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Molecular epidemiological studies on sporadic and epidemic isolates of *Acinetobacter baumannii*

PhD thesis

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1. INTRODUCTION

Currently, one of the most, if not the most serious problem modern medicine is facing is the continuous rise of bacteria that are resistant to different antimicrobials. After having introduced the first antimicrobial agents into daily medical practice, for a short while, the discovery of the new compounds allowed us to successfully compete with this trend. However, the genes responsible for antibiotic resistance were already present, albeit at very low levels, before the introduction of antibiotics. The selective pressure created by the often imprudent use of these "magic bullets", not only in humans but also in companion and food animals and in the environment, have been responsible for providing an edge for the resistant cells and clones over susceptible ones. The increasing mobility of people and goods, food and animals across the globe is another factor significantly contributing to the spread of resistant strains. Modern, scientific antibacterial therapy has started only over the last 60-70 years and by now become one of the utmost essentials of modern medicine. However, as stated by Hawkey: "As bacteria are thought to have evolved 3500 million years ago, 60 years is but ‘a second in a day’ in evolutionary time".

One of the most important nosocomial pathogen which has been added recently to the Infectious Diseases Society of America's dangerous pathogens hit-list is *A. baumannii.* The emergence of multi drug resistance (MDR) among *A. baumannii* strains has been described worldwide. This prompted several microbiological and epidemiological studies leading to the formulation of

interventions and infection control measures to prevent the impact of this organism on health case.

A. baumannii can easily be transmitted from patient-to- patient. In fact, there is a definite risk for a new patient acquiring the bacterium once admitted to the same hospital unit or ward occupied by patients colonized by acinetobacter. In the hospital setting, *A. baumannii* infections are present in two forms: sporadic infections and epidemics. Strains causing these outbreaks often exhibit extensive resistance. Epidemic strains of *A. baumannii* are highly clonal. The MDR strains causing major epidemics in hospitals all over the world represent a relatively few "successful", "particularly fit to the task" clones, i.e. genetically related strains. The best-studied ones are called (pan-) European clones I-II and III. Recently five more lineages have been identified and named as worldwide lineages (WW1 to WW8). The first three represent the European clonal lineages.

One of the most interesting features of *A. baumannii* is the ease by which it can acquire resistance to various antibiotics. Mutations, new gene acquisitions, up-regulating expression of existing genes (should those be genes of drug-destroying enzymes or efflux pumps), or losing them, like in the case of porins, all may contribute to the broad spectrum resistance of these strains. Resistance of this organism can affect practically any drugs used in clinical practice. As a result of the rapid acquisition of resistance genes to different and multiple classes of antibiotics, several drugs have already been eliminated from treatment options for *A. baumannii* infections such as penicillins, cephalosporins, aminoglycosides, quinolones and tetracyclines.

This thesis, based on published and unpublished results, describes our findings on the resistance of *A. baumannii,* particularly against β-lactam and aminoglycoside drugs, among strains isolated in Abu Dhabi Emirate.

2. AIMS AND OBJECTIVES

The general, long term aims of our studies were to provide the first detailed insight into the molecular epidemiology of *A. baumannii* in the Abu Dhabi Emirate, UAE. By this way we anticipated to generate data not only for local use (i.e. to aid infection control practices in local hospitals) but also to provide data for a better understanding of the regional and global dynamics of spread of MDR *A. baumannii.*

The specific objectives were

- to compile a representative group of clinical isolates of *A. baumannii*
- to establish their antibiotic resistance
- to study the molecular background of the resistance phenotype, particularly as far as resistance to β-lactams and aminoglycosides are concerned
- to reveal any existing clonality among the strains with establishing indicators of inter-hospital transfers
- to compare the resistance and genetic make-up of sporadic and epidemic isolates

- and finally to investigate the presence of any of the fast-spreading metallo-beta lactamases, ESBLs and ribosomal methylases among local isolates

3. MATERIALS AND METHODS

3.1 Strain collection

Between March and November 2008, clinically relevant, non-repeat isolates of *A. baumannii* strains had been collected from five different hospitals in Abu Dhabi Emirate: Tawam, Al Ain, Mafraq, Sheik Khalifa and Al Rahba Hospitals, respectively. In order to look for the presence of MBLs, from 2009 till 2010 a second collection of *A. baumannii* strains had been compiled from Tawam hospital. This group contained clinically relevant carbapenem resistant isolates, only.

