Aus dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

# Molecular and functional typing of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with emphasis on multi-drug resistant *Acinetobacter baumannii*

**Inaugural-Dissertation**
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
**Stefanie Müller**
Tierärztin aus Jena

Berlin 2017
Journal-Nr.: 3964

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## ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| *A. baumannii* | *Acinetobacter baumannii* |
| *A. haemolyticus* | *Acinetobacter haemolyticus* |
| *Acb*-complex | *Acinetobacter calcoaceticus -Acinetobacter baumannii* complex |
| AFLP | amplified fragment length polymorphism |
| AMC | amoxicillin/clavulanic acid |
| AME | aminoglycoside modifying enzyme |
| AMP | ampicillin |
| AMPS | ampicillin sulbactam |
| AST | Antimicrobial susceptibility testing |
| ATCC | American Type Culture Collection |
| BLAST® | Basic Local Alignment Search Tool |
| BHI | Brain Heart Infusion Broth |
| bp | base pair |
| CAZ | ceftazidime |
| CC | clonal complex |
| cfu | colony forming units |
| CHDL | carbapenem-hydrolyzing class D beta-lactamase |
| CIP | ciprofloxacin |
| ci plot | confidence interval plot |
| CO | colistin |
| COL S+ | Columbia agar supplemented with 5% sheep blood |
| CLSI | Clinical Laboratory and Standards Institute |
| CR | cefpirome |
| CTX | cefotaxime |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxynucleosid Triphosphate |
| DSMZ | German Collection of Microorganisms and Cell Cultures |
| EC | european clone |
| *E. coli* | *Escherichia coli* |
| ENR | enrofloxacin |
| ESBL | extended-spectrum beta-lactamase |

| | |
|---|---|
| GM | gentamicin |
| *gyrA* | DNA gyrase subunit A |
| *gyrB* | DNA gyrase subunit B |
| IMT | Institute of Microbiology and Epizootics |
| IC | international clone |
| ICU | intensive care unit |
| IGS | intergenic spacer |
| IL | interleukin |
| IMT | Institute of Microbiology and Epizootics |
| IP | imipenem |
| IS | insertion sequence |
| IDSA | Infectious Diseases Society of America |
| kbp | kilobase pair |
| LB | Luria Bertani medium |
| LPS | lipopolysaccharide |
| MAPK | mitogen activated protein kinase |
| MBL | metallo-beta-lactamase |
| MCG | Maximum Common Genome |
| MDR | multi-drug resistant |
| $Mg^{2+}$ | magnesium 2+ ions |
| MIC | minimum inhibitory concentration |
| MLST | multi locus sequence typing |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| MRSA | methicillin resistant *Staphylococcus aureus* |
| MSSA | methicillin susceptible *Staphylococcus aureus* |
| N | nitrofurantoin |
| NCBI | National Centre for Biotechnology Information |
| NF-KB | Nuclear Factor Kappa B |
| OMP | outer membrane protein |
| OXA | oxacillinase |
| PAMP | pathogen associated molecular pattern |
| *parC* | topoisomerase IV subunit A |
| *parE* | topoisomerase IV subunit B |

| | |
|---|---|
| PB | polymyxin B |
| PBS | phosphate buffered saline |
| PBP2 | penicillin binding protein 2 |
| PCR | polymerase chain reaction |
| PFGE | pulsed field gel electrophoresis |
| PIP | piperacillin |
| PRR | pathogen recognition receptor |
| PX | cefpodoxime |
| QRDR | quinolone resistance determining region |
| RFLP | restriction fragment length polymorphism |
| RI | rifampicin |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| *rpoB* | RNA polymerase beta-subunit |
| SNP | single nucleotide polymorphism |
| sRNA | small RNA |
| ST | sequence type |
| TE | tetracycline |
| TLR | toll-like receptor |
| TNF-α | tumor necrosis factor alpha |
| TTC | triphenyltetrazoliumchloride |
| T/S | trimethoprim/sulfamethoxazole |
| VAP | ventilator associated pulmonia |
| WGS | whole genome sequencing |
| XDR | extensively-drug resistant |
| USA | United States of America |

## INTRODUCTION

Less than a hundred years ago, simple bacterial infections were one of the greatest challenge for human and veterinary medicine, causing life-threatening conditions even in case of simple, superficial wound infections in healthy individuals. Besides the establishment of hygiene and disinfection measures, the introduction of penicillin for the treatment of bacterial infections entailed immeasurable medical advances on a global scale. Various further antimicrobial compounds have been discovered and generated to date. Unfortunately, extensive use of the new medical weapons led to the development and enrichment of antimicrobial resistances in bacteria following the discovery of novel drugs. The bacterial ability to adapt remarkably fast to novel antimicrobials has its origin in the evolutionary course of billions of years, in which bacteria had to fight naturally occurring structurally related substances [2-6]. The tremendous antimicrobial selective pressure of the last decades thus resulted in an emergence of multi-drug resistant and pan-drug resistant bacteria, capable of throwing medicine back to the pre-antibiotic era. In this regard, the Infectious Diseases Society of America (IDSA) highlighted a group of bacterial species, the 'ESKAPE pathogens', capable of escaping antimicrobial effects and thus posing a particular threat to human and animal health [2, 7].

Of these, *Acinetobacter* (*A.*) *baumannii* is gaining increasing attention, since this bacterial species acquired antimicrobial resistances within a remarkably short period of time, now possessing an armamentarium of mechanisms for resistance against all currently known antimicrobials [8-11]. Specific *A. baumannii* clonal lineages moreover are associated with an extraordinary epidemic potential worldwide [12-18], and have already been detected even in animal populations [19-21], illustrating the zoonotic potential of these emerging pathogens. Yet essential questions regarding the origin and evolution of multi-drug resistant epidemic *A. baumannii* have not been answered [18]. Knowledge on *A. baumannii* of animal origin is particularly lacking, although multi-drug resistance, nosocomial spread, and the potential of zoonotic transmission have been reported in these isolates [19-25].

Due to its close relatedness to *A. pittii*, *A. calcoaceticus,* and *A. nosocomialis*, *A. baumannii* has been grouped together with these species into the so called *A. calcoaceticus-A. baumannii* (*Acb*)- complex, for which reliable species identification can only be achieved by molecular techniques [13, 14, 26]. Therefore, routine diagnostic laboratories usually perform species identification only to the *Acb*-complex level. This is clearly not acceptable, as the

*Acb*-complex species differ in their pathogenicity, their ability to survive and persist in the environment, and their tendency to develop multi-drug resistance. Fast and reliable species identification is thus of utmost importance in order to contain the epidemic spread of multi-drug resistant *A. baumannii* clones by implementing appropriate infection treatment and hygiene management procedures.

However, since it has been known for years that the presence of antimicrobial selective pressure plays a crucial role in the emergence of multi-drug resistant bacteria, the underlying antimicrobial stress response mechanisms on bacterial cell level have been subject to recent research. In this regard, it has been shown that bacteria inter alia increase expression of error-prone DNA polymerases in reaction to DNA damaging stress conditions, resulting in enhanced mutation rates [27-30], which might in turn equip bacteria with altered metabolic, virulence and resistance traits. It can be assumed that antimicrobials like quinolones, which directly interfere with bacterial DNA molecules, trigger stress response induced mutagenesis, notably facilitating the emergence of novel bacterial clones.

Consequently, the aims of this work were to

I Develop a reliable, fast, and cost-efficient method for identification of the *Acinetobacter calcoaceticus- Acinetobacter baumannii* (*Acb*)- complex species suitable for routine use;

II Determine the occurrence of *A. baumannii* in veterinary clinical specimens and the proportion of multi-drug resistant isolates among *A. baumannii* of animal origin;

III Comparatively analyze the diversity and relatedness of human and animal *A. baumannii* isolates;

IV Compare fluoroquinolone sensitive *A. baumannii* wild-type and derived resistant mutant isolates on genomic and functional level in order to gain insight in cellular alterations associated with fluoroquinolone selective pressure.

# LITERATURE REVIEW

## I General characteristics and taxonomy of *Acinetobacter* spp.

Species belonging to the Genus *Acinetobacter* are Gram-negative, strictly aerobic, coccoid non-fermenting bacteria, which show oxidase negative but catalase positive reactions [12, 31, 32]. Cells have a size from 0.9-1.6 x 1.5-2.5 µm, occur in pairs and chains of variable length and do not form spores [33]. Most *Acinetobacter* sp. grow undemandingly on complex media [33]. Although the name *Acinetobacter* derives from the greek word for “non-motile” (akinos), twitching motility has been described for different *Acinetobacter* species since the 1970s [34-39]. Table 1 shows the systematic classification of *Acinetobacter* species.

Table 1: Systematic classification of *Acinetobacter* species

| | |
|---|---|
| Kingdom | Bacteria |
| Phylum | Proteobacteria |
| Class | Gamma-Proteobacteria |
| Order | Pseudomonadales |
| Family | Moraxeallaceae |
| Genus | *Acinetobacter* |

*Acinetobacter* taxonomy is rather confusing since it underwent many changes during the last decades. The first *Acinetobacter* species was described as early as 1911, and was named *Micrococcos calcoaceticus* [40]. Starting in 1953, Brisou and Prévot conducted several studies on taxonomy of members of the genus *Achromobacter* and in 1954 suggested the generic name *Acinetobacter* for oxidase-negative as well as oxidase-positive bacteria [41-43]. In 1971, it was recommended that the genus *Acinetobacter* contains only oxidase-negative isolates, following further analysis of the nutritional demands of *Acinetobacter* spp. by Baumann [32, 40, 44]. A new classification criterion was established in 1972 by Juni, who showed that isolates of different bacterial species (with similar phenotypic properties) are able to transform auxotrophic mutants of *Acinetobacter* BD413 to prototrophy [32, 45]. Based on this transformation ability, species of other genera were also assigned to the genus *Acinetobacter*, such as *Achromobacter haemolyticus,* which is now known as *Acinetobacter haemolyticus*

[46], or *Moraxella lwoffii* var. *brevis*, which was renamed to *Acinetobacter lwoffii* [47]. The genus *Acinetobacter* was first listed in Bergey's Manual On Systematic Bacteriology in 1984, still consisting of only one species: *A. calcoaceticus* in two varieties (var. *anitratus* and var. *lwoffii*) [48]. Two years later, Bouvet and Grimont performed a study based on DNA-DNA hybridization and were able to differentiate between twelve hybridization groups, of which six were given species names (*A. calcoaceticus*, *A. lwoffii*, *A. haemolyticus*, *A. johnsonii*, *A. junii* and *A. baumannii*) [46]. This was the first description of *A. baumannii*, which evolved into a severe nosocomial pathogen, displaying various antimicrobial resistances [13, 14]. In addition to the six named species, Bouvet and Grimont identified six so-called genomic species, which could not be delineated to unique species due to a lack of specific phenotypic properties by a close genotypical relatedness [46]. In 1989, Tjernberg and Ursing [49] and Bouvet and Jean Jean [50] simultaneously conducted DNA-DNA hybridization studies, describing more genomic species. For a better understanding, the discovered genomic species were numbered – using the same numbers in both studies. For this reason, the additives TU and BJ were included in the genomic species designations. Amplified fragment length polymorphism (AFLP) emerged as a new method for further discrimination of genomic species. Based on this, it was assumed that *A*. genomic species 13BJ and *A*. genomic species 14TU are indistinguishable, which led to their unification into *A.* genomic species 13BJ/14TU [51]. Just few years later, Janssen et al. questioned this unification again [52], illustrating the confusing history of *Acinetobacter* taxonomy. In the following years, various typing methods, such as 16S-, 16S-23S intergenic spacer or *rpoB* sequence analysis, multi locus sequence typing (MLST), pulsed field gel electrophoresis (PFGE), and more recently MALDI-tof MS analysis, were established, promoting the description of novel named and unnamed *Acinetobacter* species [53-61]. Moreover, various unnamed genomic species were given proper names, for example, *A. pittii,* which was formerly known as *A.* genomic species 3, or *A. nosocomialis*, formerly known as *A*. genomic species 13TU [62]. Table 2 lists all 44 currently named *Acinetobacter* species. Of these, *A. baumannii*, *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* are notably closely related on the phenotypical as well as on the genotypical level [49, 50, 62, 63], and have thus been grouped into the *A. calcoaceticus- A. baumannii* (*Acb*)- complex [26]. Reliable species identification of these four species can only be achieved by molecular typing methods [13, 14]. Two further species, which are *A*. genomic species between 1 and 3, and *A*. genomic species close to 13TU (corresponds to *A. seifertii*), were identified by Gerner-Smidt and colleagues in 1993 as being closely related to the *Acb*- complex [61, 62, 64]. Besides the pathogenic species *A. baumannii*, *A. pittii* and *A. nosocomialis*, only few other

*Acinetobacter* spp. such as *A. bereziniae*, *A. guillouiae*, *A. ursingii*, *A. schindleri*, *A. lwoffii*, *A. parvus*, *A. junii*, *A. johnsonii*, *A. radioresistens,* and *A. seifertii* have been isolated from human clinical specimens [13, 61, 65-70].

Table 2: Current *Acinetobacter* spp. with valid species names

| *Acinetobacter* species | year of first description | reference |
|---|---|---|
| *Acinetobacter apis* | 2014 | Kim et al. [71] |
| *Acinetobacter baumannii* | 1986 | Bouvet and Grimont [72] |
| *Acinetobacter baylyi* | 2003 | Carr et al. [56] |
| *Acinetobacter beijerinckii* | 2009 | Nemec et. al [73] |
| *Acinetobacter bereziniae* | 2010 | Nemec et. al [68] |
| *Acinetobacter bohemicus* | 2015 | Krizova et al. [74] |
| *Acinetobacter boissieri* | 2013 | Àlvarez-Pérez et al. [75] |
| *Acinetobacter bouvetii* | 2003 | Carr et al. [56] |
| *Acinetobacter brisouii* | 2011 | Anandham et al. [76] |
| *Acinetobacter calcoaceticus* | 1911/ 1968 | Baumann et al. [40] |
| *Acinetobacter dijkshoorniae* | 2016 | Cosgaya et al. [77] |
| *Acinetobacter gandensis* | 2014 | Smet et al. [78] |
| *Acinetobacter gerneri* | 2003 | Carr et al. [56] |
| *Acinetobacter grimontii* | 2003 | Carr et al. [56] |
| *Acinetobacter guangdongensis* | 2014 | Feng et al. [79] |
| *Acinetobacter guillouiae* | 2010 | Nemec et al. [68] |
| *Acinetobacter gyllenbergii* | 2009 | Nemec et al. [73] |
| *Acinetobacter haemolyticus* | 1963/ 1986 | Bouvet and Grimont [46] |
| *Acinetobacter harbinensis* | 2014 | Li et al. [80] |
| *Acinetobacter indicus* | 2012 | Malhotra et al. [81] |
| *Acinetobacter johnsonii* | 1986 | Bouvet and Grimont [46] |
| *Acinetobacter junii* | 1986 | Bouvet and Grimont [46] |
| *Acinetobacter kookii* | 2013 | Choi et al. [82] |
| *Acinetobacter lwoffii* | 1940/ 1954 | Brisou and Prévot [72] |
| *Acinetobacter marinus* | 2007 | Yoon et al. [83] |
| *Acinetobacter nectaris* | 2013 | Àlvarez-Pérez et al. [75] |
| *Acinetobacter nosocomialis* | 2011 | Nemec et al. [62] |
| *Acinetobacter pakistanensis* | 2015 | Abbas et al. [84] |
| *Acinetobacter parvus* | 2003 | Nemec et al. [66] |
| *Acinetobacter pittii* | 2011 | Nemec et al. [62] |
| *Acinetobacter puyangensis* | 2013 | Li et al. [85] |

Table 2: Continued

| *Acinetobacter* species | year of first description | reference |
|---|---|---|
| *Acinetobacter qingfengensis* | 2013 | Li et al. [86] |
| *Acinetobacter radioresistens* | 1988 | Nishimura et al. [87] |
| *Acinetobacter rudis* | 2011 | Vaz-Moreira et al. [88] |
| *Acinetobacter schindleri* | 2001 | Nemec et al. [65] |
| *Acinetobacter seifertii* | 2015 | Nemec et al. [61] |
| *Acinetobacter seohaensis* | 2007 | Yoon et al. [83] |
| *Acinetobacter soli* | 2009 | Kim et al. [58] |
| *Acinetobacter tandoii* | 2003 | Carr et al. [56] |
| *Acinetobacter tjernbergiae* | 2003 | Carr et al. [56] |
| *Acinetobacter towneri* | 2003 | Carr et al. [56] |
| *Acinetobacter ursingii* | 2001 | Nemec et al. [65] |
| *Acinetobacter variabilis* | 2015 | Krizova et al. [89] |
| *Acinetobacter venetianus* | 2009 | Vaneechoutte et al. [57] |

List of currently named *Acinetobacter* spp., according to http://www.bacterio.net/acinetobacter.html with addition of *Acinetobacter dijkshoorniae* [77]

## II *A. baumannii* as a nosocomial pathogen in human medicine

### 1 Clinical relevance and treatment

Opportunistic pathogens like *A. baumannii* cause infections in immunocompromised patients, who often suffer from a variety of underlying diseases [13, 14, 90]. Main clinical manifestations of *A. baumannii* infections include pneumonia, urinary tract and bloodstream infections, wound infections, and meningitis [13, 14, 32, 90]. Infections of other tissues like endocarditis or keratitis are frequently reported [14, 32]. Prolonged hospitalization, previous antimicrobial treatment, recent surgery and indwelling medical devices, such as venous and urinary tract catheters or intubation, have been identified as predisposing factors favoring *A. baumannii* infection [13, 14, 91-93]. Community-acquired *A. baumannii* infections have only rarely been reported in humans living in tropical climate zones and usually exhibit a severe clinical course with reported mortality rates of 40-64% [14, 94-98]. Patients developing such community-acquired infections also show different comorbidities like alcohol abuse, diabetes mellitus or chronic obstructive pulmonary disease (COPD) [96, 97]. The fact that *A. baumannii* is most frequently isolated from severely ill patients complicates the assessment of the impact of

*A. baumannii* infections on mortality. It is furthermore problematic to differentiate between colonization and infection, and mortality rates associated with *A. baumannii* infection are still under debate. The methodological heterogeneity of the conducted studies contributes to contradictory results [13, 14, 90, 99-101], and reported mortality rates vary significantly [90, 99, 101-107]. Nevertheless, it seems conclusive that the severity of infection correlates with mortality rates, which can be as high as 60.9% in *A. baumannii* ventilator associated pneumonia (VAP) [107] or 72.7% in meningitis patients [104]. Falagas et al. compared nine case-control and cohort studies, comparing outcomes of patients colonized or infected with *A. baumannii* to matched patients, from whom *A. baumannii* had not been isolated [99]. The reported attributable mortalities ranged from 7.8% to 23% and from 10% to 43% [99]. In a study conducted within the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) program in the United States, mortality in 111 patients suffering from an *A. baumannii* bloodstream infection was compared to 2952 cases of bloodstream infection caused by other Gram-negative bacteria [108]. The reported mortality rates were comparable (32% for *A. baumannii* bloodstream infection and 28% for bloodstream infections due to other Gram-negatives) [108]. There is moreover strong indication that mortality is higher in infections with carbapenem resistant *A. baumannii* isolates compared to infections with carbapenem susceptible isolates [92].

Until the 1970s, *Acinetobacter* infections could be treated with common antimicrobials, since most strains were still susceptible to antibiotics [11, 96]. Meanwhile, multi-drug resistant (resistance against ≥ 3 antimicrobial classes [109]), extensively-drug resistant (resistance against all but ≤ 2 antimicrobial classes [109]), and pan-drug resistant (resistance against all antimicrobial classes [92]) isolates are reported frequently [13-15, 110]. Carbapenems were considered the antimicrobial of choice for treatment of multi-drug resistant *A. baumannii* infections and still are in carbapenem susceptible isolates, but frequent application resulted in widely distributed resistances against this drug [11, 111, 112]. Carbapenem resistance in *A. baumannii* is often associated with resistance against aminoglycosides and fluoroquinolones [11]. Fortunately, susceptibility against polymyxins and tigecycline (a glycylcycline) is usually also maintained in extensively-drug resistant isolates, leaving these drugs as treatment options [11, 111, 112]. Polymyxins like colistin are comparatively old antimicrobials that have been used less frequently due to their nephrotoxicity [111, 113]. Comparison of infection treatment with colistin and imipenem did not reveal differences in mortality rates, suggesting colistin can be used as an antimicrobial agent in carbapenem

resistant *A. baumannii* isolates [114]. In order to overcome suboptimal plasma concentrations and to benefit from synergistic effects, polymyxins are usually administered in a combination therapy, often with rifampicin, tigecycline or carbapenems [11, 111, 112, 115-117]. Although a synergistic effect of combination of colistin with second agents was observed in *in vitro* studies, clinical trials could not always confirm a positive effect [112, 117, 118]. There has however been proof that higher microbiological eradication can be achieved in patients receiving colistin combination therapy, which might reduce the risk of bacterial regrowth [118, 119]. Combination therapy might moreover be considered in case of infections with colistin resistant *A. baumannii*. Hong et al. showed that synergistic effects lowered the colistin MIC levels in colistin resistant *A. baumannii* to susceptible ranges in 61% of patients treated with colistin + rifampicin or colistin + meropenem [115]. Another factor that favors polymyxin combination therapy is the occurrence of colistin heteroresistance with existence of two bacterial subpopulations: one being susceptible to colistin and one being resistant [120]. Although there is a consensus that polymyxins should represent the backbone of MDR and XDR *A. baumannii* therapy, clinicians often prefer agents like tigecycline or sulbactam, in mono- or combination therapy due to lower side effects [92]. Therapy with tigecycline remains an option for therapy of colistin resistant isolates and in patients with kidney disease. Recent comparison of tigecyline and colistin-based therapy of *A. baumannii* pneumonia showed no significant difference in mortality rates, hospital stay, or recurrence of infection [121]. Nevertheless, patients seem to benefit from combination therapy compared to monotherapy [121]. A high infection-related mortality rate of 56% was reported in patients suffering from bloodstream infections with tigecycline susceptible *A. baumannii* and receiving monotherapy [122]. This may be due to low tigecyline serum concentrations after the initial peak following drug administration [123, 124]. For this reason, tigecycline monotherapy should not be chosen for treatment of *A. baumannii* bloodstream infections, at least with the currently recommended dose [11, 112], but might be considered for surgical site infections [111]. A further possible second agent in polymyxin combination therapy is fosfomycin [112]. Combination of colistin + fosfomycin entailed lower mortality than fosfomycin monotherapy in carbapenem resistant *A. baumannii* [112, 125].

