa23*-positive strain the
182 contig containing *oxa23* was identified, then all these contigs were aligned. The alignment was used

183 to group strains by similarity of the sequence surrounding *oxa23*. Where appropriate, the presence
184 of insertion sequences IS*Aba1* and IS*Aba125* surrounding the *oxa23* gene were confirmed by PCR
185 using combinations of primers ISAba1-B (54), OXA-23-F and OXA-23-R (55), and ISAba125-F (5'-
186 TAAAACTATTCATGAGCGCC-3'). To obtain the complete sequences of the prophages containing
187 *oxa23*, contigs were aligned against the phiOXA sequence from strain AB5075-UW (accession no.
188 CP008706.1). PCRs with primers Phi-F (5' – CGT TGT TGG GCT TCT AGT GC – 3') and OXA-23-R (55)
189 were used to confirm the contig joins either side of the IS*Aba1* insertion sequence.
190
191 *Bacteriophage induction*
192 Bacterial cultures were grown overnight in LB at 37°C and shaking at 180 rpm. Overnight cultures
193 were diluted to an OD_{600} of approximately 0.05 using pre-warmed LB, then incubated at 37°C and
194 180 rpm until the OD_{600} reached 0.2. Cultures were then divided to generate two treatment cultures
195 and two control cultures per strain. Mitomycin C was added to a final concentration of 2 µg/mL to
196 the treatment cultures before they were wrapped in foil to block out light, then both treatment and
197 control cultures were incubated at 37°C. The OD_{600} of cultures was recorded every 30 minutes to
198 identify a marked drop in the optical density in the mitomycin C-treated cultures, representing
199 bacteriophage induction. Once this was observed, all cultures were centrifuged at 10,000 x *g* for 5
200 minutes, filter sterilised through a 0.22 µm filter, and treated with DNase (TURBO DNase, Invitrogen)
201 and RNase A (Thermo Scientific) to remove all bacteria and free nucleic acid from the cell lysate. The
202 presence of intact bacteriophage carrying *oxa23* in the bacterial cell lysate was determined by PCR
203 using primers OXAphi-F (5' – GGAAATGCGGTCAGAAATGC – 3') situated within *oxa23* and OXAphi-R
204 (5' – TGGACCCTGTAGATTTTGCC – 3') situated within a phage tail protein gene, giving a 1,032 bp
205 product size. PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds,
206 55°C for 30 seconds, and 72°C for 1 minutes, with a final extension of 72°C for 5 minutes. A 1 µL
207 culture volume from a phiOXA-positive strain was used as a positive control.
208

209 *Bacteriophage purification and sequencing*

210 Clarified lysates were prepared from batch cultures of A35 treated with mitomycin C by
211 centrifugation at 10,000 x *g* for 10 minutes and filter sterilization (0.22 µm). Bacteriophages were
212 precipitated by addition of NaCl and polyethylene glycol 8000 to final concentrations of 1M and 10%
213 (w/v), respectively. After storage overnight at 4°C, precipitate was recovered by centrifugation at
214 11,000 x *g* for 15 minutes at 4°C and pellets resuspended in SM buffer (50 mM Tric-Cl, 8 mM $MgSO_4$,
215 100 mM NaCl, pH 7.5). Residual polyethylene glycol was removed by the addition of an equal volume
216 of chloroform and the aqueous phase recovered after centrifugation at 3,000 x *g* for 10 minutes at
217 4°C. For the extraction of bacteriophage genomic DNA, samples were treated with DNase I and
218 RNase A (Sigma Aldrich, UK) for 1 hour at 37°C, before the addition of EDTA, SDS and proteinase K to
219 final concentrations of 20 mM, 0.5% (w/v) and 50 µg/mL, respectively (56). DNA was then purified
220 using phenol:chloroform:isoamyl alcohol extraction. Preparation of libraries and sequencing of DNA
221 was performed at the Genomic Services and Development Unit (Public Health England) using an
222 Illumina HiSeq1000 and 100 bp paired end reads. Assembly was performed using SPAdes version
223 3.11.1 (32). Sequence reads were mapped to the *de novo* assembled contigs and the A35 genome
224 assembly using BWA-MEM (57) and assembly statistics obtained using QUAST (34) and Qualimap
225 (53). Annotation was performed as previously described.