Upon receiving the strains the identity of the isolates was confirmed by a gene specific PCR i.e. bla_{OXA51}.

3.2. Antibiotic sensitivity testing

The antibiotic disc diffusion tests were performed by the Kirby-Bauer method following the CLSI standards with discs purchased from MAST. Quantitative assays for different antibiotics were conducted by E-tests (Biomérieux) according to the manufacturers instructions. In certain cases minimal inhibitory concentrations (MIC) were determined by microdilution carried out according to CLSI standards. For all assays

Escherichia coli ATCC 25922 was used as a pan-susceptible control.

3.3. Polymerase Chain Reaction (PCR)

Four-five colonies grown overnight on TSA plates were suspended in 200 µl of PCR quality distilled water (Sigma), vortexed for 10 seconds and centrifuged for 2 minutes at 13,000 rpm. The supernatant was used as the sample in PCR reactions. PCR reactions were performed for 30-40 cycles at various annealing temperatures depending upon the melting temperature of the primer set used. The reactions were performed using the Applied Biosystems 2700 thermocycler.

3.4. Plasmid profile analysis

For routine detection and isolation of large and small plasmids the method of *Kado and Liu 1981* was used with minor modifications. The strains were inoculated on a half TSA plates overnight at 37^{o}C. Next day cells were collected and suspended gently into 250 µl of lysing solution. The lysed cells were incubated at 60° C in a thermoblock for 45 minutes. After the incubation period one volume of 1:1 phenol-chloroform was added. The solution was emulsified by gentle shaking. The emulsion was broken by centrifugation at 13,000 rpm for 15 minutes. The top aqueous layer (approximately 60 µl) was transferred to clean tubes. Samples were subjected to electrophoresis for 3 hours at 120 V in 0.8% agarose gel. The gels were stained with ethidium bromide, de-stained

and scanned using the Biometra gel documentation system.

3.5. Detection of megaplasmids by S1 nuclease digestion

This method used to isolate very large plasmids. In brief, the strains were grown overnight in 15 ml TSB at 37° C. Next day, approximately 4 x10^9 cells were collected by centrifugation. The cells were washed and re-suspended in 500 µl of EC. The cell suspensions were mixed with an equal volume of 1% PFGE sample preparation agarose containing 20 µg/ml RNase and 1 mg/ml lysozyme, and immediately transferred to 1 ml syringes.

After solidifying, the plugs were sliced into 1 mm thick slices. To lyse the cells the plugs were incubated in 1 ml of EC buffer at 37° C for 1 hour. Subsequently, the plugs were incubated at 50° C overnight in ES buffer supplemented with 1mg/ml proteinase K. The ES buffer and the proteinase were inactivated by washing the plugs in 1ml of 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma) prepared in TE buffer. In order to linearize the circular megaplasmids, the incorporated DNA within the plugs was digested with 1 U of S1 nuclease in 200 µl of S1 buffer for 10 min at 37° C. The plugs were loaded into wells of a 1% horizontal agarose gel. Lambda concatamer was used as size marker loaded into the two lateral wells of each gel.

3.6. Conjugation

For conjugation experiments *E. coli* RAZ (Na-azid resistant in-house derivative of the rifampin resistant J53) and an in-house derivative of *A. baumannii* BM4547 rif (kindly provided by Dr. Patrice Nordmann, Paris) and also made resistant to Na-azid (BM4547 RAZ) were used as recipients. Conjugation was attempted by combining cultures of the donor and recipient in 1: 5 ratios.