## 2 Epidemiology

The rapid increase in occurrence of multi-drug resistant *A. baumannii* isolates is partly based on the remarkable ability of the pathogen to survive within the hospital setting. Adaption to various environmental conditions facilitates survival of *A. baumannii* on abiotic surfaces up to five months, and biofilm formation helps to colonize medical devices [126-128]. Prolonged survival implies prolonged exposure to antimicrobial selective pressure and promotes nosocomial spread via hospital equipment, staff, and colonized patients [13, 14, 93, 129]. There are some clonal lineages which are particularly associated with multi-drug resistance and epidemic spread worldwide, namely European clones (EC) or international clones (IC) I to III [12, 15-17]. While the delineation of IC I-III was made by amplified fragment length polymorphism (AFLP), the gold standard for grouping bacterial isolates into clonal lineages is currently multi locus sequence typing (MLST) [130]. Two MLST schemes, PubMLST (or Oxford) and Pasteur MLST, utilizing different housekeeping genes, have been established for *A. baumannii* [15, 131, 132]. In 2013, the PubMLST and Pasteur MLST databases already contained 287 and 176 sequence types (STs) grouped into 21 and 20 clonal complexes (CC) [130]. PubMLST CC1 and PasteurMLST CC109 correspond to IC I, CC2 and CC92 correspond to IC II, whereas CC3 and CC110 represent IC III [130]. Further typing methods like the DiversiLab™ method, which is based on repetitive extragenic palindromic PCR (rep-PCR) [130, 133], allowed additional delineation of outbreak clones [130]. While IC I and II circulate worldwide, other lineages still show a geographic restriction, like IC III or Pasteur's CC10, which are mainly found in Europe [130]. International clone II moreover is particularly associated with carbapenem resistance [134-136].

Surveillance data from the National Healthcare Safety Network showed that *A. baumannii* was one of the ten most common pathogens causing health care associated infections in the USA from 2006-2007 [137]. Overall, *A. baumannii* accounted for 3% of the recorded infections (8.4% of ventilator associated pneumonia, 2.2% of central line bloodstream infections, 1.2% of catheter-associated urinary tract infections, and 0.6% of surgical site infections), with an average carbapenem resistance rate of 33% (resistance rates varied among institutions) [137]. Susceptibility data registered in the Surveillance Network® database revealed an increase of isolates resistant against carbapenems and ≥ 2 further antimicrobial classes from 20.6% in 2002 to 49.2% in 2008 [138]. Furthermore, the rate of MDR *A. baumannii* in the USA increased from 32.1% in 1999 to 51% in 2010 [139]. Accordingly, in a recent study from Poland,

extensive-drug resistance was observed in 80.8% (101/125) of the collected *A. baumannii* isolates, with higher rates in the intensive care units (ICU) than in the non ICU wards (93.9% and 30.8%) [140]. Nevertheless, carbapenem and multi-drug resistance rates vary within distinct geographical regions. In Europe the lowest carbapenem resistance rates have been observed in Scandinavia, while rates are particularly high in Southern and Eastern Europe. In 2012, the European Antimicrobial Resistance Surveillance Network (EARS-Net) observed carbapenem resistance rates in *Acinetobacter* spp. of more than 79% in Italy, Greece, Portugal, and Romania, and rates of less than 7% in Germany, Netherlands, France, United Kingdom, and Norway [141]. Moreover, more than 70% of the analyzed isolates from Greece and Italy were also resistant against fluoroquinolones and aminoglycosides, but less than 4.2% of the isolates from Germany, Netherlands, France, United Kingdom and Norway [141]. In a separate surveillance study by Schleicher et al., a decrease in Germany in imipenem susceptibility, from 96% to 76% within 5 years (2005-2009) was reported, which corresponds to a carbapenem resistance rate of 24% [134]. Since the number of hospitals included in the latter study was lower (n=15), the higher rate might be due to regional variations, for example due to regional outbreaks.

Although various classification methods allow for a better understanding of *A. baumannii* global epidemiology, its natural habitat has not yet been identified [13, 14, 18]. It has been suggested that the hospital setting itself might constitute a potential reservoir for the epidemic *A. baumannii* lineages [13, 96, 142]. There have been some indications that the hospital setting might have served as a novel ecological niche after the spread of specific *A. baumannii* lineages from tropical or sub-tropical climate zones to colder climatic areas. Firstly, *A. baumannii* infections have a higher prevalence at warmer temperatures, for example during the summer months [14, 143, 144]. Secondly, community-acquired infections have to date only been reported within tropical and sub-tropical climate zones (only once in North America) [18, 97]. This is consistent with the fact that *A. baumannii* has frequently been isolated from environmental sources in the tropics and sub-tropics, whereas in Europe only during the warmer months [145-149]. Thirdly, *A. baumannii* isolates obtained from hospitals show a higher clonality [13, 15, 142], suggesting they might derive from few ancestral lineages.

## III *A. baumannii* in veterinary medicine

### 1 Livestock

There are only few studies that display information regarding *A. baumannii* of animal origin. In particular, data concerning the occurrence of *Acinetobacter* spp. and *A. baumannii* in livestock are lacking. A significant question is whether *Acinetobacter* spp. are part of the microbiota of livestock, and to which extent this may serve as an infection source for MDR *A. baumannii* in humans. In fact, *Acinetobacter* spp. can be isolated from feces, nose, rumen, urine and raw milk samples from cattle and other animals [150-158]. Holman et al. recently showed that *Acinetobacter* spp. account for 17.5% of the nasopharyngeal microbiota of cows [157]. However, *Acinetobacter* spp. differ in their pathogenicity [159], and infections, for example with *A. bereziniae*, *A. guilliae*, *A. haemolyticus,* or *A. radioresistens* rarely occur in humans and usually show a benign clinical course [18]. It should nevertheless be elucidated to which extent antimicrobial resistance genes are distributed among the nonpathogenic *Acinetobacter* spp., since horizontal transfer of such genes to *A. baumannii* or vice versa could occur [160].

The OXA-23 carbapenemase has already been detected in *A*. genomic species 15TU isolates from dairy cattle and in isolates of a putative novel *Acinetobacter* spp. obtained from horses [161, 162]. OXA-23 is very common in *A. baumannii* and has also been identified in isolates from cats, cattle, a pig, and fowl [20, 21, 163]. Al Bayssari et al. could moreover assign one of the bovine OXA-23 producing *A. baumannii* isolates to ST2, which belongs to the International Clone II [163]. Carbapenemase producing *A. baumannii* could also be isolated from bovine fecal samples in the USA (OXA-497) [164] and from a pig suffering from pneumonia and sepsis [165]. In the latter case, the New-Delhi-metallo beta-lactamase NDM-1 was located on a plasmid, underlining the probability of horizontal exchange of resistance genes even among animal *A. baumannii* isolates. However, livestock can serve as an infection source for humans via direct contact, consumption of animal derived food, or environmental contamination with animal feces [166-172]. In this regard, Hamouda et al. investigated *A. baumannii* isolates obtained from food-producing animals in 2008 and from animals slaughtered for human consumption in 2011 [173, 174]. The examined isolates were susceptible to antimicrobials and lacked important features typical for isolates displaying multi-drug resistance, suggesting they were exposed only to low antimicrobial selective pressure. Furthermore, there was no indication of an epidemiological link to the human IC I-III [173, 174]. Antimicrobial resistance

genes are, as mentioned, nevertheless present in *A. baumannii* isolates from livestock, and sulfonamide resistant *A. baumannii* have been detected in soil after manure fertilization in the United Kingdom and in the Czech Republic [146, 175]. Environmental spread of antimicrobial resistant *Acinetobacter* isolates originating from livestock farms, as it is known to be the case for ESBL-producing *E. coli* [176], thus seems likely.

## 2 Companion animals

In the following, companion animals are understood to include horses, dogs and cats. First reports of *Acinetobacter* spp. in horses go back to the 1970s and 1980s [177-182]. In 1993 and 1995, *Acinetobacter* spp. were isolated from horses suffering from keratitis and lower respiratory tract infection [183-185]. A few years later, Vaneechoutte et al. observed resistances against several classes of antimicrobials in *A. baumannii* isolates from vascular catheter tips of hospitalized horses. Because only one isolate could be obtained in pure culture from a case of thrombophlebitis, this study could not prove an association of *A. baumannii* isolation with disease [186]. Resistance against antimicrobials also occurred in equine *A. baumannii* isolates investigated by Brosnahan in 2008 [187]. These isolates derived from horses displaying typical predisposing factors for *A. baumannii* infections: prolonged hospitalization, antimicrobial treatment, and underlying diseases like respiratory tract, bloodstream, uterine, or ophthalmological infections [187]. Similarly, Jokisalo et al. reported a case of *A. baumannii* infection in a six-month-old horse which underwent intensive previous antimicrobial treatment [188]. This report, as well as a case of neonatal encephalopathy in a 48-hour-old foal due to *Acinetobacter* infection [189], indicate that there are no age limits for *Acinetobacter* infections in horses. Since most case reports lack information regarding species identification methods, it should be kept in mind that these reports might also be based on infections with *Acinetobacter* spp. other than *A. baumannii*.

The first systematical investigation of *A. baumannii* isolates from horses, dogs, and cats was performed by Endimiani et al. in 2011, who applied molecular methods established for human isolates [190]. Of the 19 investigated isolates, 17 could be assigned to MLST ST12 and ST15, which correspond to the human IC I and II. Resistances against gentamicin, ciprofloxacin and carbapenems were also reported and were based on resistance genes commonly identified in human *A. baumannii* isolates [190]. Additionally, the authors evaluated metadata of the

respective patients including age, underlying diseases, and occurrence of predisposing factors. They conclude that i) the same clonal lineages occur in animals and humans, ii) several cases of *A. baumannii* infection were hospital acquired, iii) animals and humans are exposed to the same predisposing factors, and iv) the role of animals in the spread of *A. baumannii* needs to be further determined. Accordingly to the finding that animal and human *A. baumannii* isolates share molecular features such as resistance genes, Abbott et al. showed that the class 1 integron in an equine MDR isolate shared significant homologies with the one from a human isolate [191]. Class 1 integrons are involved in horizontal gene transfer and might favor the exchange of genetic material between human and animal isolates. Moreover, Zordan et al. could assign *A. baumannii* isolates obtained from dogs and cats to the IC I-III [19], as it was also the case in recent studies from Portugal and Germany [20, 21]. This iterated evidence for presence of human IC I-III in the animal *A. baumannii* population encourages the question of zoonotic transmission. Indeed, there are further epidemiological similarities between human and animal *A. baumannii* isolates. In a retrospective analysis of cases of *A. baumannii* infection in 17 dogs and two cats, Francey et al. assumed nosocomial spread within an animal clinic [22]. Most cases occurred within two main outbreaks, in animals that were hospitalized for seven days on average and were treated with indwelling devices. 16 out of 19 patients underwent surgery and all but one received antimicrobial treatment [22]. A new epidemiologically unrelated outbreak of *A. baumannii* infections occurred in the same veterinary clinic in Switzerland and was reported by Boerlin et al. only one year later in 2001 [24]. PFGE analysis clustered the investigated isolates in two distinct major types. The first major type disappeared after proper hygiene management procedures, while transmission to a critically ill hospitalized horse in a nearby horse clinic could be observed during the second outbreak (represented by the second PFGE major type) [24]. Furthermore, three isolates had unique PFGE patterns, which were thus considered epidemiologically unrelated and showed a higher antimicrobial susceptibility than the outbreak isolates [24].

Infections with *A. baumannii* however occur primarily in animals suffering from underlying diseases as is also the case for human patients [13, 14, 24, 187, 190]. Attributable mortality rates are still under debate in human medicine, and respective data are particularly lacking in veterinary medicine. A case of necrotizing fasciitis in a cat due to *A. baumannii* infection [192] demonstrates that the pathogen's ability to cause death in animals. A venous catheter, which was administered to the cat during treatment of obstipation, probably served as the port of entry for *A. baumannii*. Within the following days, the patient died of septic shock [192]. The

course of infection is however linked to the respective treatment options, which are reduced in case of occurrence of antimicrobial resistances. Resistance genes previously identified in human isolates have already been detected in *A. baumannii* of animal origin [21, 165, 190], causing resistances against several classes of antimicrobials as well as multi-drug resistance [19, 21, 23, 24, 187]. In a retrospective study in 2009, Black et al. analyzed the medical records of all canine patients admitted to a veterinary teaching hospital over the course of six months. The data analysis revealed that *A. baumannii* accounted for only 7% of canine infections due to Gram-negative bacteria, but for 21% of the multi-drug resistant isolates [23]. *A. baumannii* was thus the Gram-negative species accounting for the highest proportion of multi-drug resistant isolates in their study [23]. Similar to this, van Spijk et al. found that 23 out of 24 (96%) equine *A. baumannii* isolates obtained within the same clinic were multi-drug resistant [25]. Furthermore carbapenemase, OXA-23 in particular, producing *A. baumannii* isolates belonging to the IC I and IC II, could be obtained from cats suffering from urinary tract infection [20, 21]. Taken together, *A. baumannii* isolates of companion animal origin i) cause infections in animals displaying typical predisposing factors known from human medicine, ii) are often multi-drug resistant, iii) can be transmitted within and between veterinary clinics, and iv) belong to the same outbreak clones as human isolates.

## IV Possible transmission of *A. baumannii* between humans and animals

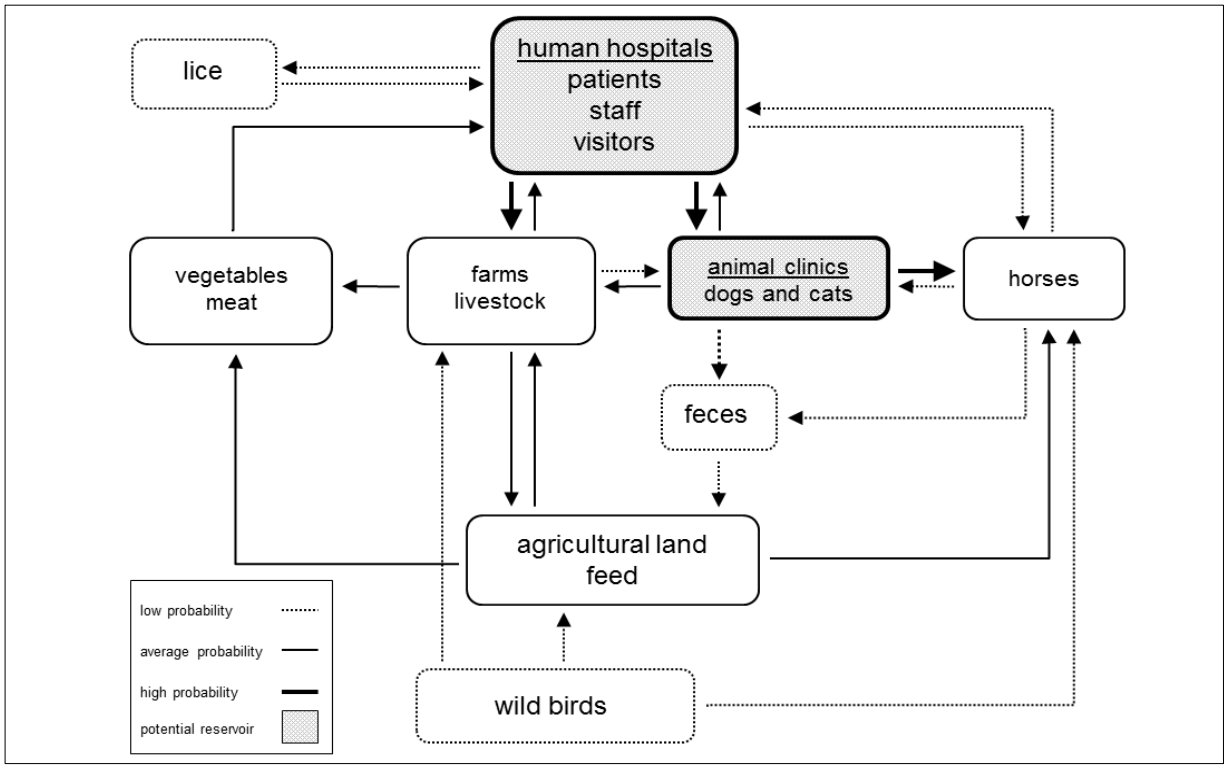
As mentioned, there has been evidence that the same *A. baumannii* outbreak clones occur in both humans and animals [19, 21, 190]. However, it remains to be elucidated if and to what extent *A. baumannii* is transmitted from animals to humans or vice versa. The fact that the pathogen's natural habitat has not yet been identified complicates the situation. Several studies investigated the occurrence of *A. baumannii* in the environment and were able to detect isolates in soil in Hong Kong and the United Kingdom [146, 147], on fish and shrimp farms in Asia [145], on vegetables from supermarkets, greengrocers and private gardens [148], as well as in water samples in Brazil [149]. Byrne-Bailey et al. moreover identified sulfonamide resistant *A. baumannii* in pig-slurry fertilized soil and soil leachate, indicating a possible transmission route to the environment [146]. It is noteworthy that slurry is assumed to be a major emission source for ESBL-producing *E. coli* besides exhaust air from stables, which might contribute to pathogen dissemination [172, 193-195]. Contact with contaminated soil, plants or vegetables might serve as a possible infection source for humans. Berlau et al.

suggest the introduction of *A. baumannii* into the hospital setting as being due to contaminated vegetables [148]. Moreover, contamination of field surroundings enables further spread of bacteria. Wild animals like rodents or birds search these fields for food (insects, worms, amphibians, seeds), and heavy rainfalls wash out the bacteria from fields into rivers and lakes. Insects, wild animals, and wild birds might in turn reintroduce the bacteria onto farms, riding stables, and private properties, serving as an infection source for livestock, companion animals, and humans. In fact, *A. baumannii* have been isolated from wild bird feces [196] and Muller et al. assume isolation of *A. baumannii* in a falcon as being due to hunted wild birds [197]. It has also been shown that wild birds can be carriers of multi-drug resistant bacteria such as ESBL-producing Enterobactericaeae [198, 199]. Once livestock is infected or colonized with *A. baumannii*, this may not alone cause spread to the environment, but the bacteria may also be transmitted to humans and carnivore pets via meat and dairy products. A recent study from Switzerland isolated *A. baumannii* from raw meat samples with a prevalence of 25.0%, mostly derived from poultry meat samples [200]. Isolates of this study showed resistances against several classes of antimicrobials, especially third and fourth generation cephalosporines. Furthermore, some isolates belonged to the clonal complexes CC32 and CC79, which are known to cause nosocomial infections in humans [200-202]. Accordingly, Rafei et al. detected *A. baumannii* with a prevalence of 6.9% in water samples, 2.7% in raw milk samples, 14.3% in cheese samples, 8.0% in cow meat and 7.7% in samples from living animals [203]. These studies suggest animals and derivative food products as potential sources of human *A. baumannii* infections. However, Hamouda et al. investigated *A. baumannii* isolates deriving from livestock and could not assign these to the IC I-III [173, 174]. In addition, Rafei et al. and Lupo et al. could allocate *A. baumannii* isolates only sporadically to epidemic clonal lineages, whereas a high diversity was observed for the majority of isolates [200, 203]. Moreover, although the analyzed isolates showed resistances against antimicrobials, they were not significantly associated with multi-drug resistance. These findings indicate that the epidemic multi-drug resistant clonal *A. baumannii* lineages do not currently circulate intensively within livestock and the environment, although carbapenemase producing *A. baumannii* have been detected in livestock [163-165].