226

227 **<u>Results</u>**

228 Analysis of the antibiotic susceptibilities of the Egyptian isolates showed that, as expected due to the
229 isolates being selected for their carbapenem resistance, they were all resistant to imipenem and
230 meropenem (Table 1). The majority of Egyptian isolates belonged to one of three major well-
231 supported clades based upon core gene sequences, corresponding to Pasteur MLST clonal complex
232 (CC^{PAS}) 1 (13 isolates), CC^{PAS}2 (24 isolates) and ST^{PAS}158 (10 isolates) (Table 1, Fig. 1). CC^{PAS}1 isolates
233 belong to International Clone (IC) 1, while CC^{PAS}2 isolates belong to IC2 (21, 58). In addition, two
234 isolates belonged to ST^{PAS}15, which are members of IC4, and two isolates belonged to ST^{PAS}25, which

235 are members of IC7 (Table 1). All isolates belonging to the ICs carried the *oxaAb* allele previously
236 shown to be associated with their respective IC, with isolates in CC^{PAS}1 (IC1) carrying *oxaAb*(69),
237 isolates in CC^{PAS}2 (IC2) carrying *oxaAb*(66), isolates in ST^{PAS}15 (IC4) carrying *oxaAb*(51), and isolates in
238 ST^{PAS}25 (IC7) carrying the *oxaAb*(64) allele (59). Isolates in ST^{PAS}158 carried *oxaAb*(65) alleles, which
239 are usually associated with CC^{PAS}79 and IC5. However, it should be noted that the *oxaAb*(65) allele in
240 the ST^{PAS}158 isolates differed from the original *oxaAb*(65) allele (GenBank accession no. AY750908)
241 by 3 silent substitutions (T90C, C636T and A663G). We compared our ST^{PAS}158 isolates with other
242 published or publicly available data, which demonstrated that this particular *oxaAb*(65) variant is a
243 feature of ST^{PAS}158 isolates in general (Table 2), and is distinct from CC^{PAS}79 (IC5) isolates. Isolates
244 from CC^{PAS}1 and CC^{PAS}2 were more diverse than ST^{PAS}158 isolates. This was evident in gene
245 conservation analysis with 3,069 genes shared by 90% of ST^{PAS}158 isolates, whereas only 2,394 genes
246 were shared by 90% of CC^{PAS}2 isolates, and 2,600 genes shared by 90% of CC^{PAS}1 isolates. For all 3 of
247 the major clades identified, the isolates from AMUH did not form their own specific sub-clades, but
248 were interspersed with the strains both from other Middle Eastern countries as well as with the
249 globally distributed strains (Figure 1). Interestingly a similar pattern was observed with respect to
250 the accessory genome (Figure 2). Based upon their accessory genomes, CC^{PAS}1, CC^{PAS}2 and ST^{PAS}158
251 isolates clustered together. The only exception was isolate 11a which clustered with the CC^{PAS}2
252 isolates (the grey dot found on the right-hand edge of the orange CC^{PAS}2 cluster in Figure 2A).
253 However, this is not too surprising given that of all the non-CC^{PAS}2 isolates, 11a is most closely
254 related to CC^{PAS}2 at the core genome level (Figure 1). The accessory genome clusters did not show
255 any geographic signal (Figure 2B), in agreement with the lack of geographical signal in the core gene
256 tree (Figure 1). Together these data demonstrate two points: firstly that there are both multiple
257 circulating clonal lineages, and multiple circulating sub-lineages within each clonal lineage, that are
258 responsible for infecting patients in AMUH, and secondly that while the accessory genome is shared
259 across isolates from several different countries within a clonal lineage, there is little sharing of the
260 accessory genome between clonal lineages.

261

262 All 54 isolates carried an *oxa23* gene (Table 1). In addition, 6 isolates also carried a *bla*$_{NDM-1}$ gene, and

263 one isolate carried *bla*$_{NDM-2}$. The *bla*$_{NDM}$ genes were not clustered in one particular bacterial sequence

264 type, with 3 of the *bla*$_{NDM-1}$ genes located in CC^{PAS}1 isolates (in one ST) while the other 3 were located

265 in CC^{PAS}2 isolates (across 2 STs) (Table 1, Figure 1). While complete transposons could not be

266 identified due to the limitations of short-read sequence data, all *bla*$_{NDM-1}$ genes had an IS*Aba125*

267 insertion sequence upstream and a *ble* bleomycin resistance gene followed by a *trpF*