The mixture was incubated without shaking for 4 hours, and then it was centrifuged at 3500 rpm for 15 minutes. The pellet was re-suspended in 200 µl TSB and added as a drop to the center of TSA plate without any antibiotics. The next day, the growth was harvested, washed twice, re-suspended in 3 ml of PBS, and serially diluted and plated onto plates containing 100 µg/ml Na-azid and the appropriate antibiotics. Next day colonies were collected and subjected to repeated antibiotic susceptibility tests, plasmid electrophoresis, PCR and, if necessary, to ERIC and PFGE to confirm that indeed, transconjugants, instead of mutants of the donor or recipient were obtained.

3.7. Molecular fingerprinting of the isolates

Macrorestriction analysis of the strains by pulsed field gel electrophoresis (PFGE). Macrorestriction analysis of the strains was carried out by pulsed field gel electrophoresis (PFGE) with minor modification of the method described by Seifert *et al.* 1994. Following the plug preparation, chromosomal DNA in the blocks was

digested with 40 U ApaI restriction enzymes (New England Biolabs) overnight at 25°C. After digestion, the plugs were washed in 250 µl of 0.5X TBE for 5 min and were transferred into wells of a 1.4% horizontal agarose gel (PFGE running agarose, Sigma). Lambda concatamer (New England Biolabs) was used as size marker loaded into the two lateral wells of each gel. The macrorestriction patterns were compared according to Dice similarity index (1–1% tolerance interval) using the GelCompare II software (Applied Math, Sint-Martens-Latem, Belgium). A pulsotype was arbitrarily defined as a cluster of strains exhibiting macrorestriction banding patterns with ≥ 80% similarity.

Multilocus Sequence Typing (MLST) of the isolates was carried out routinely by the method of *Bartual et al. (2005)*. Genomic DNA was prepared by boiling 4-5 colonies in 200 µl of sterile water. PCRs for the seven housekeeping genes, *gltA, gyrB, gdhB, recA, cpn60, gpi*, and *rpoD*, were performed in 25-µl volumes.

The products were purified using the Exo-SAP (Usb) and were sequenced in both directions using 3130X genetic analyzer (Applied Biosystems). The sequence results were analyzed using the online BLAST (*http://www.ncbi.nlm.nih.gov/BLAST/*). The isolates were then assigned to sequence types (STs) using the tools on the *A. baumannii* MLST webpage.

The Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) approach was used to quickly verify differences between donor, recipient and transconjugants or transformants. It was carried out by using the ERIC2 primer. Comparison

between amplicon patterns was done by visual inspection.

3.8 Cloning of *bla*$_{NDM}$

Genomic DNA was purified using the Qia Amp DNA mini kit (Qiagen), and partially digested with HindIII (New England Biolabs) for 30 seconds, 1, 2, 4, and 10 minutes. The resulting fragments were purified using the PCR purification kit (Qiagen). The purified product was then ligated into pUC19 using the T4 DNA ligase (NEB) overnight at 16° C. The ligation mixture was transformed into *E. coli* DH5α competent cells. The cultures were plated onto plates containing 8 µg/L ceftazidime. Next day, the colonies were screened for *bla*$_{NDM}$ by PCR, and the positive clones were digested with HindIII to estimate the size of the insert.

Of a clone carrying the plasmid p132LigB with an insert of ca. 10 kb containing the *bla*$_{NDM}$ gene was identified. A 3675-bp long partial sequence of the insert covering the flanking regions of the *bla*$_{NDM}$ gene (GenBank accession number JN112341) was generated by primer walking and subsequently confirmed by direct, bidirectional sequencing with primers ASndm1 (5'-GTCGCAAAGCCCAGCTTCGCA-3') and ASndm2 (5'-GCCTCGCATTTGCGGGGTTTTTA-3').

3.9. Sequencing

PCR products were purified either by the EXO-SAP or PCR purification kit (Qiagen) prior to sequencing. Sequencing was done using the Big Dye Cycle

Terminator V.3.1 (Applied Biosystems) according to the manufacturer's instructions. The samples were sequenced in both directions using the 3130X Genetic Analyzer (Applied Biosystems). Sequences were analysed by using the MEGA4 (http://www.megasoftware.net/mega4/mega.html) and Clone Manager (Sci-Ed software) softwares.