However, Belmonte et al. and Pailhoriés et al. observed *A. baumannii* carrier rates of 6.5% and 8.5% in pets on Reunion Island admitted to veterinary clinics [204, 205]. Belmonte et al. also showed that eight out of nine obtained *A. baumannii* isolates were closely related, although they derived from animals sampled in a distance of 40 km. An environmental infection

source, like stray dogs or arthopods, was thus assumed [204, 205]. Transmission by insects seems to be a very likely scenario, since *Acinetobacter* spp. and *A. baumannii* in particular have been isolated from human and animal lice as well as ticks worldwide [206-211]. In 2015, several cases of pneumonia on a mink farm due to *A. baumannii* infection were reported [212]. Since no infection source could be detected, it was suggested that fleas might have served as the vector of infection. Infections with *A. baumannii* belonging to epidemic clonal lineages like the IC I-III are nevertheless often hospitalacquired - in human as well as in veterinary medicine [13, 14, 24, 192]. Because the prevalence of multi-drug resistance and the clonality are higher among hospital *A. baumannii* isolates [142, 213], it has been assumed that epidemic clonal lineages have their natural habitat within the hospital setting itself [13, 96, 142]. It can be speculated that the ancestors of the respective clonal lineage were introduced into the hospital setting, for example by contaminated food or colonized patients, and adapted to the present environmental conditions. Accumulation of antimicrobial resistances happened due to the present selective pressure and the remarkable plasticity of the *A. baumannii* genome. Despite the adaption to the hospital setting, bacteria can still be disseminated to the environment, for example by means of sewage [214]. Considering this possibility Turano et al., were able to detect OXA-23 producing *A. baumannii* it in water samples in Brazil [149]. The ever-closer contact between animals and their owners, accompanied by improved veterinary intensive care medicine, is facilitating new transmission pathways. Hospital adapted *A. baumannii* lineages might hence spill over from human into veterinary companion animal medicine. This scenario could explain why these lineages are isolated from horses, dogs, and cats [19, 21, 190], but only rarely from livestock. Transmission of the epidemic *A. baumannii* lineages between humans and companion animals could thus take place independently of the epidemiology of *A. baumannii* from livestock and wild life. Yet, considering the fact that the *A. baumannii* genome shows a high plasticity, it seems possible that the human epidemic lineages will be transmitted to livestock with subsequent adaption, further spread to the environment, and reinfection of humans and companion animals.

Figure 1: Possible transmission pathways for *A. baumannii* (Müller et al. 2014 [215])




## V Quinolones – mechanisms of action and resistance

### 1 Mechanisms of action

The history of quinolones goes back to the 1960s, when Lesher et al. described nalidixic acid, the first agent of this class [216, 217]. In 1987, ciprofloxacin was introduced, being the first quinolone effective in treating infections outside the urinary tract due to an additional 6-fluoro group [218-220]. Based on its chemical structure, this second generation of quinolones was named fluoroquinolones and exhibited a wider antimicrobial spectrum and better pharmacokinetic properties [217, 219]. Although quinolones are broad spectrum antimicrobials with activity against Gram-negative and Gram-positive pathogens [218], fluoroquinolones still have limitations in their effectiveness against Gram-positive and anaerobic bacteria [218, 221]. Third generation quinolones like moxifloxacin or the veterinary pradofloxacin, however, show a better activity against Gram-positive bacteria and fourth generation quinolones (e.g. gareoxacin) finally also against anaerobes [218, 219, 222, 223].

Nevertheless, ciprofloxacin still seems to be the most active quinolone against Gram-negative bacteria [219]. Meanwhile, quinolones are used to treat a variety of infections, including urinary and lower respiratory tract infections, skin and soft tissue infections, and sexually transmitted diseases [220]. Enrofloxacin is a commonly prescribed fluoroquinolone in veterinary medicine, which is quickly metabolized to ciprofloxacin after administration [224-226].

Quinolones act by interfering in the bacterial replication by targeting two type II topoisomerases, namely DNA gyrase and topoisomerase IV [217, 218, 220, 227]. DNA gyrase has a tetramer structure consisting of two molecules of each, subunit A (encoded by *gyrA*) and subunit B (encoded by *gyrB*) [217, 218, 228, 229], and is an essential enzyme occurring only in prokaryotic cells, making it a good drug target [218]. The complete DNA gyrase tetramer introduces negative supercoils into DNA strands, which enables binding of the RNA polymerase and reduces the torsions in front of replication forks and transcription complexes [217, 218, 220, 230-233]. Topoisomerase IV also consists of two subunit A and two subunit B molecules, which are encoded by *parC* and *parE* [217, 234]. In difference to the DNA gyrase, topoisomerase IV is mainly involved in division of daughter chromosomes after replication, in order to facilitate cell division. It moreover supports negative supercoiling by relaxing positive supercoils, and resolves knots in the DNA structure [217, 218, 234, 235]. Action of both enzymes causes DNA double-strand breaks, which allow to solve the supercoiling before these breaks are resealed [220, 227].

DNA gyrase however displays the main target for quinolones in Gram-negative bacteria, while topoisomerase is the main target in Gram-positive cells [219, 227]. After accumulation of the drug in the bacterial cell, stable ternary complexes called ‘cleavage complexes’ between antimicrobial, topoisomerase enzyme, and DNA form, which block movement of replication forks and transcription complexes and increase the number of DNA breaks [220, 228, 236-239]. Although quinolones can directly bind to the topoisomerase enzymes in some bacterial species (e.g. in *Escherichia coli*), DNA seems to increase binding affinity of the quinolones [227, 240, 241]. Moreover, a contribution of magnesium ions to quinolone binding has been suggested [242, 243]. It has been shown that $Mg^{2+}$ ions mediate the interaction between moxifloxacin and topoisomerase IV in *A. baumannii* by formation of hydrogen bonds within the quinolone resistance-determining region (QRDR) of *parC* [244]. While the general effect of quinolones is bacteriostatic via inhibition of cell growth, high quinolone concentrations have been reported to cause DNA fragmentation, leading to rapid cell death [228]. DNA damage

has moreover, been shown to induce mutagenesis in *A. baumannii*, mainly due to upregulation of expression of error prone DNA polymerases [27, 28, 30]. In difference to replicative high-fidelity DNA polymerases, error prone DNA polymerases have a relatively open active center and thus facilitate mutagenesis, which in turn enhances mutagenic evolution [28, 29]. In 2014 MacGuire et al. showed that, as a consequence of ciprofloxacin selective pressure, *A. baumannii* cells divide into two subpopulations, of which one shows induction of DNA damage response and the other does not [27]. Cells that did not demonstrate induction of DNA damage response showed a survival advantage. These cells likely have a higher ability to perform conjugation, because DNA damage gene products can repress conjugational genes [27, 245]. In contrast, cells with induction of DNA damage response had decreased survival rates, likely due to reduced conjugational properties but increased mutagenesis [27]. It was thus assumed that formation of subpopulations might represent a bimodal survival strategy of *A. baumannii* under DNA damaging conditions. While one subpopulation had a better survival and can probably acquire foreign genetic material, the other subpopulation promoted fast adaption to the new environmental conditions by increased mutagenesis [27]. Considering these findings, administration of fluoroquinolones might serve as a driving force of *A. baumannii* evolution.

## 2 Resistances against quinolones

While decreased drug influx and increased efflux are contributing to resistance against quinolones, target site modifications in DNA gyrase and topoisomerase IV by development of specific mutations play the major role [217-219]. Such mutations occur particularly in so called quinolone resistance determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE*, which encompass the domains that bind to DNA [220, 227]. As a consequence, drug affinity to the target is reduced [227, 246, 247]. Mutations in *gyrA*, *gyrB* and *parC* have been reported in quinolone resistant *A. baumannii* isolates, which occur at S83 and Gly81 in *gyrA* [248, 249], E679, D644 and A677 in *gyrB* [239] and S80, Glu84, Gly78 in *parC* [239, 250]. The prevalence of *gyrA* and *parC* mutations is actually higher than the prevalence of *gyrB* mutations [227]. Phenotypic resistance is based on development of these specific mutations, which occur usually initially at the preferred target enzyme (DNA gyrase in the case of Gram-negative bacteria) and lead to an up to 10-fold increase in MIC values [219, 220]. Mutations in the second target enzyme (topoisomerase IV) occur in a second step and although they do not

influence the resistance phenotype, they can increase the MIC values significantly from 10-fold to 100-fold [219, 220, 250]. While genetic mutations confer high level resistance against quinolones, other mechanisms like efflux pumps cause only low level resistance [217, 219]. Nevertheless, these mechanisms can mediate survival of bacteria in sublethal quinolone concentrations and therefore enhance the probability of development of specific mutations [217, 218]. In this regard, loss or decreased expression of porine like proteins has been reported in *A. baumannii* [251-254], whereas efflux pumps AdeABC, AdeFGH and AdeIJK do mediate fluoroquinolone efflux in cases of overexpression [253, 255-257]. A further mechanism of quinolone resistance is acquisition of horizontally transferable plasmid-mediated quinolone resistance genes (*qnr*), of which *qnrA*, *qnrB* and *qnrS* have been detected in *A. baumannii* [258-260]. *Qnr* genes encode a pentapeptide repeat protein that interacts with DNA gyrase and topoisomerase IV, leading to destabilization of the cleavage complex. As a consequence, the quinolone is being released, DNA religated, and the active topoisomerases are regenerated [220, 261, 262].

## VI Mechanisms of antimicrobial resistance in *A. baumannii*

### 1 Intrinsic resistances and efflux pumps

There are five general mechanisms which are known to mediate resistances against antimicrobial substances in bacteria. These mechanisms are decreased influx of the drug, increased efflux of the drug, bacterial drug modification or cleavage, target site modifications, and utilization of alternate metabolic pathways. Cell envelope structure and chromosomally encoded efflux pumps or specific hydrolyzing enzymes constitute the most common intrinsic resistance mechanisms. The rather small number of porin-like membrane proteins in *A. baumannii* leads to a decreased membrane permeability compared to other Gram-negative bacteria and thus complicates drug influx [252, 263]. Antimicrobial stress can lead to downregulation of expression of porin-like membrane proteins and thus antimicrobial resistance [251, 252, 263, 264]. Only few porin-like membrane proteins have been identified in *A. baumannii*, which are CarO, a 33- to 36-kDa OMP, a 43-kDa protein similar to OprD from *P. aeruginosa* and OmpW [251-254]. In *A. baumannii*, Sulbactam takes its antimicrobial effect by binding to the penicillin-binding protein 2 (PBP2), and downregulation of this protein can cause resistance against this drug [265, 266]. Moreover, three families of efflux pumps, which

can confer resistance to antimicrobials, have been found to be present in *A. baumannii*: RND-family (resistance-nodulation-cell division), MATE-family (multi-drug and toxic compound extrusion), and MFS-family (major facilitator superfamily) efflux pumps [267]. To date the highest impact on antimicrobial resistance is attributed to the RND-family efflux pumps AdeABC, AdeFGH, and AdeIJK [255, 268-270].

Most commonly distributed is AdeABC, which is present in approximately 80% of the *A. baumannii* clinical isolates [255, 268, 270]. Genomic presence of this efflux pump causes only a low level of drug efflux and resistance is dependent on pump overexpression due to mutations in the regulator genes or insertion of insertion sequences upstream of the operon [267, 271-275]. The substrate spectrum of AdeABC is wide, including aminoglycosides, tetracyclines and tigecycline, chloramphenicol, ciprofloxacin, erythromycin, trimethoprim, meropenem, netilmicin, and ethidium bromid [257, 268, 270, 272, 276]. Similarly, the AdeFGH and AdeIJK efflux pumps also show a broad substrate spectrum and confer resistances only in case of overexpression [255, 256]. AdeFGH mediates efflux of fluoroquinolones, tetracycline and tigecycline, chloramphenicol, clindamycin, trimethoprim, sulfamethoxazole, sodium dodecyl sulfate, and dyes such as ethidium bromide, safranin O, and acridine orange [255]. AdeIJK is able to efflux beta-lactams (excluding carbapenems), chloramphenicol, tetracycline, erythromycin, lincosamids, flouroquinolones, fusidic acid, novobiocin, rifampicin, trimethoprim, sodium dodecyl sulfate, safranin, acridine, and pyronine [253, 256]. In contrast to AdeABC, the two pumps AdeFGH and AdeIJK are not regulated by a two-component system, but by transcriptional regulators named AdeL and AdeN [255, 277].

The similarly chromosomally encoded pumps AbeM and AbeS belong to the MATE-superfamily and are responsible for decreased susceptibility against fluoroquinolones, aminoglycosides, erythromycin, chloramphenicol, and trimethoprim as well as other chemicals [278, 279]. Efflux pumps belonging to the MFS-family are also commonly distributed among *A. baumannii*. One member of this family is AmvA, conferring resistance against detergents, disinfectants, dyes, and erythromycin in case of overexpression [280, 281]. Of greater importance however are the tetracycline (tet) efflux pumps, which are often located on transferable elements and are thus usually acquired [252, 280, 282-284]. Another efflux pump found in *A. baumannii* is CraA, which is the cause for intrinsic chloramphenicol resistance [285]. In contrast to the tet pumps, CraA is chromosomally encoded, just like CmlA, an efflux pump described by Fournier et al. in 2006 [286]. Beyond regulation of porin-like proteins and

efflux pumps, *A. baumannii* possesses two different kinds of chromosomally encoded beta-lactamases, which belong to the Ambler Classes C and D and confer resistance against different beta-lactam antibiotics [287].

## 2 Resistances against beta-lactam antibiotics

In principle, beta-lactam resistance can be mediated by non-enzymatic and enzymatic mechanisms. Loss of porins [251, 288], overexpression of efflux pumps [268] and modifications of penicillin-binding proteins [265, 289] are the non-enzymatic mechanisms, which have been described to date. Beta-lactam hydrolyzing enzymes, known as beta-lactamases, have been grouped into the four categories, Ambler Class A, B, C and D, due to sequence similarity of their encoding genes [267, 287, 290]. While the majority of beta-lactamases must be acquired by horizontal gene transfer, *Acinetobacter* derived cephalosporinases (ADCs), which belong to Ambler Class C beta-lactamases (AmpC), are intrinsically occurring in *A. baumannii* [287], as it is also the case for some Ambler Class D beta-lactamases (oxacillinases). These intrinsic oxacillinases have been grouped into the OXA-51-like cluster (consisting of OXA-51, -64, -65, -66, -68, -69, -70, -71, -78, -79, -80, -82 and -143) [268, 287, 291]. Intrinsic beta-lactamases in *A. baumannii* nevertheless exhibit only low efficiency and do not reduce the effect of beta-lactams to a clinically relevant level [253]. The situation changes with presence of insertion sequences (IS), especially ISAba1, upstream of the respective genes, which causes overexpression of the affected enzymes, and hence decreased antimicrobial susceptibility [253, 287, 291-294].

Ambler Class A enzymes include several subtypes of extended spectrum beta-lactamases (ESBLs), which are able to hydrolyze penams, cephems, third and fourth generation cephalosporins as well as monobactams. Nevertheless, these ESBLs can still be inhibited by beta-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam) [290, 295, 296]. Representatives of this class of beta-lactamases that have been identified in *A. baumannii* are: *Pseudomonas* extended resistance (PER), Vietnamese extended-spectrum beta-lactamase (VEB), Sulphydril variable (SHV), Temoneira (TEM), cefotaxime-hydrolyzing beta-lactamase (CTX-M) and RTG (subgroup of carbenecillin hydrolyzing beta-lactamase (CARB)) [297, 298]. Additionally, Guiana extended-spectrum beta-lactamases (GES) and *Klebsiella pneumoniae* carbapenemase (KPC) have also been reported in *A. baumannii* isolates and are able to

hydrolyze all beta-lactam antibiotics, including carbapenems [253, 267, 290, 299-302]. Ambler Class B beta-lactamases are also known as metallo-beta-lactamases (MBLs) because divalent cations are required for hydrolytic inactivation of the beta-lactam substrates [267, 290]. Four different subtypes of MBL enzymes have been detected in *A. baumannii*, which are imipenemases (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), Seoul imipenemase (SIM), and New Delhi metallo-beta-lactamase (NDM) [253, 267, 287, 290, 303, 304]. Although MBLs are highly potent enzymes conferring high-level resistance against all carbapenems except aztreonam, Ambler Class D beta-lactamases or oxacillinases (OXAs) occur most frequently in *A. baumannii* isolates [267]. Because oxacillinases are able to hydrolyze ampicillin, cefalotin, and carbapenems [8], they are often designated carbapenem-hydrolyzing class D beta-lactamases (CHDLs). CHDLs are, like MBLs, not significantly susceptible to inhibition by clavulanic acid or tazobactam [8, 287]. OXAs identified in *A. baumannii* display a high variability, leading to a subgrouping into four main clusters: OXA-51-like, OXA-40/24-like, OXA-58-like, and OXA-23-like [267, 268, 294, 305]. Of these, OXA-23 was the first one to be described and is disseminated worldwide [8, 287, 291]. Resistance against beta-lactams based on OXA-23-like enzymes is dependent on the presence of ISAba1, as is also the case for OXA-58-like and OXA-51-like genes [267, 290-292, 294]. Occurrence of oxacillinases seems to be associated with specific *A. baumannii* clonal lineages [11, 291]. OXA-23 is reported predominantly in IC I and CC92 *A. baumannii* isolates and OXA-24/40-like enzymes are most frequently identified in IC II and ST56 isolates, whereas OXA-58-like enzymes can be found in IC I-III and various further STs [291].

## 3 Resistances against other antimicrobial classes

The antimicrobial effect of aminoglycosides is based on their binding to the 16S rRNA and thus interference with protein biosynthesis. Resistance against these drugs is mediated by four main mechanisms: decreased membrane permeability, active efflux, alterations of the target side, and enzymatic drug modifications [8]. Of these, aminoglycoside-modifying enzymes (AMEs) are of greatest importance and can be classified by their mechanism of action into phosphotransferases, acetyltransferases, and nucleotidyltransferases [10, 306]. The three AMEs which are most frequently isolated from *A. baumannii* are AAC(3')-Ia, APH(3')-VI and AAC(6')-Ib [10]. All three genes differ in their aminoglycoside substrate spectrum [10]. Unlike AMEs, 16S rRNA methylases like ArmA and RmtB are causing alterations at the

aminoglycoside binding site [8, 10]. Because these enzymes do not affect the antimicrobial molecule but its target site, they cause cross-resistance against all aminoglycosides [8, 10, 307]. Resistance against tetracyclines and glycylcyclines (tigecycline), which also inhibit bacterial protein biosynthesis, is mediated by substrate specific efflux pumps belonging to the MFS superfamily [252, 280]. Of these, Tet(A), Tet(B), Tet(M) and TetA(39) could be detected in *A. baumannii* [10, 282-284, 308-310]. While Tet(A) and Tet(M) confer resistance only against tetracycline, TetA(39) is able to mediate efflux of tetracycline and doxycycline and Tet(B) of tetracycline, doxycycline as well as minocycline [252, 280, 282, 308-310]. While tet efflux pumps are limited to tetracyclines, the RND-family multi-drug efflux pumps AdeABC and AdeIJK can mediate resistance of tetracyclines and tigecycline [8, 10, 311, 312]. Although AdeFGH is assumed to be able to cause tigecycline efflux, it does not seem to be able to cause resistance [8]. The novel resistance gene *trm* furthermore was suggested to be a further mechanism for tigecyline resistance [313], and a very recent study by Li et al. shows that presence of *abrp* is associated with decreased susceptibility against tetracycline, tigecycline, chloramphenicol, and fosfomycin in *A. baumannii* [314].

Furthermore, two representatives of polymyxins, which are colistin (polymyxin E) and polymyxin B, are currently available [113]. Polymyxins exert their antimicrobial effect by binding to lipid A, a LPS component, and thus disturbing the outer membrane structure of Gram-negative bacteria [10, 315]. This mechanism is anticipated by bacterial modification of lipid A due to mutations in the *pmrABC* operon [10, 315-317]. Complete depletion of lipid A is based on inactivation of lpxA, lpxC, or lpxD by mutations or insertion of ISAba11 [10, 318, 319]. Nevertheless, the prevalence of colistin resistance is rather low in clinical *A. baumannii* isolates (for example, 5.3% in the USA compared to 33-58% carbapenem resistance [320]), but it might occur more frequently with increased application of the drug [11]. In difference to the previously described antimicrobial classes, the bactericidal effect of rifampicin is achieved by binding to the beta-subunit of the RNA polymerase subunit B (*rpoB*), by which bacterial transcription is blocked [10, 321]. One mechanism of rifampicin resistance is, again, modification of the drug target molecule. These RNA polymerase modifications are caused by amino acid substitutions due to mutations within the active site of the enzyme [10, 321, 322]. A study from Thailand moreover identified *rpoB* mutations outside the active site in rifampicin resistant *A. baumannii* isolates [323]. The ADP-ribosyltransferase ARR-2 has also been associated with rifampicin resistance [253, 324] and since the *arr-2* gene can be located on integrons, rifampicin resistance is very likely transferable at least between *A. baumannii* isolates [324].