268 phosphoribosylanthranilate isomerase gene downstream, as has typically been found in *A.*

269 *baumannii* in other studies (60). In two isolates (A86 and A6135) where longer contigs containing the

270 *bla*$_{NDM-1}$ gene were assembled, it appears likely that the *bla*$_{NDM-1}$ gene is carried on a transposon

271 similar to ΔTn*125* as described by Bonnin *et al* (61) as an IS*Aba14* fragment was detected following

272 the *dct* gene. However, further investigation using technology such as long-read sequencing is

273 required to completely resolve these mobile elements. The *bla*$_{NDM-2}$ gene in isolate A70 was located

274 within the previously described transposon Tn*125* (60). The *oxa23* gene was located on a variety of

275 different mobile genetic elements, with 11 different structures identified (Table 1, Figure 3). Several

276 of these structures were found in multiple isolates: structure A, representing Tn*2006* (62), was the

277 most common and was found in 21 isolates, 18 of which belonged to CC^{PAS}2, 2 belonged to CC^{PAS}25,

278 and one to CC^{PAS}15; structure B was found exclusively in three ST^{PAS}158 isolates; structure C in one

279 ST^{PAS}158 and two CC^{PAS}2 isolates; and structure F in four CC^{PAS}2 isolates and appeared to be borne on

280 the chromosome (Table 1, Figure 3). Of particular concern, in 8 of the 13 CC^{PAS}1 strains carrying

281 *oxa23*, the carbapenemase gene was located in prophage called phiOXA. This prophage has been

282 identified only once before in the CC^{PAS}1 isolate AB5075-UW, derived from a strain isolated in 2008

283 from a US soldier at the Walter Reed Army Medical Centre, USA, but to our knowledge has not been

284 shown to be viable (63). In order to determine whether phiOXA can form viable viral particles that

285 contain the *oxaAb* gene, four isolates encoding phiOXA (A8-T, A21, A35 and A39) and one isolate

286 that did not (A18) were treated with mitomycin C to induce bacteriophage, followed by DNase and

287 RNase treatment to remove any DNA that is not contained within a virus particle. Then a PCR for
288 *oxa23*, with an extended initial denaturation phase to lyse bacteriophage particles, was used to
289 identify the carriage of the antibiotic resistance gene by the bacteriophage. Cultures of three of the
290 four isolates tested (A8-T, A35 and A39) that had been treated with mitomycin C were found to have
291 produced intact bacteriophage carrying *oxa23*. No PCR products for *oxa23* were detected for these
292 strains when they were not induced, nor for isolate A18 (phiOXA negative) with either the presence
293 or absence of mitomycin C treatment. To confirm these data, virions from cultures of A35 exposed
294 to mitomycin C were purified and sequenced. Two contigs corresponding to phiOXA and a second
295 predicted prophage were identified. Due to extremely high coverage of the second prophage, an
296 assembly was performed using a random subset of 10% of the paired end reads. Alignment of the
297 complete dataset showed that the majority of sequence reads mapped to this prophage (89%)
298 yielding a coverage of 5,651X. For phiOXA-A35, a contig was identified in all assemblies, regardless of
299 the proportion of reads employed, representing 6% of the total reads and a coverage of 477X.
300 Comparison of this contig to the AB5075-UW genome using BLASTN showed 100% coverage and
301 identity to the phiOXA prophage in this strain and annotation confirmed the presence of the *oxa23*
302 carbapenemase. Collectively, these data demonstrate that the phiOXA prophage in these isolates
303 can be induced and form intact bacteriophage particles, and that these bacteriophages carry the
304 *oxa23* gene. Further work is required to identify a susceptible host for phiOXA-A35 in order to
305 demonstrate lysogenic conversion to a carbapenem resistant phenotype.
306
307 Analysis of the sequence of phiOXA-A35 showed it is identical to the bacteriophage/prophage
308 reported in strain AB5075-UW (Figure 4), with mean read coverage of 22 (s.d. = 7). The phiOXA-A35
309 prophage consists of a contiguous 32 kb region comprising 48 open reading frames (ORFs) with the
310 attL site residing within a tRNA-Leu, as was seen previously in strain AB5075-UW. The genomic
311 architecture of phiOXA-A35 is similar to that of members of the *Peduovirinae*, a widespread
312 subfamily of temperate bacteriophages that infect ɣ- and β-proteobacteria and includes *Escherichia*