3.10. Southern blot

Plasmid gels (0.8-1.2%), prepared either by the methods of Kado or by S1 digestion were stained by ethidium bromide, photographed and scanned using the Biometra gel documentation system with rulers placed around the gel for subsequent identification of bands. The gels were capillary-transferred to Hybond N+ membranes (Amersham) by soaking overnight in 20X SSC. The next day the membranes were UV cross-linked at 70,000 micro-joules and the hybridization was performed by using the DIG DNA labeling and detection kit (Roche).

3.11. Gene expression assay

In all assays targeting 16S ribosomal RNA genes was used as a constitutively expressed control. Bacterial RNA was isolated using the Trizol Max bacterial RNA Isolation kit (Invitrogen Life Technologies). 2.5 µg of the RNA was DNase-treated using 2-3 units of amplification grade DNase I (Invitrogen Life Technologies). One µl of the DNase-treated RNA samples were tested in PCR using 16S primers to

confirm the absence of contaminating DNA prior to cDNA synthesis.

This was followed by cDNA synthesis using Moloney Murine Leukemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies) in the presence of 40 U of RNasin RNase inhibitor (Promega). cDNAs were amplified using the Qiagen Taq polymerase kit (Qiagen). PCR reactions were performed for 20-30 cycles at various annealing temperatures depending on the melting temperature of the primer set used.

3.12. Statistical analysis

The assessment of the association of specific genes and features with sporadic and epidemic isolates was carried out by the likelihood score test. As members of the epidemic clones represented closely related isolates they were re-weighted by the weight equal to the inverse of the size of the particular clone. The calculations were done using the Logistic Regression Module of the SPSS 19.0 program (Hosmer and Lemeshow 2000) by Professor Nico Nagelkerke (FMHS, UAEU).

3.13. Arbitrary definitions used

Strains were considered "*multidrug resistant"* if they were non-susceptible to carbapenems and to at least two non-beta lactam classes. A "*sensitive*" isolate was susceptible to carbapenems and to at least 3 other classes, while a "*resistant isolate*", i.e. strains in between, were carbapenem susceptible, but non-susceptible to 3 other classes. A strain was regarded to

have an epidemic potential ("epidemic strain" for this study) if at least one other isolate recovered during the study period exhibited the same PFGE profile (with ≥85% similarity in the banding pattern) and carried the same *bla*$_{\text{OXA51-like}}$ gene. Strains clustered by these features were regarded as clones, while all other isolates were considered sporadic.

4. RESULTS

4.1. Molecular epidemiology of *A. baumannii* in Abu Dhabi hospitals

After eliminating repeated and clinically non relevant isolates, altogether 110 *A. baumannii* strains recovered from Abu Dhabi hospitals between March and November, 2008 were included in the study. Based on the banding patterns obtained by PFGE, and by using an arbitrarily chosen 85% similarity threshold, 14 clusters (A-N) containing minimum two isolates were identified. The clusters altogether contained 87 strains while 23 isolates formed singletons.

In order to establish groups containing isolates likely to represent real clones, this typing method was complemented by determining the allelic type of the *bla*$_{\text{OXA51-like}}$ enzymes indigenous to all *A. baumannii* isolates. Only those strains were considered to form a clone, which, beyond belonging to the same macrorestriction group, also carried the same allele of the gene indicating that they are member of the same lineage.

We found that 38 strains belonging to 5 clusters carried *bla*$_{\text{OXA69,}}$ 14 isolates grouped into 2 clusters carried *bla*$_{\text{OXA64}}$ and 28 strains forming four clusters had *bla*$_{\text{OXA66.}}$ It was noteworthy that clones carrying the same *bla*$_{\text{OXA51-like}}$ allele exhibited, albeit not identical, but similar PFGE patterns.

Based on these criteria, seven isolates, originally recorded among cluster-forming strains, were re-assigned to the singletons' pool. As a result, we had 80 isolates forming 11 clones what we considered to have epidemic potentials as their representative have been repeatedly isolated during the study period. There were single clones with 19, 12 and 11 members, and two-two clones with 10, 4, 3 and 2 strains, each, respectively. These isolates will be referred to as ***"epidemic"*** strains. On the contrary, the 30 singletons, as representing the only isolates recovered exhibiting their respective types, will be called ***"sporadic"*** isolates in this thesis.