Table 3: *A. baumannii* resistance genes for selected antimicrobial classes

| antimicrobial class | mechanism of resistance | protein name | gene name |
|---|---|---|---|
| beta-lactams | AmpC | ADC | *blaADC* |
| | ESBL | SHV | *blaSHV* |
| | ESBL | PER | *blaPER* |
| | ESBL | CTX-M | *blaCTX-M* |
| | ESBL | TEM | *blaTEM* |
| | ESBL | GES | *blaGES* |
| | ESBL | KPC | *blaKPC* |
| | MBL | VIM | *blaVIM* |
| | MBL | SIM | *blaSIM* |
| | MBL | IMP | *blaIMP* |
| | MBL | NDM | *blaNDM* |
| | OXA | OXA-51-like cluster | *blaOXA-51-like* |
| | OXA | OXA-23-like cluster | *bla-OXA-23-like* |
| | OXA | OXA-24/40-like cluster | *bla-OXA24/40-like* |
| | OXA | OXA-58-like cluster | *bla-58-like* |
| fluoroquinolones | target site modification | DNA gyrase alpha subunit | *gyrA* |
| | target site modification | DNA gyrase beta subunit | *gyrB* |
| | target site modification | topoisomerase IV alpha subunit | *parC* |
| | target site modification | topoisomerase IV beta subunit | *parE* |
| | protects target site | plasmid-mediated quinolone resistance determinants | *qnrA* |
| | protects target site | plasmid-mediated quinolone resistance determinants | *qnrB* |
| | protects target site | plasmid-mediated quinolone resistance determinants | *qnrS* |
| aminoglycosides | drug modification (phosphotransferases) | APH(3′)-Ia | *aphA1* |
| | drug modification (phosphotransferases) | APH(3′)-IIb | |
| | drug modification (phosphotransferases) | APH(3′)-VIa | *aphA6* |
| | drug modification (acetyltransferase) | AAC(3)-Ia | *aacC1* |
| | drug modification (acetyltransferase) | AAC(3)-IIa | *aacC2* |
| | drug modification (acetyltransferase) | AAC(6′)-Ib | *aacA4* |

Table 3: Continued

| antimicrobial class | mechanism of resistance | protein name | gene name |
|---|---|---|---|
| aminoglycosides | drug modification (nucleotidyltransferase) | ANT(2″)-Ia | *aadB* |
| | drug modification (nucleotidyltransferase) | ANT(3″)-Ia | *aadA1* |
| | target site modification (16S RNA methylase) | ArmA | *armA* |
| | target site modification (16S RNA methylase) | RmtB | *rmtB* |
| tetracyclines/ tigecycline | efflux pump | Tet(A) | *tet(A)* |
| | efflux pump | Tet(B) | *tet(B)* |
| | efflux pump | Tet(M) | *tet(M)* |
| | efflux pump | TetA(39) | *tetA(39)* |
| polymyxins | target site modification | PmrABC | *pmrABC* |
| | loss of lipid A | LpxA | *lpxA* |
| | loss of lipid A | LpxC | *lpxC* |
| | loss of lipid A | LlpxD | *lpxD* |
| rifampicin | target site modification | RNA polymerase beta subunit | *rpoB* |
| | target site modification (ADP-ribosyltransferase) | ARR-2 | *arr-2* |

## VII Immune defense during *A. baumannii* infection

While epidemiology and resistance mechanisms have extensively been studied in recent years, the mechanisms underlying immune defense during *A. baumannii* infections are not well understood. In principle, immune cells recognize bacterial pathogens through pattern recognition receptors (PRRs), which bind to pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins or flagellins [325, 326]. Toll-like receptors (TLRs) are a group of PRRs that activate signal transduction pathways by binding to PAMPs. Activation of these pathways results in activation of Nuclear Factor Kappa B (NF-ĸB) and mitogen activated protein kinases (MAPKs), and therefore among other things production of proinflammatory cytokines and chemokines [326, 327]. As a global transcriptional regulator, NF-ĸB is involved in various cellular processes, also playing an important role in immune defense. After binding of TLR-4 to LPS, the MyD88 pathway is activated, resulting in NF-ĸB translocation into the cell nucleus of most cell types, where it serves as a promotor for

expression of cytokines, chemokines, adhesions, antimicrobial peptides (AMPs), iNOS, and cyclooxygenase-2 (COX2) [328, 329]. Signaling pathways dependent on TLR-4 and its co-receptor CD14 seem to play a major role during early immune defense in case of *A. baumannii* infection, leading to increased levels of cytokines (IL-1ß, IL-6, IL-8, IL-12, IL-17), TNF-α, NF-ĸB, and MAPKs [327, 330-335]. Although macrophages are able to phagocytose and kill *A. baumannii* cells during infection, their killing efficiency is lower compared to that of neutrophils [336, 337]. Neutrophil depletion causes increased host susceptibility to *A. baumannii* infection, resulting in enhanced bacterial burdens and increased lethality [338], whereas increased neutrophil influx leads to enhanced bacterial clearance [334, 337-341]. It was thus assumed that a bactericidal function of macrophages might be of relevance only at the early stages of infections, until sufficient numbers of neutrophils are recruited to the infection site for efficient bacterial killing [336]. Moreover, mice treated with anti-NK1.1$^+$ antibodies show a reduced ability to eliminate the bacteria and decreased survival rates, suggesting also an important role for NK1.1$^+$ cells besides macrophages in neutrophil recruitment [338]. However, TLR-4 independent signaling pathways became a focus of research, because TLR-4 deficient mice still show enhanced IL-6 and TNF-α levels and bacterial loads comparable to wild-type isolates [330, 342]. Moffatt et al. furthermore showed that LPS- deficient *A. baumannii* cells are still able to activate NF-ĸB and TNF-α expression [343].

Since LPS is the major target molecule for TLR-4, this observation supports the assumption that TLR-4 independent signaling pathways are involved in immune defense during *A. baumannii* infection. A possible candidate molecule triggering alternate inflammatory processes is TLR-2. Although there have been reports that TLR-2 might not be involved in cytokine production and even might impair neutrophil influx [330, 331], there is also evidence to the contrary. In this regard, TLR-2 deficiency has been found to be associated with impaired cytokine production in bone marrow derived macrophages, recruitment of polymorphonuclear cells, and TNF-α production [327, 332]. Another approach is to investigate a possible role of TLR-9 and intracellular immune receptors (Nod-receptors) [330]. TLR-9 deficient mice have been shown to have a decreased cytokine and chemokine production during *A. baumannii* infection and significantly increased bacterial burdens in the lung [344]. Moreover, increased bacterial loads could be counted in Nod-1, Nod-2 and Rip2- deficient human lung epithelial cells infected with *A. baumanni* [345]. Participation of these receptors in immune defense seems reasonable, since there is evidence for invasion of *A. baumannii* into lung epithelial

cells [346]. Some years ago, a novel host defense mechanism has been described called neutrophil extracellular traps (NETs), by which neutrophils capture pathogens in web-like structures [347, 348]. Recently, Kamoshida et al. gained evidence that in contrast to *Pseudomonas* (*P.*) *aeruginosa*, *A. baumannii* does not induce NET formation in the host [349]. Furthermore, *P. aeruginosa* caused higher expression levels of myeloperoxidase (MPO), reactive oxygen species (ROS) and superoxide in neutrophils compared to *A. baumannii* [349]. Similar to this finding, de Breij et al. showed that *A. baumannii* induces a reduced cytokine response compared to other pathogenic *Acinetobacter* spp. [350]. Taking these results into account, the question arises whether *A. baumannii* might possess mechanisms for impairment of the host's immune response.

## MATERIALS AND METHODS

### I Materials

#### 1 Origin of *Acinetobacter* isolates

Reference isolates used in the present study are listed in table 4 and have been kindly provided by the Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität, Giessen, Germany.

Table 4: Reference isolates

| reference isolates | species | host | specimen | designation |
|---|---|---|---|---|
| COL 20820 | *A. baumannii* | human | blood | IMT30483 |
| DSMZ 1139 | *A. calcoaceticus* | human | hexadecane enrichment | IMT30485 |
| DSMZ 9308 | *A. pittii* | human | endotracheal aspirate | IMT30487 |
| ATCC 17903 | *A. nosocomialis* | human | not specified | IMT30488 |

All clinical human and animal *Acb*-complex isolates (n=642) originate from routine diagnostic laboratories. For a one-year time period starting in February 2013, all isolates that have been identified as belonging to the *Acb*-complex either by Bruker Biotyper System

(Bruker Corporation, USA), or VITEK®2 Systems (BioMeriéux, France), were collected from various clinical specimens. Human isolates (n=275) have been provided by the MVZ Labor Ravensburg GbR (Ravensburg, Germany). Animal isolates (n=367) descend from IDEXX Vet Med Labor GmbH, Division of IDEXX Laboratories (Ludwigsburg, Germany). Fluoroquinolone resistance was induced in clinical porcine *A. baumannii* isolates, which have been isolated and provided by the Institute of Hygiene and Infectious Diseases of Animals, Justus-Liebig-Universität (Giessen, Germany). The respective isolates are listed in table 5.

Table 5: Porcine *A. baumannii* isolates used for induction of fluoroquinolone resistance

| species | host | specimen | year of isolation | designation |
|---|---|---|---|---|
| *A. baumannii* | piglet | feces | 2011 | IMT31302 |
| *A. baumannii* | piglet | feces | 2011 | IMT31303 |
| *A. baumannii* | piglet | feces | 2011 | IMT31305 |

## 2 Consumables and media for bacterial cultivation

Consumables and media for bacterial cultivation are listed in Table 42.

## 3 Chemicals, enzymes and devices

All Chemicals and enzymes which have been used in the present study are listed in Table 43, devices are listed in Table 44.

## 4 Buffers and solutions

Protocols for preparation of buffers and solutions used in this work are described in the supplementary materials (cf. Appendix).

## II Methods

### 1 General methods

Cultivation and conservation of bacteria

Reference strains and clinical *Acb*-complex isolates have been provided on swabs. Swabs were spread out on COL S+ agar plates and incubated aerobically over night at 37°C before they were processed for conservation. For this purpose, 500 µl of a fresh overnight culture were mixed with 800 µl 60% glycerol and stored in cryo-conservation tubes at -80°C in the strain collection of the Institute of Microbiology and Epizootics (IMT), Freie Universität Berlin, Germany. Bacterial isolates were recovered from the conservation in -80°C stocks for each experiment by streaking out on COL S+ agar plates and aerobic incubation over night at 37°C. Overnight cultures were achieved by inoculation of a single bacterial colony, grown on COL S+ agar plates, in 5 ml Luria Bertani Broth in sterile A-tubes followed by aerobic incubation on a shaking incubator for approximately 16 hours (37°C, 200 rpm).

Isolation of chromosomal DNA

Depending on the subsequent use of the obtained DNA, two different isolation protocols were used. For application in polymerase chain reaction (PCR), sequencing of PCR amplicons or restriction fragment length polymorphism (RFLP) DNA was isolated using a heat lysis protocol. For this, a single colony of the respective bacterial isolate grown on COL S+ agar plates was suspended in 100 µl sterile 0.9% NaCl solution. The bacterial suspension was incubated at 100°C for 10 minutes and placed on ice immediately for 5 minutes, followed by centrifugation for 30 seconds at 16 000 x g. DNA for subsequent whole genome sequencing (WGS) was received using the MasterPure DNA Purification Kit for Blood II (epicenter Biotechnologies, USA) following the manufacturer's instructions. DNA concentrations were measured using the NanoDrop 1000 spectralphotometer.

## 2 Identification of species of the *Acb*-complex

### 2.1 Molecular methods

#### 2.1.1 Restriction fragment length polymorphism of the 16S-23S intergenic spacer region

In order to perform species identification by means of restriction fragment length polymorphism (RFLP), the 16S-23S intergenic spacer region (IGS) was amplified by PCR. The master mix for the PCR contained 75 ng DNA template, forward and reverse primer 0.5pmol each, deoxynucleosid triphosphates (dNTPs) 2.5 mM each, Dream Taq DNA Polymerase 0.35 U, 1x DreamTaq Green Buffer. Millipore water was added to a total reaction volume of 25 µl. This master mix was used as universal master mix for all polymerase chain reactions. For amplification of the target region, 35 cycles of denaturation at 94°C for 30 seconds followed by annealing at 56°C for 30 seconds and elongation at 72°C for 90 seconds were applied. Successful amplification was examined by agarose gel electrophoresis with 1.5 % agarose gels supplemented with Midori Green Advance (Nippon Genetics, Europe) as dye and an applied voltage of 120 V for 50 minutes in 1x TBE buffer. For amplicon size control, a 100 bp DNA ladder (Thermo Fisher Scientific, Germany) was used.

Table 6: Universal master mix for all polymerase chain reactions

| substrate | concentration | volume |
|---|---|---|
| DNA | 30 ng/µl | 2.5 µl |
| DreamTaq® Green Buffer | 10x | 2.5 µl |
| forward primer | 10 pmol | 0.5 µl |
| reverse primer | 10 pmol | 0.5 µl |
| dNTP mix of all 4 deoxynucleosid triphosphates | 2.5 mM | 0.5 µl |
| DreamTaq® Green polymerase | 5 U/µl | 0.07 µl |
| millipore water | | ad 25 µl |

Table 7: Primer sequences for amplification of the 16S-23S intergenic spacer region

| target region | primer | sequence | product size | reference |
|---|---|---|---|---|
| 16S-23S intergenic spacer | forward<br>reverse | 5′GTCGTAACAAGGTAGCCGTA3′;<br>5′GGGTTYCCCCRTTCRGAAAT3’ (Y is C or T and R is A or G). | 786 - 817 bp | Chang et al. (2005) [351] |

PCR products were subsequently digested in reference to Dolzani et al. (1995) [352]. Differing from the recommended protocol, primers which have been published by Chang et al. (2005) [351] were used for amplification of the 16S-23S intergenic spacer region. Furthermore, restriction was achieved by application of the restriction endonuclease *Mbo*II. Thus, new species-specific restriction patterns were obtained. The master mix for restriction digestion of the amplified 16S-23S IGS contained 18.0 µl PCR product, 10x Buffer B 4 µl and 2.5 U of restriction endonuclease *Mbo*II. Millipore water was added to a total reaction volume of 43.0 µl. The master mix was incubated for 90 minutes at 37°C. Digestion was terminated by incubation at 60°C for 10 minutes. Afterwards, 20 µl of the reaction mixture were separated by electrophoresis on 3.5 % agarose gels supplemented with Midori Green Advance (Nippon Genetics, Europe) (applied voltage 100 V for 60 minutes, 1x TAE buffer) and restriction patterns were analyzed for each template. For this a 100 bp DNA ladder (Thermo Fisher Scientific, Germany) was added to the agarose gel.

### 2.1.2 16S-23S intergenic spacer sequencing

For sequencing, the 16S-23S IGS region was amplified as described in 2.1.1. PCR products were sequenced by LGC genomics GmbH, Berlin, Germany. Sequences were analyzed using Geneious® 6 Software (Biomatters Limited, New Zealand) and Basic Local Alignment Search Tool (BLAST®, NCBI, USA) [353, 354]. Isolates were assigned to the *Acb*-complex species displaying the highest 16S-23S IGS sequence identity. As 16S-23S IGS sequencing was meant for confirmation of species identification previously performed by RFLP, a representative number of random samples for each of the *Acb*-complex species was sequenced and analyzed. Representative sample numbers were calculated using the following homepage: http://epitools.ausvet.com.au/ (design prevalence 0.05; unit sensitivity 1.0; population sensitivity 0.95; population size N = total number of isolates belonging to the

respective *Acb*-complex species). Furthermore, 16S-23S IGS sequencing was also performed for six isolates which displayed unique RFLP restriction patterns.

Table 8: Numbers of random samples for each *Acb*-complex species

| species | number of random samples (n) | population size (N) |
|---|---|---|
| *A. baumannii* | 51 | 221 |
| *A. pittii* | 55 | 319 |
| *A. calcoaceticus* | 42 | 75 |
| *A. nosocomialis* | 1 | 1 |

### 2.1.3 Partial RNA polymerase beta subunit (*rpoB*) sequencing

As recent research questions the reliability of species identification based on sequencing of the 16S-23S IGS region [355], a second sequencing target for confirmation of species assignment was included in the present study. Sequencing of the *rpoB* gene is considered reliable for *Acb*-complex species discrimination [206, 356, 357]. Thus, partial *rpoB* sequencing was performed for all isolates for which 16S-23S IGS sequencing was previously implemented. Amplification of the partial *rpoB* region was achieved using the universal master mix described in section 2.1.1 with primers published by Gundi et al. (2009) [356] (cf. table 9). Thermocycler conditions were adjusted to 35 cycles of denaturation at 94°C for 30 seconds, followed by annealing at 52°C for 30 seconds and elongation at 72°C for 60 seconds. Sequences were analyzed using Geneious® 6 Software and Basic Local Alignment Search Tool (BLAST®). Isolates were assigned to the *Acb*-complex species displaying the highest *rpoB* sequence identity as previously implemented for the 16S-23S IGS sequences. For all investigated *A. pittii* isolates BLAST® analysis resulted in hits displaying the same sequence identitiy for *A. pittii* and *A. calcoaceticus* database entries. For these isolates, the respective sequences were aligned to the *A. pittii* and *A. calcoaceticus* reference sequences published by Gundi et al. (2009) [356] using Geneious® 6 Software. Nine isolates showed accordingly identical sequence identities to *A. calcoaceticus* and *A. oleivorans* database entries in the BLAST® analysis and were thus aligned to the respective sequence of *A. calcoaceticus* RUH2201 [356] and *A. oleivorans* DR1 (Acc. no. CP002080.1). Isolates were assigned to the species showing the highest sequence similarity in these alignments.

Table 9: Primer sequences for partial *rpoB* amplification

| target region | primer | sequence | amplicon size | reference |
|---|---|---|---|---|
| *rpoB* | forward<br>reverse | 5'TAYCGYAAAGAYTTGAAAGAAG3'<br>5'CMACACCYTTGTTMCCRTGA3' | 350 bp | Gundi et al. (2009) [356] |

## 2.2 Phenotypic methods

### 2.2.1 Omnilog® Phenotypic MicroArray

#### 2.2.1.1 Experimental procedure

Differences in the metabolic properties of the *Acb*-complex species were examined by means of the Omnilog® Phenotypic MicroArray system (Biolog, USA). Experiments were carried out for the four *Acb*-complex reference isolates IMT30483 (*A. baumannii*), IMT30485 (*A. calcoaceticus*), IMT30487 (*A. pittii*) and IMT30488 (*A. nosocomialis*), following the procedures as recommended by the manufacturer. Each of the four isolates was tested in three biological replicates. Strains were streaked out from the - 80°C stocks on COL S+ agar plates and cultivated over night at 37°C. Subcultures were subsequently prepared from single colonies the next day and also incubated over night at 37°C. The inoculation medium for the experiment was freshly prepared in sterile tubes and contained 1.88 ml ddH2O, 10.0 ml IF-Oa and 120 µl Redox Dye-Mix A (Biolog, USA). The turbidimeter was adjusted to 100 % transmittance using the pure inoculation medium as blank sample. One to three single colonies of the fresh subcultures of each bacterial sample were picked using a sterile cotton swab and resuspended in the inoculation medium. The bacterial suspension was adjusted to 85 % ± 2 % transmittance using the turbidimeter, before it was transferred into a sterile plastic reservoir suitable for a multichannel sampler. 100 µl of the bacterial suspension were then pipetted in each well of the respective microtiter plate (PM01 and PM2A for carbon sources; for plate maps see http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf). Microtiter plate lids were closed immediately and plates were placed into the Omnilog® Phenotypic MicroArray machine and incubated for 48 h at 37°C.

#### 2.2.1.2 Evaluation of data

The Omnilog® Phenotypic MicroArray is based on the color change of the Biolog Redox Dye-Mix A (100x) during bacterial metabolism which enables released electrons to be transferred to the redox dye. The transferred electrons reduce the dye which changes its color from colorless towards purple. The measured intensity of the purple color is therefore proportional to the bacterial metabolic activity occurring. The Omnilog® Phenotypic MicroArray System measures the intensity of the purple color and generates appropriate bacterial growth kinetics. Data obtained from the present Omnilog® Phenotypic MicroArray experiment is supposed to reveal unambiguous differences in the metabolic properties of each of the four examined isolates suitable for species discrimination. In this context, unambiguous can be understood as positive (purple color) or negative (no purple color) metabolization. For this reason, the parameter maximum height of the growth curve (A) which is reflected by the maximum color intensity is suitable for the analysis.

For data analysis, the opm package available for the Software R Studio Version 3.1.1 was used [358]. Microtiter plate well A01 was used for data normalization, as it contains the adjusted bacterial suspension but no carbon source. Growth in well A01 reflects the basal bacterial growth in the plain inoculation medium. Thus, the calculated A value for A01 was subtracted from each of the calculated A values of the other microtiter wells. Confidence intervals of 95% were calculated for each well based on the respective normalized A values for all three biological replicates of each tested isolate. Isolates were considered variable for metabolization if they showed a 95% confidence interval that ranged values under and above 100. An isolate was considered negative for metabolization if the 95% confidence interval was located in values smaller than 100 and considered positive for metabolization if the 95% confidence interval was located in values above 100. The threshold value of 100 was selected because an indicator color change was in previous experiments only observed, when the 95% ci plots where located above this value.

### 2.2.2 Species identification based on selected carbon sources using the *Acinetobacter* test medium

Based on the Omnilog® Phenotypic MicroArray results, the carbon sources D-ribose (C04, PM01), D-malic acid (G11, PM01), citraconic acid (E03, PM2A), L-hydroxyproline (G08, PM2A) and L-ornithine (H01, PM2A) were selected for testing their suitability for phenotypic *Acb*-complex species discrimination (cf. table 10). For this purpose, an appropriate test medium (*Acinetobacter* test medium, cf. Appendix) was prepared. In addition to the reference strains IMT30483 (*A. baumannii*), IMT30485 (*A. calcoaceticus*), IMT30487 (*A. pittii*) and IMT30488 (*A. nosocomialis*) clinical isolates of each of the *Acb*-complex species were included in the experiment. In order to reflect a possible variability, clinical isolates from different host species and different clinical specimens were randomly chosen (cf. table 27).