313 phage P2 and *Pseudomonas* phage phiCTX. The genome can be divided into four modules,
314 representing genes involved in virion morphogenesis and assembly which contains the diagnostic *Q-*
315 *P-O-N-M-L* capsid gene cluster (64), lysis, replication, and control of lysogeny. This relationship is
316 further supported by phylogenetic analysis of the portal vertex and major capsid protein (Figures S1
317 and S2). Apart from a single syntenic break with IS*Aba1*, *oxa23* and a gene coding for a DUF815
318 domain protein, phiOXA is nearly identical to predicted prophage regions found in *A. baumannii*
319 strains A85, AYE, DA33382, USA15 and WCHAB005078. Comparison of these regions using VIRIDIC
320 (65) suggests that they represent a single species of temperate bacteriophage as each exhibit >95%
321 sequence similarity (66). We propose that phiOXA-A35 represents a new genus within the subfamily
322 *Peduovirinae*. A total of eight phiOXA ORFs are annotated as hypothetical proteins and whether
323 these represent additional proteins which influence the pathobiology or environmental fitness of
324 their host lysogen remains to be elucidated.

325

326 The two most likely scenarios that could explain the presence of phiOXA in multiple CC^{PAS}1 isolates
327 are i) that the bacteriophage inserted once into a CC^{PAS}1 isolate and has then spread via vertical
328 transmission, or ii) that phiOXA has independently infected multiple isolates. In order to investigate
329 this, we examined the apparent insertion site (tRNA-Leu) for all isolates included in the CC^{PAS}1 clade
330 in Figure 1. Our analyses showed that within the CC^{PAS}1 clade, isolates carrying phiOXA are not
331 monophyletic and are found in four separate sub-clades, indicating acquisition of phiOXA is likely to
332 have occurred on at least four independent occasions (Figure S3). These data, combined with the
333 demonstration that complete bacteriophage particles carrying *oxa23* are released by the bacteria,
334 suggest that phiOXA has been spreading through the CC^{PAS}1 population via horizontal transmission
335 of the bacteriophage.

336

337 **Discussion**

338 In this study we aimed to use genomics to characterise the molecular epidemiology and carbapenem
339 resistance of *A. baumannii* isolates from Alexandria, Egypt. Genome-level studies of this nature from
340 low- and middle-income countries are not common despite the fact that these countries bear the
341 highest burden of antibiotic resistance. By using genomics we can simultaneously characterise
342 antibiotic resistance genes and the genetic environment supporting them, and the fine-scale
343 epidemiological relationships between isolates. It also has the added benefit of being backward-
344 compatible with previous typing methods such as MLST. In the context of Egypt, there are a few
345 studies that have used one of the MLST schemes for *A. baumannii* – either the Pasteur scheme (22)
346 (as used in this study), or the Oxford scheme (23) – to investigate the relatedness of isolates. Where
347 studies have used MLST, the most commonly identified clonal complex is $CC^{PAS}2$ ($CC^{OX}208$). However,
348 a considerable proportion of isolates are often found to belong to less common clonal complexes, or
349 are singletons (16, 19, 27, 67). This is entirely consistent with the results from our study, where 44%
350 of isolates belonged to $CC^{PAS}2$, 24% of isolates belonged to $CC^{PAS}1$, and 19% belonged to $ST^{PAS}158$.
351 The core genome analysis we conducted demonstrated that even within MLST STs there was a lot of
352 diversity. This shows that multiple carbapenem-resistant strains are present within AMUH,
353 suggesting that rather than facing an outbreak, the bacterium is endemic. Whether patients are
354 acquiring these strains once admitted to the hospital, or whether there is widespread circulation of
355 carbapenem-resistant *A. baumannii* in the community is an open question that we hope to address
356 in the future.
357
358 While $CC^{PAS}1$ and $CC^{PAS}2$ strains are globally distributed and frequently encountered, strains
359 belonging to $ST^{PAS}158$ have been reported far less frequently and from a more focused geographic
360 area. $ST^{PAS}158$ belongs to $CC^{PAS}158$ ($CC^{OX}499$) (68) and is usually found in isolates from North Africa,
361 the Middle East and Pakistan (Table 2). Most previous studies that have identified $CC^{PAS}158$ isolates
362 have found them to carry the OxaAb variant OxaAb(65). However, in $CC^{PAS}158$ strains the *oxaAb*(65)
363 allele differs from the original allele (accession no. AY750908) by three synonymous substitutions. As