Representatives of the larger clones have been present for several months in Abu Dhabi hospitals. The majority of strains from the three large tertiary care hospitals (Tawam, SKMC and Mafraq) qualified as epidemic isolates, while from Al Ain and Rahba hospitals mostly sporadic isolates were recovered. Almost 40% (38.8%) of the epidemic strains derived from respiratory samples and 81.6% of all respiratory isolates represented types repeatedly isolated during the study period. On the other hand approximately the same percentage (36.7%) of the sporadic isolates came from wound samples, but still, the majority of the strains of this origin also (22, 66.7% of this sample type) represented epidemic isolates.

The eleven epidemic clones represented 9 core genotypes, i.e. a set of genes present in >50% of the members of the cluster. On the contrary, the 30 sporadic isolates exhibited a considerable genetic variability carrying 21 genotypes.

In general, the strains were highly resistant to antibiotics. With the exception of colistin, at least 50% of all the strains were non-susceptible to any antibiotics for which break-point values were available (i.e. this excludes tigecycline).

While non-susceptibility was common among the strains, it was significantly more frequently seen among epidemic isolates for some drugs reaching (ciprofloxacin), for several others (ceftazidime, meropenem, imipenem, gentamicin, trimethoprim-sulfamethoxazole) approaching 100%. As a consequence, 97.5 % of the epidemic strains were considered MDR compared to 20% of the sporadic isolates, i.e. a highly significant difference.

4.2. Aminoglycoside resistance associated with *armA* ribosomal methylase

The presence of the *armA* gene in several (30) isolates coding for a ribosomal methylase raised the possibility of broad-range aminoglycoside resistance among these strains. The gene was part of the core genotype in several clones as well as 2 sporadic isolates. Therefore, for these isolates, the quantitative susceptibility testing was extended to other drugs of this class (i.e. streptomycin, kanamycin, netilmicin, and spectinomycin). Of the 30 isolates (2 sporadic and 28

epidemic strains, respectively) 27 expressed uniform, high level resistance to all aminoglycosides tested.

As the presence of the *armA* gene was associated earlier with paradoxical amikacin susceptibility pattern in *A. baumannii*, we tested our isolates for this phenomenon. Indeed, all 27 strains carrying the *armA* exhibited the halo of susceptibility around a zone of resistance implying as if the bacterium resisted to a higher concentration (close to the disc) while being susceptible to a lower concentration far from the disc.

As the nature of the phenomenon suggested a concentration dependent response, it was investigated whether the expression of the genes of *armA,* or those of the efflux pumps were affected by the concentration of amikacin. When testing the cDNA of epidemic strain NM 99 by semi-quantitative RT-PCR no effect was found on the amount of specific mRNA produced in response to exposure to various concentrations of amikacin, neither in case of *armA* nor in case of any of the efflux genes tested.

As the *armA* gene may locate on a plasmid in *A. baumannii* attempts were made to conjugally transfer the gene either into *E. coli* or into *A. baumannii.* Irrespective, however the conjugation temperature used, no transconjugants were obtained.

4.3. Plasmid-localization of bla_{PER7}

One member of the collection, an epidemic strain from clone H (NM55) had a pair (NM 128) having been isolated from the same patient four months apart. As coming from the same patient and exhibiting the same

PFGE patterns, they were considered isogenic. However, while NM55 expressed the same resistance pattern as the rest of its clone, NM128 was more susceptible. It was particularly noteworthy that although both strains were resistant to carbapenems, it was only NM 55, which also exhibited resistance to ceftazidime.