Table 10: Omnilog® Phenotypic MicroArray results for selected carbon sources

| *Acb*- complex species | D-ribose | D-malic acid | citraconic acid | L-hydroxyproline | L-ornithine |
|---|---|---|---|---|---|
| *A. baumannii* (IMT30483) | + | + | + | + | + |
| *A. calcoaceticus* IMT30485) | + | - | - | - | - |
| *A. pittii (*IMT30487) | - | - | - | + | - |
| *A. nosocomialis* (IMT30488) | + | + | - | - | + |

+ = reference strain showed metabolization in Omnilog® Phenotypic Microarray
- = reference strain showed no metabolization in Omnilog® Phenotypic Microarray

Isolates were cultivated from the - 80°C stocks on COL S+ agar plates and cultivated over night at 37°C. One single colony of each bacterial isolate was inoculated in a sterile A-tube containing 5 ml of the *Acinetobacter* test medium supplemented with one of the selected carbon sources. Two independent test approaches were prepared, each containing one of the indicators TTC (triphenyltetrazolium chloride) or phenol red. Subsequent incubation was performed on a shaking incubator (37°C, 200 rpm) for 24 and 48 hours.

## 3 Analysis of human and animal clinical *Acb*-complex isolates

### 3.1 Species identification

Species identification to *Acb*-complex level was performed by either VITEK®2 Systems (BioMeriéux, France), or MALDI Biotyper® Systems, (Bruker Corporation, USA) in the diagnostic laboratories MVZ Labor Ravensburg GbR (Ravensburg, Germany) and IDEXX Vet Med Labor GmbH, Division of IDEXX Laboratories (Ludwigsburg, Germany). Further identification to species level was performed at the Institute of Microbiology and Epizootics, Freie Universität Berlin, by means of RFLP of the 16S-23S intergenic spacer region as described in chapter 2.1.1.

### 3.2 Antimicrobial susceptibility testing

Subsequent to the isolation in the diagnostic laboratories, antimicrobial susceptibility testing (AST) was performed for the collected clinical *Acb*-complex isolates using the VITEK®2 System (BioMérieux, France). For this purpose, the VITEK®2 AST-GN38 card was utilized for animal and human isolates. Human isolates were moreover analyzed using the human AST-N263 card. For evaluation of bacterial susceptibility breakpoints recommended by the Clinical Laboratory and Standards Institute (CLSI) were applied. Thus, the published guidelines CLSI M100-S26 (26th Edition, 2015) [359] for human isolates and CLSI VET01S2 (Volume 33 Number 8, July 2013) [360] for animal isolates were used. In case the VET01S2 guideline did not provide breakpoints for one of the investigated antimicrobials suitable for *Acinetobacter* spp., breakpoints were derived from the CLSI M100-S26 for *Acinetobacter* spp. isolated from humans. This concerns breakpoints for piperacillin, tetracycline, polymyxin B, tobramycin and trimethoprim/sulfamethoxazole. For ceftiofur and cefpirome (third and fourth generation cephalosporines) breakpoints for animal isolates were derived from the M100-S26 breakpoints for the corresponding human antimicrobials ceftazidime and cefepime. Equally, breakpoints for enrofloxacin were derived from the M100-S26 breakpoints for ciprofloxacin. Table 11 and table 12 are giving an overview of the tested antimicrobials and the applied breakpoints. For ampicillin, nitrofurantoin and rifampicin no breakpoints are given in both CLSI guidelines, veterinary VET01S2 and human M100-S26. For these substances the Minimum Inhibitory Concentration (MIC) values can only be directly compared between isolates without

susceptibility validation. Isolates displaying resistances against three or more tested antimicrobial classes were considered as being multi-drug resistant (MDR) as recommended by Schwarz et al. [361]. Isolates were furthermore considered as being extensively-drug resistant according to Magiorakos et al. [109]. This concerns *Acinetobacter* isolates with resistances against all but two classes of the following antimicrobials: antipseudomonal fluoroquinolones, aminoglycosides, tetracyclines, antipseudomonal carbapenems, extended-spectrum cephalosporins, folate-pathway inhibitors, penicillins + beta-lactamase inhibitors, antipseudomonal penicillins + beta-lactamase inhibitors and polymyxins. Antipseudomonal penicillins + beta-lactamase inhibitors and penicillins + beta-lactamase inhibitors were grouped together for the animal *A. baumannii* isolates, since piperacillin-tazobactam and ticarcillin-clavulanic acid are generally not used in veterinary medicine.

Table 11: Tested antimicrobial substances and respective breakpoints for animal and human *A. baumannii* isolates analyzed using the AST-GN38 panel

| antimicrobial substance | sensitive (µg/ml) | resistant (µg/ml) | guideline |
|---|---|---|---|
| amikacin[1] | ≤ 16 | ≥ 64 | CLSI VET01S2 |
| amoxicillin/clavulanic acid[1] | ≤ 8/4 | ≥ 32/16 | CLSI VET01S2 |
| cefpirome[3] | ≤ 8 | ≥ 32 | CLSI M100-S26 |
| ceftiofur[3] | ≤ 8 | ≥ 32 | CLSI M100-S26 |
| enrofloxacin[3] | ≤ 1.0 | ≥ 4 | CLSI M100-S26 |
| gentamicin[1] | ≤ 4 | ≥ 16 | CLSI VET01S2 |
| imipenem[1] | ≤ 1 | ≥ 4 | CLSI VET01S2 |
| piperacillin[2] | ≤ 16 | ≥ 128 | CLSI M100-S26 |
| polymyxin B[2] | ≤ 2 | ≥ 4 | CLSI M100-S26 |
| tetracycline[2] | ≤ 4 | ≥ 16 | CLSI M100-S26 |
| tobramycin[2] | ≤ 4 | ≥ 16 | CLSI M100-S26 |
| trimethoprim/sulfamethoxazole[2] | ≤ 2/38 | ≥ 4/76 | CLSI M100-S26 |

1: breakpoints derive from CLSI VET01S2 suitable for *Acinetobacter* spp.; 2: no breakpoints given for *Acinetobacter* spp. in CLSI VET01S2, thus breakpoints for the respective antimicrobial derive from human CLSI M100-S26 for *Acinetobacter* spp.; 3: no breakpoints given for *Acinetobacter* spp. in CLSI VET01S2, thus breakpoints for the respective antimicrobial derive from human CLSI M100-S26 for *Acinetobacter* spp. for substances within the same antimicrobial class

Table 12: Tested antimicrobial substances and respective breakpoints for human *A. baumannii* isolates analyzed using the AST-N263 panel

| antimicrobial substance | sensitive (µg/ml) | resistant (µg/ml) | guideline |
|---|---|---|---|
| ampicillin/sulbactame | ≤ 8/ 4 | ≥32/ 16 | CLSI M100-S26 |
| piperacillin/tazobactam | ≤ 16/ 4 | ≥ 128/ 4 | CLSI M100-S26 |
| cefotaxime | ≤ 8 | ≥ 64 | CLSI M100-S26 |
| ceftazidime | ≤ 8 | ≥ 32 | CLSI M100-S26 |
| ciprofloxacin | ≤ 1 | ≥ 4 | CLSI M100-S26 |
| gentamicin | ≤ 4 | ≥ 16 | CLSI M100-S26 |
| imipenem | ≤ 2 | ≥ 8 | CLSI M100-S26 |
| levofloxacin | ≤ 2 | ≥ 8 | CLSI M100-S26 |
| meropenem | ≤ 2 | ≥ 8 | CLSI M100-S26 |
| trimethoprim/sulfamethoxazole | ≤ 2/38 | ≥ 4/76 | CLSI M100-S26 |

## 3.3. Whole genome sequencing

### 3.3.1 Selection of isolates

Application of molecular typing methods like pulsed field gel electrophoresis (PFGE) or multi locus sequence typing (MLST) for all collected clinical *A. baumannii* isolates (n=221) prior to whole genome sequencing was not feasible within this work due to reasons of time and cost. This is why 23 of the collected human and animal clinical *A. baumannii* isolates were selected based on the accordance of their resistance profiles. The multi-drug resistant human isolate IMT31566 was also included as well as the porcine isolates IMT31302, IMT31303 and IMT31305 as representatives for livestock isolates. Moreover, ten published complete *A. baumannii* genomes (GenBank®, NCBI, USA) were additionally used in the analysis in order to assess the relatedness of the animal isolates to the main human outbreak clones. All selected clinical *A. baumannii* isolates and their respective resistance profiles are listed in table 28 and the selected published complete *A. baumannii* genomes are listed in table 29.

### 3.3.1 Evaluation of data

All selected *A. baumannii* isolates and published genomes (n=37) were analyzed on the basis of alignment of their Maximum Common Genome (MCG) [362], which consists of the set of genes, present in all investigated isolates. An unrooted tree was generated by means of single nucleotide polymorphisms (SNPs) within the genes of the MCG. Furthermore, the whole genome sequences were used to create a Distance Matrix based on calculation of the pairwise distances between the isolates by means of MEGA software version 6 [363]. Bioinformatical work was performed by Torsten Semmler, Robert Koch- Institute, Berlin. Moreover, MLST was performed for all sequenced human and animal *A. baumannii* isolates using the Pasteur MLST scheme [15] at the Centre for Genomic Epidemiology Server (CGE) [364], which was also used for detection of resistance genes for each investigated isolate.

## 4 Investigation of fluoroquinolone resistance in *A. baumannii*

### 4.1 Induction of fluoroquinolone resistance

Resistance to fluoroquinolones was induced in the following clinical *A. baumannii* isolates IMT313202, IMT31303 and IMT31305 (cf. table 5) by means of gradient plates.

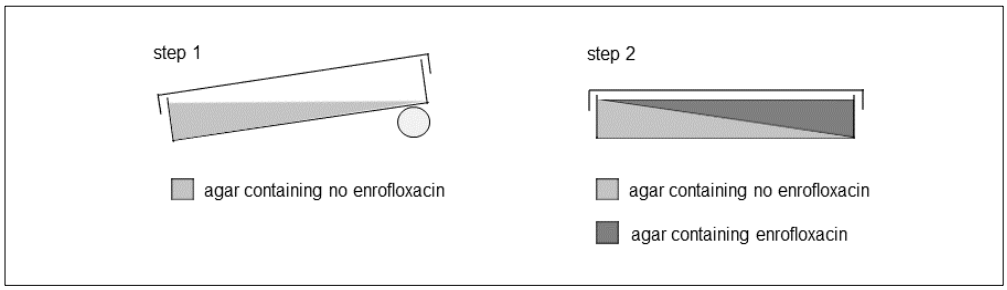
### 4.1.1 Preparation of gradient plates

Gradient plates consist of two wedged layers of solid media of which one contains an antimicrobial and one does not. Diffusion between these two layers results in formation of a concentration gradient of the antimicrobial substance. For the conducted experiments enrofloxacin was chosen as antimicrobial substance. Enrofloxacin (ENR) is a commonly used veterinary fluoroquinolone and resistance to ENR usually results in cross resistance to most fluoroquinolones, especially ciprofloxacin, marbofloxacin and levofloxacin. Preparation of gradient plates was performed as recommended by K. R. Aneja [1].

For preparation of the gradient plates, 250 ml of sterile autoclaved LB solid medium were heated and cooled down to 56°C. 15 ml were then filled into a sterile plastic petri dish for each gradient plate. Each petri dish was then placed on a glass stick with only one edge of the petri

dish bottom, until the LB medium reached room temperature and hardened, resulting in the first wedged LB layer (step 1). Subsequently, a second bottle containing 250 ml sterile autoclaved LB solid medium was heated, cooled down to 56°C and enrofloxacin was added from a stock solution to the respective necessary concentration (which is equal to the required maximum concentration of the enrofloxacin gradient). The glass stick was removed and the petri dish was placed normally on the table. 15 ml of the liquid LB medium supplemented with enrofloxacin were added on top of the first wedged layer so that a horizontal level of medium was achieved (step 2). The point of maximum enrofloxacin concentration was marked on the petri dish. Gradient plates were stored at 4°C over night to allow the enrofloxacin to diffuse between the two layers. Figure 2 illustrates the preparation of gradient plates.

Figure 2: Preparation of gradient plates (figure modified after K. R. Aneja [1])




4.1.2 Cultivation in subinhibitory fluoroquinolone concentrations

Enrofloxacin (ENR) susceptibility of the clinical porcine *A. baumannii* isolates IMT31302, IMT31303 and IMT31305 was confirmed using VITEK®2 System (AST-GN38 card, Bio Mérieux, France). All three isolates were streaked out on COL S+ agar plates and incubated over night at 37°C. A single colony of each isolate was inoculated in 5 ml Brain Heart Infusion (BHI) broth and again cultivated over night at 37°C on a shaking incubator (200 rpm). Subsequently, 100 µl of each overnight culture were evenly plated on the gradient plate containing 0.25 µg/ml ENR maximum concentration (gradient plate 1). Plates were sealed with parafilm to avoid desiccation during incubation and were incubated for several days at 37°C until colonies appeared. Single colonies of each isolate grown in the highest ENR concentration were again picked, inoculated in 5 ml BHI broth and incubated over night at 37°C on a shaking incubator (200 rpm). The next day, 100 µl of the overnight culture were plated on

the freshly prepared gradient plate containing the next highest ENR maximum concentration (gradient plate 2). Plates were again sealed with parafilm and incubated at 37°C until colonies grew in elevated ENR concentrations. The procedure was repeated with increasing ENR amounts until colonies grew in ENR concentrations higher than 4.0 µg/ml, which complies the derived breakpoint for ENR resistance (CLSI M100-S26: breakpoint for CIP resistance ≥ 4 µg/ml; cf. Table 11).

Table 13: Increasing maximum ENR concentrations on gradient plates used in this study

| gradient plate 1 | gradient plate 2 | gradient plate 3 | gradient plate 4 | gradient plate 5 | gradient plate 6 |
|---|---|---|---|---|---|
| 0.25 µg/ml | 0.5 µg/ml | 1.0 µg/ml | 2.0 µg/ml | 4.0 µg/ml | 6.0 µg/ml |

### 4.1.3 Species confirmation of fluoroquinolone resistant colonies

For each isolate, colonies grown in ENR concentrations higher than 4.0 µg/ml were considered as being ENR resistant mutants. As resistance to fluoroquinolones is mediated by genetic mutation, not by acquisition of foreign genetic material, isolates are not expected to lose resistance by cultivation lacking ENR selective pressure. Each ENR resistant mutant was enriched in BHI broth over night at 37°C on a shaking incubator (200 rpm) and immediately conserved at -80°C. Furthermore, overnight cultures were streaked out and subcultivated on COL S+ agar plates (incubation 37°C, over night). Species confirmation of these subcultures was performed by means of RFLP of the 16S-23S intergenic spacer region (cf. 2.1.1). Species identification was furthermore confirmed by whole genome sequencing of selected mutants.

## 4.2 Whole genome sequencing

In order to identify mutations in the genome of ENR resistant mutants, whole genome sequencing was performed using next generation sequencing by the Illumina MiSeq sequencing system (Illumina, USA) at the Institute of Microbiology and Epizootics, Freie Universität Berlin. Moreover, whole genome sequencing

might allow linkage of deviations in the phenotype of the ENR resistant mutants (compared to the respective wild-type isolates) to genetic alterations.

### 4.2.1 Selection of isolates

A total of 26 ENR resistant mutant isolates derived from the three ENR sensitive wild-type isolates. Whole genome sequencing could not be performed for all mutants on grounds of costs. Mutants were selected for sequencing based on their macroscopic and microscopic appearance. Isolates displaying substantial pleomorphic colony and cell morphology or isolates with substantially reduced growth rates were excluded from sequencing. Macroscopic examination revealed that for mutants ENRres4, ENRres7, ENRres10 and ENRres11 two morphologically distinct stable lineages originated from the same single gradient plate colony. For these mutants, both distinct lineages were selected for whole genome sequencing. For identification of mutations due to the enrofloxacin selective pressure, mutants have to be compared to their respective wild-type isolate. For this, the ENR sensitive wild-type isolates were also whole genome sequenced. Table 30 lists all wild-type and mutant isolates selected for whole genome sequencing.

### 4.2.2 Evaluation of data

Subsequent to whole genome sequencing, assembly and annotation of the obtained data was carried out using CLC Genomics Workbench 9.0 (CLC bio, Denmark, http://www.qiagenbioinformatics.com/products/clc-genomics-workbench/) and the RAST server (Rapid Annotations using Subsystems Technology, http://rast.nmpdr.org/ [365]). Assembly and annotation were performed by Torsten Semmler, Robert Koch-Institute, Berlin. The assembled and annotated genomes were analyzed using Geneious® 6 Software. In this regard, all contigs of one ENR resistant mutant were mapped against all contigs of the respective ENR sensitive wild-type isolate and analyzed for presence of single nucleotide polymorphisms (SNPs), insertions and deletions. Once a mutation was detected, the affected region of the wild-type isolate served as reference for alignment of the reads of the mutant isolate (creation of BAM files which contain the alignment data). Mutations were considered as confirmed, if they also occurred in the created BAM files. Three target genes *hfq*, *adeL* and *adeN*, which showed mutations in most ENR resistant mutants, were furthermore sequenced

by means of the Sanger method. Both methods, Sanger sequencing and confirmation by BAM files, produced the same results and the latter was considered reliable.

Table 14: Primer sequences and annealing temperatures for Sanger sequencing of *hfq*, *adeL*, *adeN*

| target region | primer sequence (forward/ reverse) | annealing temperature | product size | reference |
|---|---|---|---|---|
| *hfq* | 5‘- CGCAGGTAGCTTTAATATGCTTT-3‘<br>5‘- CACGACAACTTGCCAAACGT-3‘ | 62.0 °C | 699 bp | this work |
| *adeL* | 5‘- TTTCGAACTTACTCATCTGCTGA-3‘<br>5‘- GGTTTATGGAATGGACGGAGC-3‘ | 62.5 °C | 1285 bp | this work |
| *adeN* | 5‘- GCTGGGTGGAAGTGGGAAAA-3‘<br>5‘- AAGCAGTGTTAGCCGTCGTT-3‘ | 62.5 °C | 746 bp | this work |

## 4.3 Phenotypic analysis

### 4.3.1 Macroscopic and microscopic investigation

For each of the obtained ENR resistant mutant isolates, single colonies of fresh subcultures were macroscopically investigated for purity, growth rate, size, smell, color and striking deviations from common *A. baumannii* morphology. In order to examine cell morphology, a single colony of each mutant isolate was Gram-stained and investigated using the 100-fold magnification. Bacterial cells were evaluated for their Gram-staining behavior, cell arrangements, size and shape.

### 4.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was implemented using the Epsilometer test (Etest) method, (BioMérieux, France). The following antimicrobials were chosen as representatives for the respective antimicrobial class: enrofloxacin, ampicillin, piperacillin, cefpodoxime, cefpirome, gentamicin, imipenem, tetracycline, rifampicin, trimethoprim/sulfamethoxazole and colistin. MIC values were determined as recommended by the manufacturer and in correspondence with the CLSI guidelines [360]. Suitability of the Mueller-Hinton agar plates was validated

according to the CLSI guidelines [360] using the following reference isolates: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212. Antimicrobial susceptibility testing was implemented for wild-type isolates IMT31302, IMT31303, IMT31305 and all ENR resistant mutants which already had been whole genome sequenced (cf. table 30). All isolates were tested in triplicates and the mean MIC values were calculated for each tested antimicrobial.

### 4.3.3 Conjugation experiments

#### 4.3.3.1 Identification of a conjugative plasmid

A wide variety of marker genes for successful plasmid transfer is described. Several requirements are necessary for selection of a suitable marker gene, namely location on a transferable plasmid, expression by the recipient isolate and mediation of e.g. antimicrobial resistance.

##### 4.3.3.1.1 Polymerase chain reaction

Resistance to aminoglycosides was selected as the marker for plasmid transfer in the ENR sensitive wild-type isolates and their ENR resistant mutants, because all isolates were tested susceptible to this antimicrobial. Since expression of the marker gene should be assured, all clinical *A. baumannii* isolates exhibiting aminoglycoside resistance (n= 83) were screened for presence of one of the following genes: *aadB, armA, aac(6')-Ih, aac(3)-IIa* and *aphA6* by means of PCR (cf. table 15). All of these genes have been described as being encoded chromosomally or on plasmids [366-371]. For the screening PCRs the universal master mix and the thermocycler conditions were used as described in chapter 2.1.1. Annealing temperatures were modified according to the investigated target gene.