364 the *oxaAb* genes are intrinsic to *A. baumannii* and specific alleles are associated with certain
365 international clones (ICs), the gene can be used as a useful epidemiological marker to identify the IC
366 an isolate belongs to (59, 69, 70). However, under this scheme, OxaAb(65) is associated with IC5.
367 Isolates belonging to IC5 are members of $CC^{PAS}79$ and are found at particularly high frequency in
368 Latin America (15, 59, 71, 72). The allele profiles of the founder sequence types of $CC^{PAS}158$ and
369 $CC^{PAS}79$ ($ST^{PAS}158$ and $ST^{PAS}79$ respectively) are quite different, sharing only 1 of the 7 alleles (*rplB*
370 allele 4), which at the nucleotide level translates to 13 SNPs. It is therefore clear that in this instance,
371 numbering the *oxaAb* alleles based upon their amino acid sequence can mask important
372 epidemiological information and that, as suggested by Karah *et al* (68), these genes should be
373 numbered according to their nucleotide sequences as has been done for the *Acinetobacter ampC*
374 genes (73).
375
376 Previous studies of *A. baumannii* in Egypt have found that rates of carbapenem resistance are high,
377 typically >70% (17, 74), and that this is usually associated with isolates carrying the *oxa23* gene with
378 carriage frequencies reaching as high as 100% in carbapenem-resistant isolates (16-18). This was
379 reflected in our study, where *oxa23* was carried by 100% of carbapenem-resistant isolates. Reports
380 of the metallo-β-lactamases NDM-1 and NDM-2 being encoded by isolates from Egypt indicate
381 frequencies of $bla_{\text{NDM-1}}$ can typically reach up to 30% (18, 75, 76), though reports from specific
382 hospitals can occasionally report higher frequencies (16, 77). This is in line with our study where 6
383 isolates (11%) carried a $bla_{\text{NDM-1}}$ gene and only 1 isolate (2%) carried a $bla_{\text{NDM-2}}$ gene. It is possible
384 that the almost ubiquitous nature of the *oxa23* gene has reduced the selective advantage for
385 subsequent acquisition and retention of bla_{NDM} genes, limiting their spread within *A. baumannii*. The
386 *oxa23* gene in *A. baumannii* is typically carried on a transposon mobilised by insertion sequences
387 (ISs), usually IS*Aba1* (10). The IS elements are located immediately upstream of the *oxa23* gene,
388 where they provide a promotor sequence that drives high level expression of *oxa23* (13, 62). The
389 most commonly reported transposons carrying *oxa23* are Tn*2006* which is a composite transposon

390 where *oxa23* and three other genes are bracketed by two IS*Aba1* elements (62), and Tn*2008* which
391 is a one-ended transposon with a single IS*Aba1* element upstream of the *oxa23* gene (78). While the
392 limitations of short-read sequencing in enabling the assembly of transposons is well known, in our
393 study we were nevertheless able to identify a large number of different genetic arrangements
394 surrounding the *oxa23* gene. In line with what is reported in the literature, a structure likely to be
395 Tn*2006* was the most common arrangement in our isolates. However, the large number of different
396 structures we have identified involving IS*Aba125*, IS*Aba33* and IS*Aba4* in addition to IS*Aba1*
397 demonstrate that the carbapenem-resistant *A. baumannii* population in AMUH is not dominated by
398 a single mobile element that is disseminating *oxa23.* Rather, a multitude of different mobile
399 elements are hosting the gene, consistent with the apparent endemic nature of *oxa23* in the
400 bacterial population where multiple *A. baumannii* lineages co-circulate and there is the opportunity
401 for persistent transfer, re-arrangement, and selection to occur over an extended period of time.
402
403 The carriage of antibiotic resistance genes on transposons is common, and is the typical genetic
404 context for OXA-type carbapenemases in *A. baumannii*. However, in isolates belonging to $CC^{PAS}1$ in
405 our study the most commonly identified mobile element carrying *oxa23* was a bacteriophage
406 phiOXA. Reports of the carriage of antibiotic resistance genes in prophages have become more
407 common in recent years (79-81), but it is thought that this is generally a rare occurrence (82).
408 However, recent evidence from studies focusing on *A. baumannii* have suggested that carriage of
409 both virulence and antibiotic resistance genes by prophages is relatively common in this species and
410 may be a major mechanism of horizontal transfer of these genes (83-87). It was recently noted that
411 prophages appeared to be more common in IC5 isolates than in those belonging to IC1 or IC2 (88)
412 and it is an intriguing possibility that prophages may be a major factor in the evolution of different
413 international clones. The carriage of OXA-type carbapenemases in prophages has been observed
414 previously, with *oxa58* identified on a prophage in a *Proteus mirabilis* strain (89) and *oxa23*
415 identified on a prophage in *A. baumannii* strain ANC 4097 (83, 84) and on the phage phiOXA in