Comparing the genotypes of the two isolates revealed that NM 55, like the majority of the members of its clone, carried the *bla*$_{\text{PER}}$ gene while NM128 did not. As this gene codes for an ESBL with considerable 3rd generation cephalosporinase activity we assumed that its loss in NM128 might be responsible for its susceptibility to cephalosporins, while its carbapenem resistance was maintained by *bla*$_{\text{OXA-23}}$ still carried by the strain. Meanwhile, our collaborators in Edinburgh, by sequencing the gene, established that its actual allelic type was *bla*$_{\text{PER-7.}}$

We attempted to localize the gene *bla*$_{\text{PER-7}}$. Experiments to prove the possible plasmid localization of the gene by transferring it into a suitable recipient repeatedly failed. However, by conventional gel electrophoresis plasmids of around 200 kb (slightly less in NM128) were visible. In NM55 this plasmid strongly hybridized with a *bla*$_{\text{PER}}$ oligo probe indicating the localization of the gene. As simple plasmid extraction gels are unsuitable to accurately size plasmids in this molecular mass range, we assessed the size of these plasmids by S1 nuclease digested preparations separated in pulsed electric field. The gels revealed that NM 55 indeed carries a plasmid of ca. 200 kb, while the one in NM 128 was approximately 20 kb less.

4.4. *bla*$_{\text{NDM-2}}$ in *Acinetobacter baumannii*

To reveal whether the *bla*$_{\text{NDM,}}$ i.e. a rapidly emerging MBL among Enterobacteriaceae penetrated the population of *A. baumannii* strains in Abu Dhabi we screened a 155 strong pool of carbapenem resistant isolates collected between 2008 and 2010 in Tawam hospital, Al Ain, UAE. Using an in-house primer pair, two *bla*$_{\text{NDM}}$ positive strains were identified. Both strains were recovered from a 55-year old Egyptian female with a congenital single kidney.

The two strains revealed the same susceptibility profile. Both isolates carried the naturally occurring *bla*$_{\text{OXA-70}}$ gene and were positive for IS*Aba1*, which was not, however, identified upstream of the *bla*$_{\text{OXA-70}}$ nor of the *bla*$_{\text{AmpC}}$ genes. Both isolates were negative for all other carbapenemase genes tested by PCR. Attempts to detect any of the ribosomal methylases genes, not infrequently associated with NDM-producing organisms also yielded negative results.

The two isolates exhibited the same macro-restriction pattern. They belonged to the same MLST types irrespective of the typing system used, i.e. ST253 or ST103. Both of the isolates contained multiple plasmid bands ranging from 7- to 140-kb.

Attempts were made to conjugally transfer the *bla*$_{\text{NDM}}$ gene. Repeated experiments carried out at different temperatures to mobilize the gene into an *E. coli* recipient were not successful. However, when using an *A. baumannii* recipient (BM4547, kindly provided by Prof. P. Nordmann, Paris) made Na-azid resistant (BM4547-RAZ) transfer of the *bla*$_{\text{NDM}}$ gene with

concomitant increase of the carbapenem MIC was achieved. However, while the derivatives were all positive by PCR targeting *bla*$_{NDM}$ and exhibited the same PFGE pattern as the recipient, this was not accompanied by a simultaneous transfer of any detectable plasmid. The S1 nuclease digestion method to reveal any plasmids in the derivative did not yield a positive result, either.

In order to determine the genetic structure surrounding the *bla*$_{NDM}$ gene, DNA from strain AG132 was partially digested with HindIII (New England Biolabs NEB, USA) and ligated using T4 DNA ligase (NEB) into pUC19. The ligation mixture was transformed in *Escherichia coli* DH5α followed by plating onto plates containing 8 μg/L ceftazidime. A clone carrying the plasmid p132LigB with an insert of c. 10 kb containing the *bla*$_{NDM}$ gene was identified. A 3675-bp long partial sequence of the insert covering the flanking regions of *bla*$_{NDM}$ (GenBank accession number JN112341) was generated by primer walking and subsequently confirmed by direct, bidirectional sequencing.