Table 15: PCR conditions for screening of aminoglycoside resistance genes

| target region | primer sequence (forward and reverse) | annealing temperature | product size | reference |
|---|---|---|---|---|
| *aadB* | 5‘-GGGAAGAATCAATACCGCAA-3‘<br>5‘-AATTTCACCCCAAACAATCG-3‘ | 52.0 °C | 999 bp | Hamidian et al. (2012) [368] |
| *armA* | 5‘-AGGTTGTTTCCATTTCTGAG-3’<br>5‘-TCTCTTCCATTCCCTTCTCC-3‘ | 53.0 °C | 590 bp | Yamane et al. (2005) [307] |
| *aac(6’)-Ih* | 5‘-TGCCGATATCTGAATC-3‘<br>5‘-ACACCACACGTTCAG-3‘ | 62.0 °C | 407 bp | Noppe-Leclercq et al. (1999) [372] |
| *aac(3)-IIa* | 5‘-ATGCATACGCGGAAGGC-3‘<br>5‘-TGCTGGCACGATCGGAG-3‘ | 53.0 °C | 822 bp | Noppe-Leclercq et al. (1999) [372] |
| *aphA6* | 5‘-CGGAAACAGCGTTTTAGA-3‘<br>5‘-TTCCTTTTGTCAGGTC-3‘ | 53.0 °C | 716 bp | Noppe-Leclercq et al. (1999) [372] |

#### 4.3.3.1.2 Plasmid preparation

The clinical human *A. baumannii* isolate IMT31566 was tested positive for *armA* and *aphA6.* In order to further investigate the location of these genes, plasmid preparation was conducted. Subsequent to plasmid preparation, the respective PCRs for *armA* and *aphA6* were repeated for the obtained plasmid fraction. For plasmid preparation, the isolate IMT31566 was streaked out on COL S+ agar plates from the -80°C bacterial stock and incubated over night at 37°C. A single colony was inoculated the next day in 5 ml LB broth and again incubated over night at 37°C on a shaking incubator (200 rpm). 1 ml of this overnight culture was centrifuged for 5 minutes at 12000x g. The supernatant was discarded and this step was repeated. The bacterial pellet was resuspended in 20 µl TE Buffer before 100 µl freshly prepared Lysis Buffer were added. Samples were mixed by careful panning and incubated for 25 minutes at 58°C. Afterwards 2 ml phenol/chloroform/isoamylalcohol were filled from the lower phase into a fresh Eppendorf tube and 100 µl of this filling were added to the sample. The sample was very carefully manually turned upside down 100-fold, followed by centrifugation for 15 minutes at 16000x g. The plasmid fraction containing supernatant was transferred into a fresh Eppendorf tube without destroying the protein layer. PCRs for *armA* and *aphA6* were performed according to the described protocol (cf. 4.3.3.1.1) using heat isolated chromosomal DNA of IMT31566 as well as the plasmid preparation.

4.3.3.1.3 Prediction of plasmid sequence

The whole genome of IMT31566 was sequenced using illumina MiSeq at the Institute of Microbiology and Epizootics, Freie Universität Berlin. In 2014 Hamidian et al. published the sequence of the conjugative plasmid pAb-G7-2 carrying the transposon *TnaphA6* [367]. This plasmid was used as reference for plasmid sequence prediction for IMT31566. For this purpose, all contigs of IMT31566 obtained during whole genome sequencing were assembled to the sequence of pAb-G7-2. All contigs of IMT31566 which mapped to pAb-G7-2 were furthermore analyzed using Basic Local Alignment Search Tool (BLAST®). All sequences for which the calculated sequence similarity was 50% or higher by at least 25% query cover were again used as additional references for plasmid prediction. The whole genome of IMT31566 was again assembled to each of these additional references in order to identify contigs of IMT31566 which had not been detected by assembly to pAB-G7-2. Reference plasmid sequences and respective accession numbers are listed in table 31. The putative plasmid was named pAB31566.

4.3.3.2 Conjugation

In order to compare the ability to successfully acquire and express foreign plasmids between the enrofloxacin (ENR) sensitive wild-type and generated ENR resistant mutant isolates, conjugation experiments were performed.

4.3.3.2.1 Selected isolates

The fact that the gene *aphA6* has been described to be located on plasmids which are only transferable between *Acinetobacter* species complicates the distinction between plasmid donor and recipient. For this reason, the clinical *A. baumannii* isolate IMT31566 (harboring pAB31566) was not suitable as donor for the conjugation experiments with the ENR sensitive wild-type and ENR resistant mutant isolates, which belong to the same bacterial species. To ensure a reliable distinction, an *Acinetobacter* (*A.*) *haemolyticus* isolate was chosen as donor. *A. haemolyticus* shows haemolytic zones around each single colony on COL S+ agar plates, whereas *A. baumannii* does not. For this purpose, an *A. haemolyticus* isolate harboring the plasmid of interest pAB31566 had to be created for implementation as the donor isolate for the subsequent conjugation experiments. Conjugation experiment A enabled the transfer of

pAB31566 from clinical *A. baumannii* IMT31566 into the *A. haemolyticus* isolate IMT32484, which was tested negative for *aphA6* by means of the *aphA6* PCR as previously described (cf. 4.3.3.1.1). Transfer of pAB31566 to IMT32484 lead to the new isolate IMT32484*_aphA6* and was confirmed by a positive reaction in the *aphA6* PCR for IMT32484*_aphA6.* Expression of the marker gene in the new isolate IMT32484*_aphA6* was proved by successful subcultivation on COL S+ agar plates supplemented with 100 µg/ml Kanamycin. In the second conjugation experiment B, pAB31566 was supposed to be transferred between the new donor isolate IMT32484*_aphA6* and the ENR sensitive wild-type and ENR resistant mutant isolates (recipients). Due to grounds of costs and time, conjugation experiments were performed for only one ENR resistant mutant for each of the three ENR sensitive wild-type isolates. Selection of the respective mutant isolates was made randomly among the mutants which had already been whole genome sequenced. Susceptibility of the ENR sensitive wild-type and resistant mutant isolates (recipients) to kanamycin was assured by subcultivation on COL S+ agar plates supplemented with 100 µg/ml Kanamycin (no bacterial growth). Recipients were additionally tested for absence of *armA* and *aphA6* by i) alignment of their whole genome sequences to the *armA* and *aphA6* sequences from IMT31566 and ii) alignment of the respective primer sequences as listed in table 15.

Table 16: Isolates selected for conjugation experiments A and B

| conjugation experiment | transferred plasmid | donor isolate (species) | recipient isolate (species) |
|---|---|---|---|
| A | pAB31566 | IMT31566 (*A. baumannii*) | IMT32484 (*A. haemolyticus*) |
| B | pAB31566 | IMT32484*_aphA6* (*A. haemolyticus*) | IMT31302 (*A. baumannii*) |
| B | | | ENRres1 (*A. baumannii*) |
| B | | | IMT31303 (*A. baumannii*) |
| B | | | ENRres6 (*A. baumannii*) |
| B | | | IMT31305 (*A. baumannii*) |
| B | | | ENRres9 (*A. baumannii*) |

4.3.3.2.2 Filter mating and selection for transconjugants

Donor and recipient isolates were cultivated from the -80°C stock on COL S+ agar plates (incubation over night at 37°C). A single colony of each isolate was inoculated in 5 ml LB broth

an incubated over night at 37°C on a shaking incubator (200 rpm). The next day 1 ml of each overnight culture was centrifuged at 9000x g for 2.5 minutes and the supernatant was discarded. The cell pellet was washed in 500 ml LB broth (preincubated to 37°C) and centrifuged at 9000x g for 2.5 minutes. The supernatant was again discarded and the washing step was repeated. The final cell pellet was resuspended in 500 µl preincubated LB broth and the optical density was measured for each sample at a wavelength of 600nm. Based on the optical density, the bacterial suspensions were adjusted to approximately $1x10^8$ cfu/ml for each isolate as the optical density correlates with the cfu (colony forming units). 400 µl of the cfu adjusted bacterial suspension of each recipient isolate and 100 µl of the cfu adjusted bacterial suspension of the donor isolate were transferred into the same new Eppendorf tube and mixed. This recipient/donor mixture was centrifuged at 9000x g for 2.5 minutes and the supernatant was carefully discarded without touching the cell pellet. The cell pellet was again resuspended in 20 µl preincubated LB broth and the recipient/donor-solution was dropped on sterile Whatman-Paper wafers on LB agar plates (also preincubated to 37°C). Plates were subsequently incubated with the lid up at 37°C for 24h. After 24h the wafers were transferred into 10 ml preincubated (37°C) LB broth supplemented with 50 µg/ml kanamycin and incubated again for 24h at 37°C. After incubation, the solutions were diluted and 100 µl of each of the $10^{-4}$, $10^{-5}$,$10^{-6}$ dilutions were plated as duplicates onto COL S+ agar plates supplemented with 100 µg/ml kanamycin and incubated at 37°C for 24h.

Afterwards colony forming units of grown nonhaemolytic colonies (transconjugants) were counted and the cfu/ml was calculated for each recipient isolate by means of the following formula:

$$\frac{\text{cfu}}{\text{ml}} = \frac{(A * 0.01 + B * 0.1 + C * 1.0)}{3} * 10^7$$

with *A*=mean of counted cfu of douplicates of $10^{-4}$ dilution; *B*=mean of counted cfu of douplicates of $10^{-5}$ dilution; *C*=mean of counted cfu of douplicates of $10^{-6}$ dilution.

In case of confluent growth of bacterial colonies on the plates of the $10^{-4}$ dilution, only the $10^{-5}$, $10^{-6}$ dilutions were included in the cfu/ml calculation. In this case, the formula was adjusted to

$$\frac{\text{cfu}}{\text{ml}} = \frac{(B * 0.1 + C * 1.0)}{2} * 10^7$$

In order to verify the donor/recipient relation for each single experiment, the cfu adjusted bacterial suspensions were correspondingly diluted, plated on COL S+ agar plates and the cfu/ml values were calculated using the described formulas. Conjugation experiment B was performed in three biological replicates for the wild-type/mutant pairs IMT31302/ENRres1, IMT31303/ENRres6 and IMT31305/ENRres9. For further validation of the reliability of the results, the experiment was conducted in another six replicates for IMT31302/ENRres1.

4.3.3.2.3 Confirmation of plasmid uptake

Plasmid uptake and expression were confirmed by i) successful subcultivation of ten randomly chosen transconjugant colonies for each recipient isolate for replicates 1-3 on COL S+ agar plates supplemented with 100 µg/ml kanamycin ii) *aphA6* PCR for the subcultivated transconjugants of replicates 1-3. Transconjugants were furthermore confirmed as *A. baumannii* colonies by means of RFLP of the 16S-23S intergenic spacer region (cf. 2.1.1). Prior to PCR reactions DNA was isolated using the heat lysis protocol as described elsewhere (cf. 1.2).

4.3.4 Cell culture experiments

Possible alterations in the early innate immune response towards infection with the ENR resistant mutant isolates compared to their respective ENR sensitive wild-type isolates were investigated by means of the Nuclear factor-kappaB (NF-KB) reporter assay. The cell culture experiments were performed with the same isolates that were previously investigated in the conjugation experiments. The respective isolates are IMT31302/ENRres1, IMT31303/ENRres6 and IMT31305/ENRres9.

4.3.4.1 Cell lines

Two different cell lines were included in the NF-KB reporter assays: 3D4/31 is a porcine alveolar macrophage cell line, which derives from the same host species like the ENR wild-type isolates [373]. Furthermore, human THP-1 monocytes [374] were also included in the experiments in order to take a possible host specificity into account. Both cell lines have been selected for adherence and THP-1 monocytes have been activated for differentiation to

macrophages by colleagues at the Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin.

4.3.4.2 Cultivation and passaging of cell lines

Cell culture medium was prepared by supplementation of 500 ml of Iscove's Basal Medium with 50 ml fetal calve serum and 5 µg/ml puromycin prior to cell cultivation. Puromycin was used as the selective agent for adherent, NF-KB reporter gene positive cells. All buffers and solutions were preincubated to 37°C before application in cell cultivation and passaging. Due to the cellular structure of the investigated cell lines 100 % confluent monolayers cannot be achieved. For this reason, passaging was implemented as soon as cells achieved at least 80 % confluence [375]. The consumed cell culture medium was then carefully discarded without destroying the cell layer. Cells were washed with 5 ml of preincubated 1x phosphate buffered saline (PBS) prior to adding 5 ml of a 1x trypsin/EDTA solution. Cells were subsequently incubated at 37°C with 5 % $CO_2$ pressure for 5-15 minutes until cells completely detached from the bottom of the cell culture flask. The solution containing the detached cells was transferred into a 10 ml falcon tube and 2 ml of fresh cell culture medium were added for inactivation of the trypsin/EDTA solution. Cells were pelleted by centrifugation for 5 minutes at 155 x g. The supernatant was discarded and the cells were resuspended in 5 ml fresh cell culture medium. 30 µl of the cell/medium-solution were transferred into a new T25-cell culture bottle containing 5 ml of fresh cell culture medium. Cells were incubated at 37°C with 5 % $CO_2$ pressure for 7 days until a new cell monolayer was established.

4.3.4.3 Nuclear factor-kappa B (NF-KB) reporter assay

In case of binding of an antigen to the immune cell surface, different signaling pathways are activated within these cells. These signaling pathways lead to activation of NF-KB a transcriptional regulator. NF-KB is subsequently translocated to the immune cell nucleus where it is activating various target genes like cytokines, inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2, growth factors and inhibitors of apoptosis. The level of NF-KB activation is thereby proportional to the level of previous activation of the signaling pathways due to antigen binding. For quantification of the NF-KB activation following antigen exposure, the 3D4/31 and THP-1 cell lines have been modified for NF-KB reporter function due to integration of a lentiviral

luciferase (luc) using the Cignal Lenti Reporter Assay (QIAGEN, Netherlands). After modification, the *luc* expression is proportional to the NF-KB activation.

Previous to the NF-KB reporter assays, cells were investigated for confluent growth in monolayers and cell passaging was performed as described (cf. 4.3.4.2). 20 µl of the cell/medium-solution obtained after resuspension of the cell pellet during passaging were seeded into each well of a flat bottom 96-well microtiter plate. Subsequently, 80 µl of fresh cell culture medium supplemented with 5 µg/ml puromycin (preincubated to 37°C) were added to each well. Cells were incubated at 37°C with 5% $CO_2$ pressure until an at least 80% confluent cell monolayer established in all wells. Two hours before infection, the cell culture medium was discarded from each well and cells were washed with 100 µl preincubated 1x PBS. Afterwards 100 µl of fresh cell culture medium without puromycin supplementation were added to each well.

For infection of the cells, bacteria of freshly prepared COL S+ cultures were inoculated in 50 ml LB broth in 250 ml Erlenmeyer flasks to an optical density of 0.1 at 600nm wave length. Bacterial suspensions were incubated for 2 h on a shaking incubator (200 rpm) at 37°C. 2 ml of each bacterial suspension were then transferred into an Eppendorf tube and centrifuged for 2.5 minutes at 7500 x g. The supernatant was discarded and the bacterial cell pellets were resuspended in 1 ml cell culture medium without puromycin supplementation. These bacterial suspensions were adjusted to approximately 0.6 x $10^8$ cfu/ml and 100 µl of the adjusted bacterial suspensions were pipetted into the respective microtiter plate wells. This corresponds to an approximate multiplicity of infection (MOI) of 100 for the 3D4/31 cells. Since there was no data available concerning the number of macrophages per well for adherent THP-1 cells, infection was performed with the same bacterial load as for the 3D4/31 cells. Four microtiter plate wells were infected for each investigated bacterial isolate and for each of the measured time points 7 h and 19 h post infection. For each time point four wells remained uninfected. The uninfected cells were used for normalization as they represent the basal expression of *luc*. Immediately after infection the microtiter plate was centrifuged 10 minutes at 250x g in order to attach the suspended bacteria onto the cell monolayer and the plate was incubated at 37°C with 5% $CO_2$ pressure. The cell culture medium was discarded 1 h post infection and 100 µl of preincubated (37°C) cell culture medium supplemented with 50 µg/ml Gentamicin were added to each well. After 2 h of infection, the cell culture medium was again changed to 100 µl of

medium supplemented with 10 µg/ml gentamicin. The microtiter plate was afterwards incubated until the first measurement at 7h post infection (p.i.).

In order to measure the luciferase activity, a chemoluminescent reagent (Bright-Glo luciferase Assay substrate, Promega, Germany) was added to the respective wells. For this, 75 µl of cell culture medium were discarded from each of the four wells per bacterial isolate and 25 µl of the liquid Bright-Glo luciferase Assay substrate were added without touching the cell monolayer. Bright-Glo was also added to the four wells containing uninfected cells and the microtiter plate was incubated for 5 minutes. The induced chemoluminescence intensity was quantified by means of an ELISA reader using the KC4 Data Analysis Software (BioTek, USA). Directly after the first measurement, the microtiter plate was again incubated at 37°C with 5 % $CO_2$ pressure until the second measurement 19 h p.i.. Measurement 2 (19 h p.i.) was performed appropriate to measurement 1 (7 h p.i.). All isolates were tested in three biological replicates and the median was calculated for the measured values for each isolate and replicate. For normalization, the median of the uninfected cells was subtracted from each of the previously calculated medians for the infected cells. The obtained values are represented by the variable difference-median and were analyzed for normal distribution. Afterwards the Tukey Test was performed for pairwise comparison of the variable difference-median of the investigated isolates. The level of significance was determined as $p \leq 0.1$ based on the low sample size. Statistical work was performed using IBM SPSS Statistics 22 software.

# RESULTS

## I Genotypical and phenotypical analysis of isolates of the *Acb*-complex

### 1 Collection of clinical *Acb*-complex isolates

Within a one-year time-period starting in February 2013, 642 clinical *Acb*-complex isolates were collected. Of these, 275 originated from humans and 367 from animal hosts. Table 17 provides an overview of the number of *Acb*-complex isolates obtained from different clinical specimens, whereas table 18 lists host species of the animal *Acb*-complex isolates. Isolates belonging to the same bacterial species, originating from the same individual and displaying the same resistance profile, were considered as being very likely identical and were counted only once.

Table 17: Number of human and animal *Acb*-complex isolates collected from different clinical specimens

| specimen | number of human *Acb*-complex isolates | number of human *A. baumannii* isolates | number of animal *Acb*-complex isolates | number of animal *A. baumannii* isolates |
|---|---|---|---|---|
| respiratory tract | 47 | 12 | 114 | 43 |
| wound/ abscess | 66 | 13 | 108 | 54 |
| urinary tract | 57 | 10 | 31 | 19 |
| thoracic/ abdominal cavity | 4 | 1 | 8 | 6 |
| eye | 3 | 0 | 30 | 9 |
| ear | 3 | 0 | 14 | 10 |
| bloodstream | 12 | 1 | 0 | 0 |
| feces/ rectum/ anal region | 6 | 3 | 8 | 2 |
| genital tract | 5 | 0 | 10 | 3 |
| gastric/ ulcer | 12 | 2 | 1 | 1 |
| others | 7 | 0 | 44 | 16 |
| unknown | 54 | 16 | 2 | 2 |

*Acb*-complex isolates and associated metadata were collected from human and animal clinical specimens within a twelve-month period starting in February 2013 (total number of human isolates: n=275; total number of animal isolates n=367); number of *Acb*-complex isolates includes *A. baumannii, A. pittii, A. calcoaceticus* and *A. nosocomialis* isolates; identification to species level was performed by RFLP of the 16S-23S IGS by *Mbo*II

Table 18: Number of animal *Acb*-complex and *A. baumannii* isolates originating from different host species

| host species | number of *Acb*-complex isolates | number of *A. baumannii* isolates |
|---|---|---|
| dog | 205 | 106 |
| cat | 83 | 38 |
| horse | 26 | 11 |
| rabbit/ guinea pig/ chinchilla | 14 | 2 |
| reptile | 13 | 2 |
| exotic bird | 17 | 0 |
| eagle-owl | 1 | 0 |
| chicken | 2 | 1 |
| ruminants | 3 | 1 |
| lion | 1 | 1 |
| monkey | 1 | 1 |
| kangaroo | 1 | 0 |

*Acb*-complex isolates and associated metadata were collected from animal clinical specimens within a twelve-month period starting in February 2013 (total number of animal isolates n=367); number of *Acb*-complex isolates includes *A. baumannii, A. pittii, A. calcoaceticus* and *A. nosocomialis* isolates; identification to species level was performed by RFLP of the 16S-23S IGS by *MboII*

## 2 Species identification based on restriction fragment length polymorphism (RFLP) of the 16S-23S intergenic spacer region due to restriction by *Mbo*II

### 2.1 Species-specific restriction patterns of the amplified 16S-23S intergenic spacer

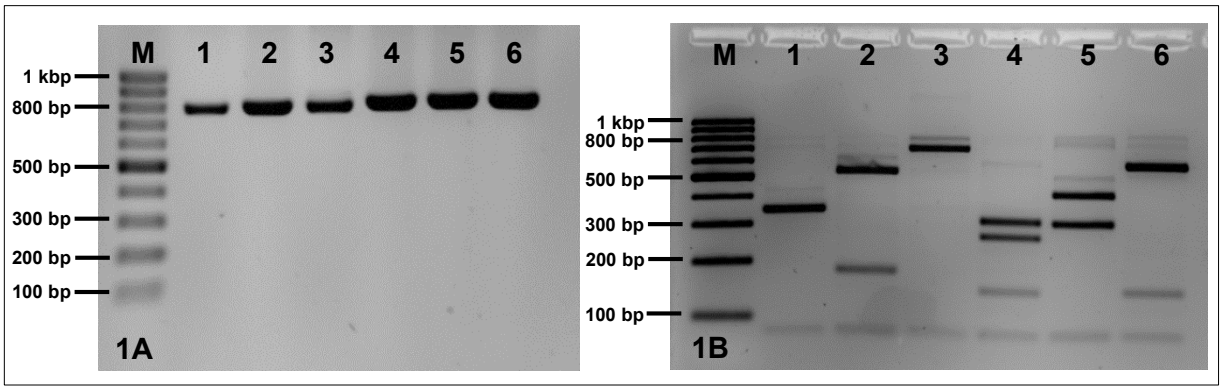
Amplification of the 16S-23S Intergenic spacer (IGS) region resulted in PCR products ranging from 786bp for *A. baumannii* to 817bp for *A. calcoaceticus*. Six different species-specific restriction patterns for the four *Acb*-complex species could be obtained (cf. figure 3). Three of these restriction patterns belong to *A. calcoaceticus* based on SNPs of the *Mbo*II restriction site (pattern 5: bp 473 G→A and bp 479 A→G and restriction pattern 6: bp 197 G→A). Fragment lengths of the species-specific restriction patterns are given in table 19.