416 isolate AB5075-UW (63). However, López-Leal *et al* (88) recently indicated that OXA carbapenemases
417 in prophages may be more widespread, with evidence for potential OXA prophage carriage in
418 approximately 25% of isolates studied. Similarly, we found *oxa23* carried on phiOXA in 15% of our
419 isolates. Moreover, these isolates were not clonally related within $CC^{PAS}1$ but were spread
420 throughout the $CC^{PAS}1$ clade, indicating that phiOXA is widely disseminated amongst $CC^{PAS}1$ isolates
421 in AMUH. Furthermore, we demonstrated that phiOXA can be induced and that the induced phage
422 particles are carrying the *oxa23* gene. It is therefore clear that in *A. baumannii* bacteriophages could
423 be a major mechanism for the mobilisation of antibiotic resistance genes including those of greatest
424 clinical concern such as the carbapenemases. As more genomic studies using long-read sequencing
425 are conducted that can properly resolve complex mobile element structures, the true magnitude of
426 bacteriophage-mediated antibiotic resistance gene carriage will be revealed.
427
428 **Authors and contributors**
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431 methodology, investigation and writing (original draft preparation, review and editing) of the study.
432 EL was involved in the investigation and writing (review and editing) of the study.
433
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436
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716 Table 1: Carbapenem susceptibility data, MLST assignments, carbapenem resistance genes and associated mobile genetic elements of Egyptian isolates.
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Strain	MICs (mg/L)		MLST (Pasteur)		Resistance genes		*oxa23* mobile element[1]
	IMI	MER	ST	CC	*oxaAb*		
1a	4	32	664	2	66	*oxa23*	A
2	8	64	1	1	69	*oxa23*, bla_{NDM-1}	nd
4	16	256	1	1	69	*oxa23*	nd
5	32	64	25	25	64	*oxa23*	A
6	8	32	1	1	69	*oxa23*	phiOXA
7-T	8	64	1	1	69	*oxa23*	phiOXA
8-T	8	64	1	1	69	*oxa23*	phiOXA
8a	16	32	15	15	51	*oxa23*	A
9	8	64	158	158	65	*oxa23*	H
10	8	32	158	158	65[2]	*oxa23*	C
10a	16	32	2	2	66	*oxa23*	A
11a	16	32	1535		65[3]	*oxa23*	E
13a	4	32	664	2	66	*oxa23*	A
14a	16	16	664	2	66	*oxa23*	A
15	8	64	25	25	64	*oxa23*	A
16	8	64	85	-	94	*oxa23*	nd
18	8	64	19	1	69	*oxa23*	J
21	32	64	1	1	69	*oxa23*, bla_{NDM-1}	phiOXA
22	8	32	2	2	66	*oxa23*	A
27	8	32	2	2	66	*oxa23*	F
30	8	32	158	158	65	*oxa23*	nd
31	16	64	15	15	51	*oxa23*	G
34	8	64	158	158	65	*oxa23*	nd
35	4	64	1	1	69	*oxa23*	phiOXA
36	8	64	158	158	65	*oxa23*	nd