Detailed sequence analysis identified that the strain carries the *bla*$_{NDM-2}$ allele which, however, did not contain the silent A→G substitution at position 468 found in the Israeli isolate. Insertion sequence ISAba125 was found upstream of the *bla*$_{NDM-2}$ gene while downstream a putative bleomycin resistance gene and a phosphoribosyl anthralinate isomerase gene were found. The sequence of the *bla*$_{NDM-2}$ gene, as well as that of its flanking regions was deposited to GenBank with accession No. JN112341

5. CONCLUSIONS

Based on our results we conclude that

1. *Acinetobacter baumannii* is well established in hospitals of Abu Dhabi both as sporadic infections as well as epidemics. The latter type represents the majority, almost 3 quarters, of the cases
2. The epidemic strains represent not only different clones, but also three different lineages (marked by bla_{OXA69}, bla_{OXA64}, and bla_{OXA66}, respectively)
3. In some cases multiple clones are simultaneously present in the same hospital (e.g. in Tawam clones D and H). On the other hand representatives of certain clones, while primarily characteristic to a "host" institution, are regularly found in other hospitals, as well
4. Epidemic strains are genetically much more uniform than their extremely heterogeneous sporadic counterparts
5. The epidemic strains contain more than twice as many resistance-related genes, as the ones isolated from sporadic cases
6. Unexpectedly in the region, the IS*Ab1*-linked bla_{OXA23} is present in the core genotype of all epidemic clones, while, also unexpectedly bla_{OXA24}, bla_{OXA58} are completely absent or rarely encountered suggesting the power of local factors affecting the type of the dominant strains
7. Aminoglycoside resistance genes are, with exceptions, more evenly distributed than most of those causing resistance to β-lactams (e.g. bla_{OXA23}, bla_{PER})

8. Nearly 100% of the local epidemic strains are non-susceptible to all first-line drugs (ceftazidime, carbapenems, ciprofloxacin, gentamicin). Sporadic isolates, as an average, are much more sensitive. Nevertheless, non-susceptibility among them also reaches a level which makes initial treatment with first line drugs highly risky
9. With the exception of one isolate, strains were all susceptible to colistin, and tigecycline is likely to be effective, as well.
10. Based on these data we conclude that in the hospitals of the region it is primarily the lack of susceptibility to broad spectrum β-lactams, ciprofloxacin and gentamicin what may provide the advantage needed to secure survival
11. *armA*, a ribosomal methylase has penetrated several clones and some sporadic isolates in the region
12. The presence of *armA* was confirmed to be on a plasmid, at least in case of two clones
13. The *armA* -related unique pattern of amikacin susceptibility (also linked to false susceptibility reporting) is not due to the increased gene expression neither in case of *armA* nor efflux pump genes *ade* or *abe*
14. Unlike initially reported, $bla_{\text{PER-7}}$ gene can also be localized on a plasmid outlining a possible path for its introduction into the species
15. Our data strongly suggest that the $bla_{\text{PER-7}}$ is within a ca. 20 kb fragment of the plasmid together with *armA* and *aac(3)-Iia*
16. We identified two isogenic strains locally carrying $bla_{\text{NDM-2}}$

17. We cloned and sequenced the gene and its surrounding revealing that both the upstream and downstream region is highly similar to what have been seen in strains carrying various alleles of the gene
18. Based on the almost complete identity of our strain (linked to Egypt) with two other isolates from the Middle East we surmise that this clone has evolved locally, and has spread to Europe providing a very recent example of pathogen trafficking

Published papers related to thesis:

1. Opazo A., Sonnevend A., Lopes B., Hamouda A., **Ghazawi** A., Pal T., Amyes S.G. (2012). Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in Acinetobacter baumannii isolated in the United Arab Emirates. *J Antimicrob Chemother*. **67:**1619-22.
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1. Szijártó V., Pal T., Nagy G., Nagy E., **Ghazawi** A., Al-Haj M., Kurdi S.E., Sonnevend A. (2012). The rapidly emerging ESBL-producing Escherichia coli O25-ST131 clone carries LPS core synthesis genes of the K-12 type. *FEMS Microbiol Lett*. **332:**131-6.
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3. Rizvi T.A., Kenyon J.C., Ali J., Aktar S.J., Phillip P.S., **Ghazawi A.**, Mustafa F., Lever A.M. (2010). Optimal Packaging of FIV Genomic RNA Depends upon a Conserved Long-range Interaction and a Palindromic Sequence within gag. *J Mol Biol.* **403:**103-19.

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