Table 19: Fragment lengths for the respective *Acb*-complex species-specific restriction patterns, based on restriction of 16S-23S IGS amplicons by *Mbo*II

| restriction pattern | species | reference isolate | fragment lengths (bp) |
|---|---|---|---|
| 1 | *A. baumannii* | COL 20820 | 82, 351[A], 353 [A] |
| 2 | *A. pittii* | DSZM 9308 | 82, 542, 174 |
| 3 | *A. nosocomialis* | ATCC 17903 | 82, 712 |
| 4 | *A. calcoaceticus* | DSZM 1139 | 7[B], 82, 145, 272, 311 |
| 5 | *A. calcoaceticus* | IMT30821 | 82, 424, 311 |
| 6 | *A. calcoaceticus* | IMT31135 | 82, 145, 574 |

[A]: fragments are represented by same band in electropherogram;
[B]: fragment too small to visualize in electropherogram

Figure 3: Electropherogram of 16S-23S IGS amplicons of *Acb*-complex species (1A) and species-specific restriction patterns based on restriction of the 16S-23S IGS amplicons by *Mbo*II (1B)




(1A) 1: *A. baumannii* (COL 20820), 2: *A. pittii* (DSZM 9308), *3: A. nosocomialis* (ATCC 17903), 4: *A. calcoaceticus* (DSZM 1139), *5: A. calcoaceticus* (IMT30821), 6: *A. calcoaceticus* (IMT31135), M: 100 bp DNA ladder (Thermo Fisher Scientific, Germany); running conditions for electropherogram: 1.5% agarose gel, 120 V, 45 min, 1x TBE buffer

(1B) 1: *A. baumannii* (COL 20820), 2: *A. pittii* (DSZM 9308), *3: A. nosocomialis* (ATCC 17903), 4: *A. calcoaceticus* (DSZM 1139), *5: A. calcoaceticus* (IMT30821), 6: *A. calcoaceticus* (IMT31135), M: 100 bp DNA ladder (Thermo Fisher Scientific, Germany); running conditions for electropherogram: 3.5% agarose gel, 100 V, 60 minutes, 1x TAE buffer

2.2 Sequence analysis of the partial RNA polymerase beta subunit (*rpoB*)

The partial RNA polymerase subunit B (*rpoB*) sequences of a representative number of random samples of the collected *Acb*-complex isolates were amplified by PCR and analyzed using the Basic Local Alignment Search Tool (BLAST®) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [353, 354, 376] and Geneious® 6 (http://www.geneious.com) [377]. Numbers of representative samples were n=51 for *A. baumannii*, n=55 for *A. pittii*, n=45 for *A. calcoaceticus* and n=11 for *A. nosocomialis.* Six isolates which showed a unique RFLP restriction pattern were also analyzed. Results of partial *rpoB* sequence analysis are illustrated in table 21. Calculated intraspecies identities are reported in table 20. Partial *rpoB* sequences of all but one (IMT31749) investigated *A. baumannii* isolates showed the highest sequence identity to *A. baumannii* database entries by BLAST® analysis (98%-99%). Further alignment to the partial *rpoB* sequence of *A. baumannii* ACICU (accession number: NC_010611.1) resulted in pairwise identities of 98.3%-100.00% for all isolates but IMT31749, which showed a pairwise identity of 86.7%. Sequence analysis of the partial *rpoB* sequences of the selected *A. calcoaceticus* isolates however did not produce results as consistent as it was the case for the *A. baumannii* isolates. BLAST® analysis of the *A. calcoaceticus* partial *rpoB* sequences showed highest sequence identities to *A. calcoaceticus* database entries (97%-99%) for most of the investigated isolates (39/45). Further alignment of the partial *rpoB* sequences of these 39 isolates to the respective sequence of *A. calcoaceticus* strain RUH2201 (published by Gundi et al. [356]) resulted in pairwise identities of 96.2%-99.7%. Six isolates were not assigned to *A. calcoaceticus*, neither in the BLAST® analysis nor in the alignment to the partial *rpoB* sequence of strain RUH2201.

However, species assignment based on RFLP and partial *rpoB* sequencing showed a high association with the *A. pittii* isolates. BLAST® analysis of the partial *rpoB* sequences resulted in identical sequence identities (97%-99%) to *A. pittii* and *A. calcoaceticus* database entries for all investigated isolates. The partial *rpoB* sequences were thus aligned to the respective *A. pittii* BIAc11 and *A. calcocaeticus* RUH2201 sequences published by Gundi et al. [356] and allocated to the species showing the highest pairwise identity. Based on this, 54 of the 55 isolates were assigned to *A. pittii* with pairwise identities of 98.4%-100.0%. IMT31062 showed higher identities to *A. calcoaceticus* RUH2201 than to *A. pittii* BIAc11 and was therefore assigned to *A. calcoaceticus*. The partial *rpoB* sequences of eleven isolates which were identified as *A. nosocomialis* based on their RFLP pattern were also analyzed by BLAST® and

aligned to the respective sequence of *A. nosocomialis* BlAc12 (published by Gundi et al. [356]). Isolate IMT33001 showed the highest sequence identity to an *A. nosocomialis* database entry in the BLAST® analysis and a pairwise identity to *A. nosocomialis* BlAc12 of 99.4%. In contrast, the other ten isolates were assigned to other *Acinetobacter* spp. by BLAST® analysis and showed pairwise identities of 94.1%-82.7% in the alignment to strain BlAc12. Furthermore, BLAST® analysis of the partial *rpoB* sequences of the isolates showing unique RFLP restriction patterns assigned three isolates to *A. pittii*, two isolates to *A. baumannii* and one to *A. nosocomialis*.

2.3 Sequence analysis of the 16S-23S intergenic spacer (IGS)

In addition to the partial *rpoB* sequences, 16S-23S intergenic spacer (IGS) sequences were amplified by PCR and analyzed using Basic Local Alignment Search Tool (BLAST®) and Geneious® 6. Table 21 also summarizes the results obtained from 16S-23S IGS sequencing. Similar to the partial *rpoB* sequencing results, analysis of the *A. baumannii* 16S-23S IGS sequences assigned all isolates but IMT31749 to *A. baumannii* using BLAST® with sequence identities of 98%-100%. Pairwise identities in the alignments to the 16S-23S IGS sequence of *A. baumannii* ACICU ranged from 96.9% to 100.0% for all isolates but IMT31749. A slightly lower proportion of *A. calcoaceticus* isolates showed corresponding results for species assignments by RFLP and 16S-23S IGS sequencing. For 39 of the 45 investigated *A. calcoaceticus* isolates, BLAST® analysis of the 16S-23S IGS sequences resulted in highest sequence identities to *A. calcoaceticus* database entries. Moreover, pairwise identities of 96.3%-99.5% were calculated for the alignments to the 16S-23S IGS sequence of *A. calcoaceticus* DSMZ 1139. Six isolates showed highest sequence identities to other *Acinetobacter* spp. in the BLAST® analysis with pairwise identities to the 16S-23S IGS sequence of *A. calcoaceticus* DSMZ 1139 of less than 95.5%.

Of the investigated 55 *A. pittii* isolates, all but two could be assigned to *A. pittii* by means of BLAST® (identities ranging from 97%-100%). Subsequent alignment to the 16S-23S IGS sequence of *A. pittii* DSMZ 9308 resulted in pairwise identities of 96.8%-100.0%. The remaining two isolates were assigned to other *Acinetobacter* spp. with less than 92.0% pairwise identity in the alignment to *A. pittii* DSMZ 9308. Furthermore, 16S-23S IGS sequencing allocated only five of the eleven analyzed *A. nosocomialis* isolates to *A. nosocomialis* using BLAST® (99% sequence identity). For these five isolates pairwise

identities of 96.1%-96.5% were calculated in the alignment to the 16S-23S IGS sequence of *A. nosocomialis* ATCC 17903. Furthermore, four isolates showed highest identities to *A. baumannii* database entries in the BLAST® analysis and in the pairwise alignments. Analysis of the six isolates with unique RFLP restriction patterns assigned three isolates to *A. pittii*, one each to *A. nosocomialis*, *A. baumannii* and to a non *Acb*-complex *Acinetobacter* spp.

## 2.4 Comparison of species assignment based on RFLP with MboII of the 16S-23S intergenic spacer (IGS) sequence, partial rpoB sequencing and 16S-23S IGS sequencing

Of the 51 isolates which had been assigned to *A. baumannii* by means of RFLP, all but one isolate (98.04%) were also assigned to *A. baumannii* by sequencing of their partial *rpoB* and 16S-23S IGS region. IMT31749 was identified as *A. baumannii* using RFLP, as *A. genomospecies* 20 by partial *rpoB* sequencing and as *A. pittii* based by 16S-23S IGS sequencing and was thus considered as being not typeable by the applied methods. 39 of the 45 isolates allocated to *A. calcoaceticus* by RFLP were also assigned to *A. calcoaceticus* by partial *rpoB* and 16S-23S IGS sequencing (86.67%). Species assignment of the remaining six isolates did produce different results in the three applied methods and the isolates were considered as being not typeable. A proportion of 94.54% of the isolates identified as *A. pittii* by RFLP were also allocated to this species by partial *rpoB* and 16S-23S IGS sequencing. Only three of the 55 isolates were considered as being not typeable (5.45%), because analysis of the investigated genetic regions did not produce consistent results. Sequencing of the two target genes, moreover, could not confirm species identification by RFLP for the eleven isolates showing an *A. nosocomialis* species-specific restriction pattern. Since none of the assumed *A. nosocomialis* isolates produced consistent results in the applied methods, all were considered as being not typeable, as it was also the case for the six isolates displaying unique RFLP restriction patterns. Table 32 lists the respective partial *rpoB* and 16S-23S IGS sequencing results for all isolates that were considered as being not typeable.

Table 20: Intraspecies sequence identities of the partial *rpoB* and 16S-23S intergenic spacer (IGS) sequences of clinical *Acb*-complex isolates based on BLAST® analysis and reference alignments

| target gene | *Acb*-complex species based on RFLP | intraspecies identity in BLAST® analysis | reference isolate | pairwise identity to reference sequence |
|---|---|---|---|---|
| partial *rpoB* | *A. baumannii* | 98%-99 % | *A. baumannii* ACICU | 98.0%-100.0% |
| 16S-23S IGS | *A. baumannii* | 97%-100% | *A. baumannii* ACICU | 96.9%-100.0% |
| partial *rpoB* | *A. pittii* | 97%-99% | *A. pittii* BIAc11 | 98.4%-100.0% |
| 16S-23S IGS | *A. pittii* | 97%-100% | *A. pittii* DSMZ 9308 | 98.6%-100.0% |
| partial *rpoB* | *A. calcocaceticus* | 97%-99% | *A. calcoaceticus* RUH2201 | 96.5%-99.7% |
| 16S-23S IGS | *A. calcocaceticus* | 98%-99% | *A. calcoaceticus* DSMZ 1139 | 98.0%-99.0% |

Numbers of random samples for partial *rpoB* and 16S-23S sequencing: *A. baumannii* n=51, *A. pittii* n=55, *A. calcoaceticus* n=45; since there was no clinical *A. nosocomialis* isolate collected in the present study, there is no data given in the table concerning intraspecies identities of partial *rpoB* and 16S-23S IGS sequences for *A. nosocomialis*; alignments to reference isolates were done using Geneious® 6; partial *rpoB* reference sequences of *A. pittii* BIAc11 and *A. calcoaceticus* RUH2201 were published by Gundi et al. [356]; *A. baumannii* ACICU accession number: NC_010611.1

Table 21: Summary of results obtained from partial *rpoB* and 16S-23S IGS sequencing of a representative number of random samples of *Acb*-complex isolates

| species based on RFLP | number of samples | target | number of samples assigned to *A. baumannii* | number of samples assigned to *A. calcoaceticus* | number of samples assigned to *A. pittii* | number of samples assigned to *A. nosocomialis* | number of samples assigned to non *Acb*-complex species |
|---|---|---|---|---|---|---|---|
| *A. baumannii* | 51 | partial *rpoB* | 50 (98.04%) | 0 | 1 (1.96%) | 0 | 0 |
| *A. calcoaceticus* | 45 | partial *rpoB* | 0 | 39 (86.67%) | 4 (8.89%) | 0 | 2 (4.44%) |
| *A. pittii* | 55 | partial *rpoB* | 0 | 1 (1.82%) | 54 (98.18%) | 0 | 0 |
| *A. nosocomialis* | 11 | partial *rpoB* | 0 | 0 | 7 (63.64%) | 1 (9.09%) | 3 (27.27%) |
| *A. baumannii* | 51 | 16S-23S IGS | 50 (98.04%) | 0 | 1 (1.96%) | 0 | 0 |
| *A. calcoaceticus* | 45 | 16S-23S IGS | 4 (8.89%) | 39 (86.67%) | 0 | 0 | 2 (4.44%) |
| *A. pittii* | 55 | 16S-23S IGS | 1 (1.82%) | 0 | 53 (96.39%) | 0 | 1 (1.82%) |
| *A. nosocomialis* | 11 | 16S-23S IGS | 4 (36.36%) | 0 | 0 | 5 (45.45%) | 2 (18.18%) |
| *A. baumannii* | 51 | partial *rpoB* +16S-23S IGS | 50 (98.04%) | 0 | 0 | 0 | 1 [A] (1.96%) |
| *A. calcoaceticus* | 45 | partial *rpoB* +16S-23S IGS | 0 | 39 (86.67%) | 0 | 0 | 6 [A] (13.33%) |
| *A. pittii* | 55 | partial *rpoB* +16S-23S IGS | 0 | 0 | 52 (94.55%) | 0 | 3 [A] (5.45%) |
| *A. nosocomialis* | 11 | partial *rpoB* +16S-23S IGS | 0 | 0 | 0 | 0 | 11 [A] (100%) |

Collected clinical *Acb*-complex isolates were identified to species level by restriction fragment length polymorphism (RFLP) of 16S-23S intergenic spacer (IGS) amplicons by *MboII*; species identification was verified by means of partial *rpoB* and 16S-23S IGS sequencing of a representative number of random samples (n=51 for *A. baumannii*, n=45 for *A. calcocaeticus*, n=55 for *A. pittii*, n=11 for *A. nosocomialis*); [A]: isolates were moreover considered as being not typeable if species assignment by the applied methods did not produce consistent results

## 2.5 Species distribution among clinical *Acb*-complex isolates of human and animal origin

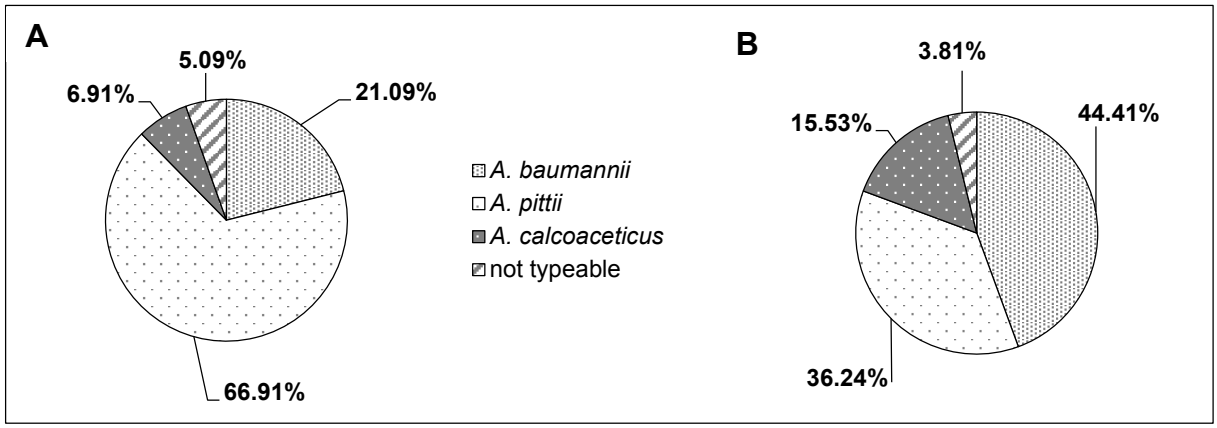
Species identification for the 642 collected *Acb*-complex isolates was performed using the 16S-23S intergenic spacer RFLP method as described (cf. II.2.1.1). *A. pittii* was the predominant *Acb*-complex species among the 275 human isolates. In difference, *A. baumannii* was the predominant *Acb*-complex species among the 367 animal isolates, while *A. pittii* constituted for a slightly smaller proportion. Furthermore, *A. calcoaceticus* accounted for proportions of only 6.91% and 15.53% in human and animal *Acb*-complex isolates (cf. table 22 and figure 4). Isolates belonging to *A. nosocomialis* could not be obtained, neither from human nor animal hosts.

Table 22: Number of collected clinical isolates belonging to the respective *Acb*-complex species

| | *A. baumannii* | *A. pittii* | *A. calcoaceticus* | *A. nosocomialis* | not typeable | total |
|---|---|---|---|---|---|---|
| human isolates | 58 (21.09%) | 184 (66.91%) | 19 (6.91%) | 0 | 14 (5.09%) | 275 |
| animal isolates | 163 (44.41%) | 133 (36.24%) | 57 (15.53%) | 0 | 14 (3.81%) | 367 |
| total | 221 | 317 | 76 | 0 | 28 | 642 |

*Acb*-complex isolates were collected from various clinical specimens within a twelve-month period starting in February 2013; species identification was performed by restriction fragment length polymorphism (RFLP) of 16S-23S IGS amplicons by *Mbo*II; isolates were considered as being not typeable, if species assignment by RFLP of the 16S-23S IGS, partial *rpoB* and 16S-23S IGS sequencing did not produce consistent results

Figure 4: Species distribution among clinical human (A) and animal (B) *Acb*-complex isolates




*Acb*-complex isolates were obtained within a twelve-month period starting in February 2013 and derived from different host species and various clincial specimens; number of human *Acb*-complex isolates: n=275; number of animal *Acb*-complex isolates: n=367; species assignment was performed by restriction fragment length polymorphism (RFLP) of 16S-23S IGS amplicons by *Mbo*II and verified by partial *rpoB* and 16S-23S IGS sequencing; isolates were considered as being not typeable if species assignment by the applied methods did not produce consistent results

## 3 Phenotypic species identification

### 3.1 Phenotyping of *Acb*-complex reference strains by Omnilog® Phenotypic MicroArray

The four *Acb*-complex reference isolates IMT30483 (*A. baumannii*), IMT30485 (*A. calcoaceticus*), IMT30487 (*A. pittii*) and IMT30488 (*A. nosocomialis*) were tested for their ability to metabolize various carbon sources. Isolates were considered variable for metabolization if they showed a large 95% confidence interval that ranged in values under and above 100 (measured intensity of dye). An isolate was considered negative for metabolization if the 95% confidence interval was located in values smaller than 100, and considered positive for metabolization if the 95% confidence interval was located in values above 100. Table 33 gives the assessment of the ability of the four reference isolates to metabolize the tested carbon sources. In case two or more reference isolates differed in their metabolic abilities, the respective substrates were considered as being possibly suitable for *Acb*-complex species discrimination. These substrates were D-saccharic acid, D-ribose, D-aspartic acid, alpha-keto-butyric acid, alpha-hydroxy-butyric acid, bromo-succinic acid, propionic acid, mucic acid, L-threonine, alanine-glycine, D- malic acid and glucuronamide (corresponding to microtiter plate wells A04, C04, D02, D07, E07, F06, F07, F08, G04, G06, G11 and H07) for Omnilog® Phenotypic MicroArray microtiter plate PM01. The following substrates were

additionally identified from microtiter plate PM2A: butyric acid, caproic acid, citraconic acid, D-citramalic acid, 4-hydroxy-benzoic acid, alpha-keto-valeric acid, D-ribono-1,4-lactone, L-hydroxyproline, L-isoleucine, L-ornithin and D, D-carnitine (corresponding to microtiter plate wells D12, E02, E03, E04, E07, E10, F07, G08, G09, H01 and H05). The 95% confidence interval plots of the respective selected substrates are shown in figure 5 for PM01 and in figure 6 for PM2A.