39	4	64	1	1	69	*oxa23*	phiOXA
40	32	64	1	1	69	*oxa23, bla*$_{NDM-1}$	nd
41	16	32	1	1	69	*oxa23*	phiOXA
42	32	64	158	158	65	*oxa23*	B
43	16	64	158	158	65	*oxa23*	B
44	8	64	158	158	65	*oxa23*	B
45	32	32	158	158	65	*oxa23*	nd
46	8	16	1	1	69[4]	*oxa23*	phiOXA
64	8	64	664	2	66	*oxa23*	A
68	8	32	158	158	65	*oxa23*	D
69	8	32	2	2	66	*oxa23*	C
70	16	32	103	-	70	*oxa23, bla*$_{NDM-2}$	nd
71	8	32	2	2	66	*oxa23*	F
72	16	64	2	2	66	*oxa23*	A
73	8	64	2	2	66	*oxa23*	A
74	8	32	664	2	66	*oxa23*	A
75	8	16	2	2	66	*oxa23*	F
78	8	16	570	2	+	*oxa23, bla*$_{NDM-1}$	A
82	64	64	2	2	66	*oxa23*	C
83	64	64	2	2	66	*oxa23*	A
84	64	64	2	2	66	*oxa23*	A
85	64	32	2	2	66	*oxa23*	A
86	64	>256	570	2	66	*oxa23, bla*$_{NDM-1}$	A
87	64	64	1	1	69	*oxa23*	I
88	64	64	2	2	66	*oxa23*	A
89	64	64	2	2	66	*oxa23*	F
92	32	64	2	2	66[5]	*oxa23*	A
5910	128	>256	2	2	66	*oxa23*	A
6135	8	32	600	2	66	*oxa23, bla*$_{NDM-1}$	A

735 [1]nd, not determined – the Illumina sequence data was not able to resolve contigs showing the genetic environment of *oxa23* in these isolates. [2]Contig break

736 giving incomplete gene, with 243/243 amino match to *oxaAb*(65). [3]Contig break giving incomplete gene, with 265/266 amino acid match to *oxaAb*(65).

737 [4]Contig break giving incomplete gene, with 266/266 amino acid match to *oxaAb*(69). [5]Contig break giving incomplete gene, with 266/266 amino acid match

738 to *oxaAb*(66). +, *oxaAb* gene was not identified in genome sequence but was positive by PCR.

739 Table 2: $CC^{PAS}158$ and $CC^{OX}499$ isolates reported in the literature or in public databases.

Isolate[a]	**ST^{PAS}**	**ST^{OX}**	**Country**	**Year**	***oxaAb***[b]	**Accession no.**	**Ref.**
10 isolates	158	499[f]	Egypt	2010-15	65*		This study
1309; 2226C	158	-	Turkey	2009	-	-	PubMLST
2313; AA-014	158	960	Iraq	2008	65*	GCA_000335595	(90)
3826; 778944; ABC002	158	1717	Egypt	2012	-	-	PubMLST
K50	158	-	Kuwait	2008	65*	OHJL00000000	(91)
Unnamed	158	-	Lebanon	2013	65*	-	(92)
Ab-Pak-Pesh-01	158	-	Pakistan	2015	65*	SMUB01000000	(68)
Ab-Pak-Pesh-07	158	-	Pakistan	2015	65*	QQPV00000000	(68)
Ab-Pak-Pesh-28	158	-	Pakistan	2015	65*	QQPZ00000000	(68)
AMA 341	158	499	Denmark[c]	2012	65[d]	SAMN03160609	(93)
Two isolates	158	-	Kuwait	2011-12	-	-	(94)
ACB69C	158	-	Turkey	2009-11	-	-	(95)
30 isolates	158	-	Kuwait	2007-08	66	-	(96)
7 isolates	158	499	Tunisia	2008-09	-	-	(97)
1830; J17	342	-	China	2011	-	-	(98)
3840; ACIN00151	342	1776	USA	2016	694	PubMLST[e]	PubMLST
2178; A.baumannii64	615	-	Egypt	2012	-	-	(16)
2180; A.baumannii85	615	-	Egypt	2013	-	-	(16)
2182; A.baumannii108	618	-	Egypt	2013	-	-	(16)
3950; TR112	1241	-	Turkey	2016	-	-	PubMLST
8 isolates	-	499	Egypt	2015	-	-	(27)
2 isolates	-	499	Saudi Arabia	2011-13	-	-	(19)
1 isolate	-	499	Kuwait	2011-13	-	-	(19)

740

741 [a]If an isolate is known by more than one name, all are provided separated by semicolons. [b]The

742 *oxaAb*(65*) alleles differ from the original *oxaAb*(65) sequence by 3 silent substitutions. [c]This isolate

743 was likely imported from Egypt. [d]Authors do not state whether the nucleotide sequence differs from

744 the original *oxaAb*(65) sequence. [e]This genome is available through the PubMLST website. [f]One

745 isolate did not have its ST^{OX} determined.

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751 Figure 1: Core gene tree of all isolates. In the centre is the core gene tree generated in FastTree (37)
752 using a core gene alignment output from Roary (36). The tree is scaled by genetic distance, and
753 branch labels indicate level of support based upon the Shimodaira-Hasegawa test using 1,000
754 resamples. Leaves are labelled with isolate names or SRA accession numbers, and are colour coded
755 to highlight the three major Pasteur MLST scheme clonal complex or sequence types identified in
756 this study. The ST/CC of isolates that are not coloured can be seen in Table 1 . The outer solid
757 coloured ring indicates the geographic source of the isolates. The outer rings of shapes indicate β-
758 lactamases and phiOXA encoded by the isolates.

759

760 Figure 2: Clustering of isolates by similarity of their non-core genomes using PANINI. Each dot
761 represents an isolate, and the distance between isolates indicates the similarity of their accessory
762 genomes. (A) the network is coloured according to the MLST data as in figure 1 (CC1 is purple, CC2 is
763 orange, ST158 is green, and other STs are grey). (B) the network is coloured according to the country
764 of origin of the isolates as in Figure 1 (Egypt is red, Saudi Arabia is dark blue, Iraq is light blue, the
765 UAE is green, and other countries outside the Middle East are grey).

766

767 Figure 3: Genetic environments surrounding *oxa23* genes. Arrows represent genes, which are colour-
768 coded by their type. Unlabelled grey genes represent hypothetical proteins. The size of the genes
769 and the distances between them are drawn to scale. Vertical grey boxes indicate homology between
770 sequences ranging between 73% and 100% identity (BLASTn). The diagram was created using Easyfig
771 (99) and annotated in Adobe Photoshop.

772

773 Figure 4: Schematic genome map of phiOXA-A35 and related prophages. Prophages are orientated
774 as they appear in their host genome. Arrows depict open reading frames and are coloured according
775 to function. Homologs to gene products in *Escherichia* phage P2 are indicated in parentheses. ORFs
776 encoding hypothetical proteins are shown as black outlines. The tRNA-Leu, representing the attL

777 site, is shown as a dark red rectangle. Shading between entries represents the percent identity

778 (BLASTn) from 90% (light grey) to 100% (dark grey). The map was constructed using Easyfig (99) and

779 annotated in Adobe Illustrator.

780

781 Figure S1: Maximum-likelihood phylogenetic tree created from portal vertex protein sequences of

782 members of the subfamily *Peduovirinae*. Sequences were aligned with Clustal Omega and trees

783 constructed using IQTree v1.6.12 with the LG + G4 substitution model IQTree v1.6.12 with

784 ModelFinder, SH-aLRT test and ultrafast bootstrap (1000 replicates). Enterobacteria phage mEp237

785 (JQ182730) was used as an outgroup to root the tree. Branch length is proportional to the number of

786 substitutions per site (see scale bar). Members of virus genera are denoted by coloured blocks. An

787 asterisk (*) adjacent to a genus name indicate proposed genera that at the time of writing were yet

788 to be ratified by the International Committee on the Taxonomy of Viruses (100).

789

790 Figure S2: Maximum-likelihood phylogenetic tree created from major capsid protein sequences of

791 members of the subfamily *Peduovirinae*. Sequences were aligned with Clustal Omega and trees

792 constructed using IQTree v1.6.12 with the WAG + G4 substitution model, SH-aLRT test and ultrafast

793 bootstrap (1000 replicates). Enterobacteria phage mEp237 (JQ182730) was used as an outgroup to

794 root the tree. Branch length is proportional to the number of substitutions per site (see scale bar).

795 Members of virus genera are denoted by coloured blocks. An asterisk (*) adjacent to a genus name

796 indicate proposed genera that at the time of writing were yet to be ratified by the International

797 Committee on the Taxonomy of Viruses (100).

798

799 Figure S3: Distribution of different inserted elements at the tRNA-Leu site in $CC^{PAS}1$ isolates. The tree

800 is the same as that shown in Figure 1, retaining only those leaves representing $CC^{PAS}1$ isolates.

801 Leaves are annotated with the type of insertion found at tRNA-Leu: orange indicates phiOXA, pale

802 blue indicates no insertion, and pale pink indicates an insertion other than phiOXA is present. In the

803 case of strain AYE the insertion is bacteriophage RPHR (101) which is identical to phiOXA except for

804 the IS*Aba1*-*oxa23* insertion (see Figure 4), and in isolates 6322619 and 6322626 this represents the

805 insertion of an unrelated uncharacterised element.