Table 23: Metabolic patterns of *Acb*-complex reference isolates for selected substrates of Omnilog® Phenotypic MicroArray microtiter plate PM01

| | PM01 microtiter plate wells | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| isolate | A04 | C04 | D02 | D07 | E07 | F06 | F07 | F08 | G04 | G06 | G11 | H07 |
| IMT30483 (*A. baumannii*) | + | + | - | + | + | + | + | + | - | - | + | v |
| IMT30485 (A. c*alcoaceticus*) | + | + | + | - | - | - | - | v | - | + | - | + |
| IMT30488 (*A. nosocomialis*) | - | - | - | + | + | - | - | - | + | + | - | - |
| IMT30487 (*A. pittii*) | + | + | - | - | - | + | + | + | - | + | + | - |

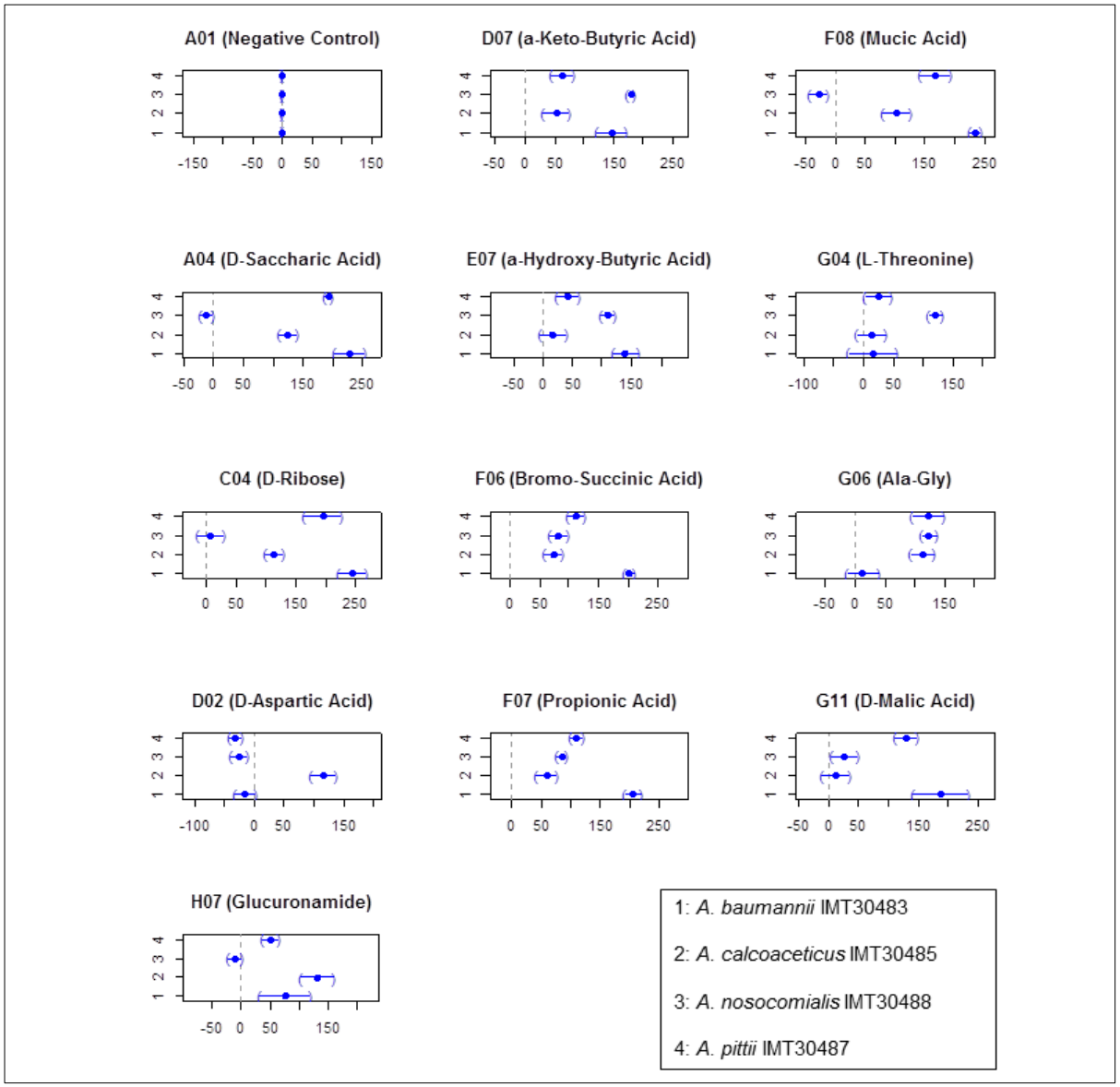
Assessment of metabolic properties according to results obtained from Omnilog® Phenotypic MicroArray for *Acb*-complex reference isolates IMT30483, IMT30485, IMT30488 and IMT30487 tested utilizing microtiter plate PM01 (48h of incubation at 37°C); substrates were selected because the tested reference isolates showed different metabolization capabilities and thus substrates might be suitable for species discrimination; listed wells represent substrates in correspondence to the PM01 microtiter plate layout for Omnilog® Phenotypic MicroArray; +: positive metabolization; -: no metabolization; v: variable metabolization

Table 24: Metabolic patterns of *Acb*-complex reference isolates for selected substrates of Omnilog® Phenotypic MicroArray microtiter plate PM2A

| | PM2A microtiter plate wells | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| isolate | D12 | E02 | E03 | E04 | E07 | E10 | F07 | G08 | G09 | H01 | H05 |
| IMT30483 (*A. baumannii*) | + | + | + | + | + | + | + | + | - | + | + |
| IMT30485 (A. c*alcoaceticus*) | + | + | - | - | + | - | - | - | - | - | - |
| IMT30488 (*A. nosocomialis*) | - | - | - | - | - | + | - | + | + | - | v |
| IMT30487 (*A. pittii*) | + | + | - | - | + | v | + | - | - | + | + |

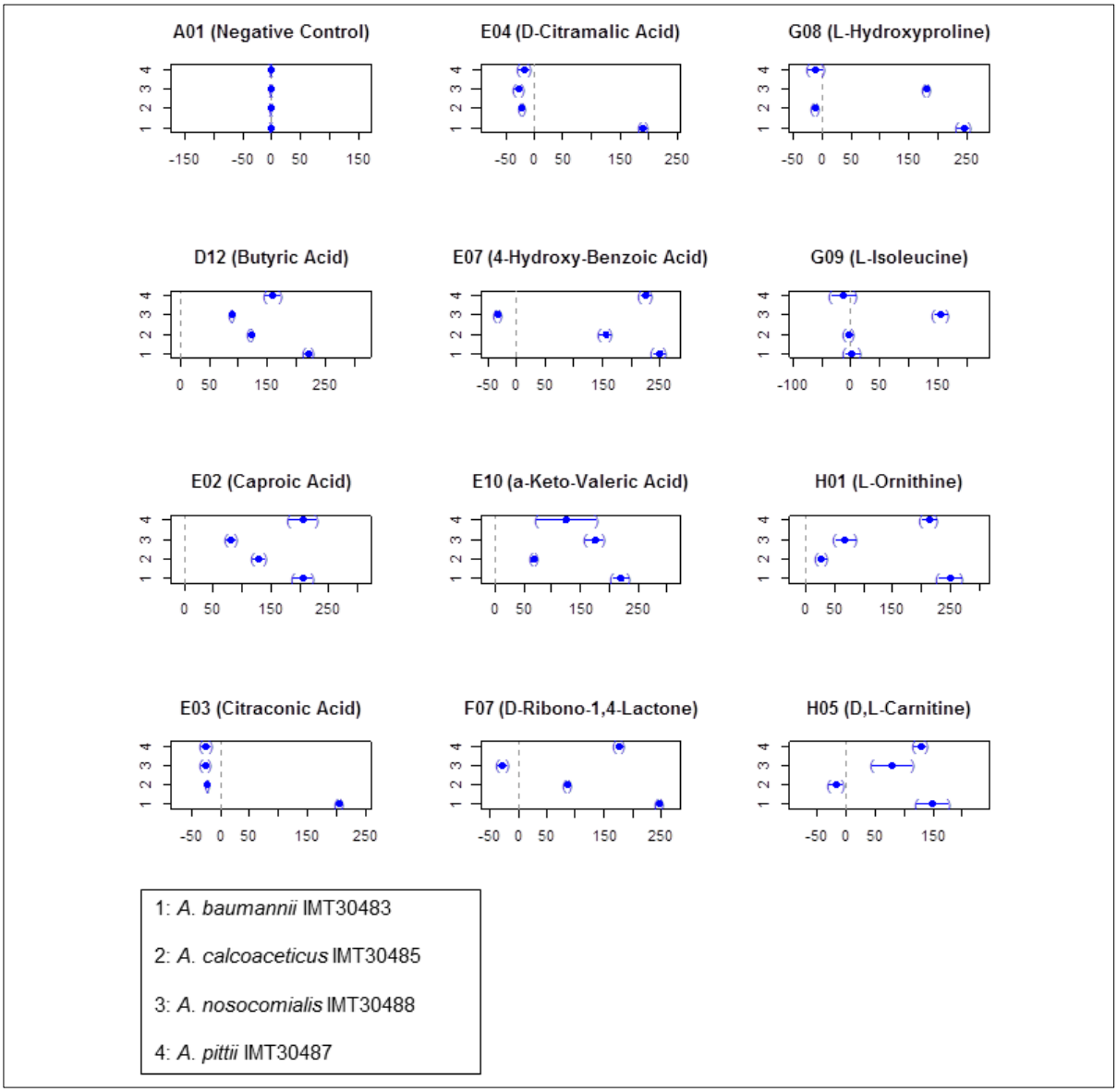
Assessment of metabolic properties according to results obtained from Omnilog® Phenotypic MicroArray for *Acb*-complex reference isolates IMT30483, IMT30485, IMT30488 and IMT30487 tested utilizing microtiter plate PM2A (48h of incubation at 37°C); specific substrates were selected due to deviations in the metabolic properties of the *Acb*-complex species, which are probably suitable for species discrimination; listed wells represent substrates in correspondence to the PM01 microtiter plate layout for Omnilog® Phenotypic MicroArray; +: positive metabolization; -: no metabolization; v: variable metabolization

Figure 5: 95% confidence interval plots for selected substrates from Omnilog® Phenotypic MicroArray microtiter plate PM01




95% confidence interval plots were generated for the four *Acb*-complex reference isolates which were investigated for their metabolic properties by Omnilog® Phenotypic MicroArray using microtiter plate PM01 for carbon sources (48h of incubation at 37°C); specific substrates were selected due to deviations in the metabolic properties of the *Acb*-complex species, which are probably suitable for species discrimination; assessment of metabolic properties is based on 95% confidence intervals which represent positive metabolization when located above the threshold value of 100, no metabolization when located under the threshold value of 100, and variable metabolization when located around the threshold value of 100.

Figure 6: 95% confidence interval plots for selected substrates from Omnilog® Phenotypic MicroArray microtiter plate PM2A




95% confidence interval plots were generated for the four *Acb*-complex reference isolates, which were investigated for their metabolic properties by Omnilog® Phenotypic Microarray using microtiter plate PM2A for carbon sources (48h of incubation at 37°C); specific substrates were selected due to deviations in the metabolic properties of the *Acb*-complex species, which are probably suitable for species discrimination; assessment of suitability is based on 95% confidence intervals which represent positive metabolization when located above the threshold value of 100, no metabolization when located under the threshold value of 100 and variable metabolization when located around the threshold value of 100

## 3.2 Phenotypic species identification of clinical and reference *Acb*-complex isolates utilizing the *Acinetobacter* test medium

Four clinical *A. baumannii*, three clinical *A. pittii* and two clinical *A. calcoaceticus* isolates were investigated for their ability to metabolize D-ribose (C04, PM01), D-malic acid (G11, PM01), citraconic acid (E03, PM2A), L-hydroxyproline (G08, PM2A) and L-ornithine (H01, PM2A). Clinical isolates were randomly chosen and numbers of random samples correspond to the clinical importance of the respective *Acb*-complex species. The four reference isolates IMT30483, IMT30487, IMT30485 and IMT30488 were also included in the experiments. Table 25 summarizes the different metabolic properties of the four reference isolates according to the Omnilog® Phenotypic MicroArray results. The investigated clinical *Acb*-complex isolates are expected to behave in correspondence to the reference isolate of the respective species.

Table 25: Different metabolic properties of *Acb*-complex reference isolates for selected substrates suitable for species discrimination

| | selected substrates | | | | |
|---|---|---|---|---|---|
| *Acb*- complex species | D-ribose | D-malic acid | citraconic acid | L-hydroxyproline | L-ornithine |
| *A. baumannii* (IMT30483) | + | + | + | + | + |
| *A. calcoaceticus* (IMT30485) | + | - | - | - | - |
| *A. nosocomialis* (IMT30488) | + | + | - | + | - |
| *A. pittii* (IMT30487) | - | + | - | - | + |

Substrates were selected based on 95% confidence interval plots generated for *Acb*-complex reference isolates IMT30483, IMT30485, IMT30488 and IMT30487 for all substrates tested by Omnilog® Phenotypic MicroArray microtiter plates PM01 and PM2A; species identification can be achieved by combined testing of the metabolic properties of the respective isolates for the selected substrates; +: positive metabolization, -: no metabolization

All isolates were tested by the use of two different indicators (TTC and phenolred) and incubated for 24 hours and 48 hours. Incubation for 48 hours showed the same results as it was the case for incubation for 24 hours. TTC indicated no metabolization for D-malic acid and citraconic acid but phenol red showed a positive reaction (color change from red to yellow) for

all isolates. The following results derive from 24h incubation with phenol red as indicator. Table 34 provides an overview of the metabolic properties as determined utilizing the *Acinetobacter* test medium. In general, the obtained results were not consistent with the results obtained from Omnilog® Phenotypic MicroArray for the four *Acb*-complex reference isolates and also differed among isolates of the same bacterial species. For example, all investigated isolates were tested positive for D-malic acid and citraconic acid metabolization, although *A. calcoaceticus* and *A. pittii* isolates were expected to be negative (cf. table 34). *A. nosocomialis* should not be able to metabolize citraconic but was tested positive. Furthermore, four of the five *A. baumannii* isolates metabolized L-hydroxyproline and two of three *A. calcoaceticus* isolates were tested negative for L-hydroxyproline and L-ornithine, while one was tested positive. All four *A. pittii* isolates were moreover tested positive for D-ribose metabolization, although they were expected to be negative.

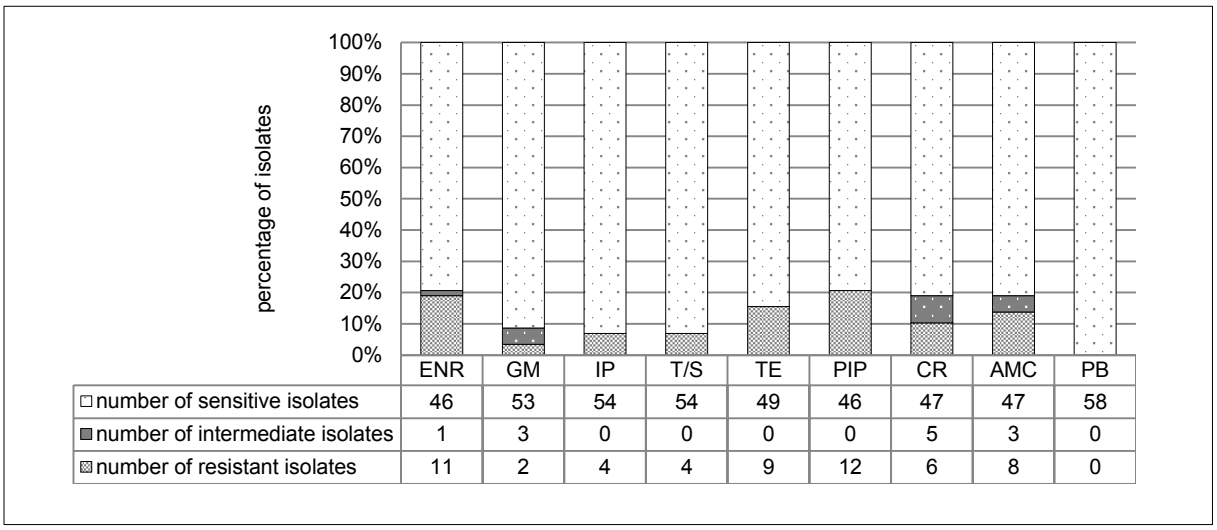
## II Analysis of human and animal clinical *Acb*-complex isolates

### 1 Antimicrobial susceptibility of human and animal *A. baumannii* isolates

All collected 221 *A. baumannii* isolates (human isolates: n=58; animal isolates: n=163) were investigated for their susceptibility against several classes of antimicrobials. Resistance rates for human isolates against human antimicrobials (tested utilizing the VITEK®2 AST-N263 panel) were as follows: ceftazidime (CAZ) 13.79%, cefotaxime (CTX) 15.52%, ciprofloxacin (CIP) 18.97%, gentamicin (GM) 3.45%, imipenem (IP) 6.90%, trimethoprim/sulfamethoxazole (T/S) 6.09% and ampicillin/sulbactam (AMPS) 3.45%. Furthermore, the following resistance rates were determined for the animal *A. baumannii* isolates (tested utilizing the VITEK®2 AST-GN38 panel): cefpirome (CR) 11.66%, enrofloxacin (ENR) 46.63%, GM 51.53%, tetracycline (TE) 46.63%, IP 3.07%, T/S 32.52%, amoxicillin/clavulanic acid (AMC) 34.36% and piperacillin (PIP) 42.94%. Only the canine isolate IMT31959 was resistant against polymyxin B (PB). Resistance rates of human *A. baumannii* isolates against veterinary antimicrobials (tested also utilizing the VITEK®2 AST-GN38 panel) were: CR 10.34%, ENR 18.97%, GM 3.45%, TE 15.52%, IP 6.90%, T/S 6.90%, AMC 13.79% and PIP 20.69%. All human isolates were susceptible against polymyxin B. All human and animal isolates were resistant against ceftiofur with MIC values of ≥ 8 µg/ml. Numbers of resistant, intermediate and sensitive isolates for the respective antimicrobials can be found in figure 7 and figure 9 for human isolates and in figure

8 for animal isolates. An isolate was considered as being multi-drug resistant if it exhibited resistances against three or more classes of antimicrobials [361]. A higher proportion of animal *A. baumannii* (50.92%) isolates exhibited multi-drug resistance compared to human *A. baumannii* isolates (15.52%) (cf. figure 10). Ten isolates were furthermore considered as being extensively drug resistant, corresponding to 4.91% of the animal isolates (8/163) and 3.45% of the human isolates (2/58). The term extensively drug-resistant was used according to Magiorakos et al. [109] for isolates that were susceptible against two or less of the following antimicrobials: ENR, GM, IP, TE, T/S, CR, AMC, PIP and PB. Resistance profiles of the animal XDR *A. baumannii* isolates are listed in table 35.

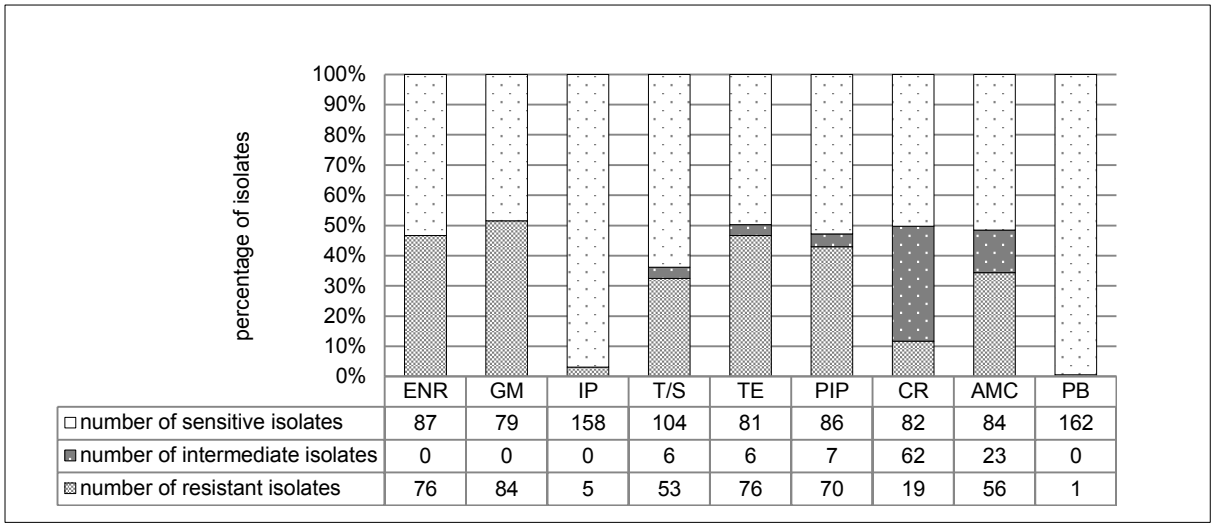
Figure 7: Antimicrobial resistances in clinical human *A. baumannii* isolates using the VITEK®2 panel for veterinary antimicrobials




| | ENR | GM | IP | T/S | TE | PIP | CR | AMC | PB |
|---|---|---|---|---|---|---|---|---|---|
| number of sensitive isolates | 46 | 53 | 54 | 54 | 49 | 46 | 47 | 47 | 58 |
| number of intermediate isolates | 1 | 3 | 0 | 0 | 0 | 0 | 5 | 3 | 0 |
| number of resistant isolates | 11 | 2 | 4 | 4 | 9 | 12 | 6 | 8 | 0 |

Clinical human *A. baumannii* isolates (n=58) derive from various specimens and have been tested for their resistance pattern using the VITEK®2 system (BioMeriéux, France) by means of the VITEK®2 antimicrobial susceptibility panel for Gram-negative bacteria (AST-GN38, developed for veterinary use); assessment of resistance was made according to the breakpoints given in the CLSI guidelines M100-S26 and VET01S2 for *Acinetobacter* spp.; ENR: enrofloxacin, GM: gentamicin, IP: imipenem, T/S: trimethoprim/sulfamethoxazole, TE: tetracycline, CR: cefpirome, AMC: amoxicillin/clavulanic acid, PB: polymyxin B

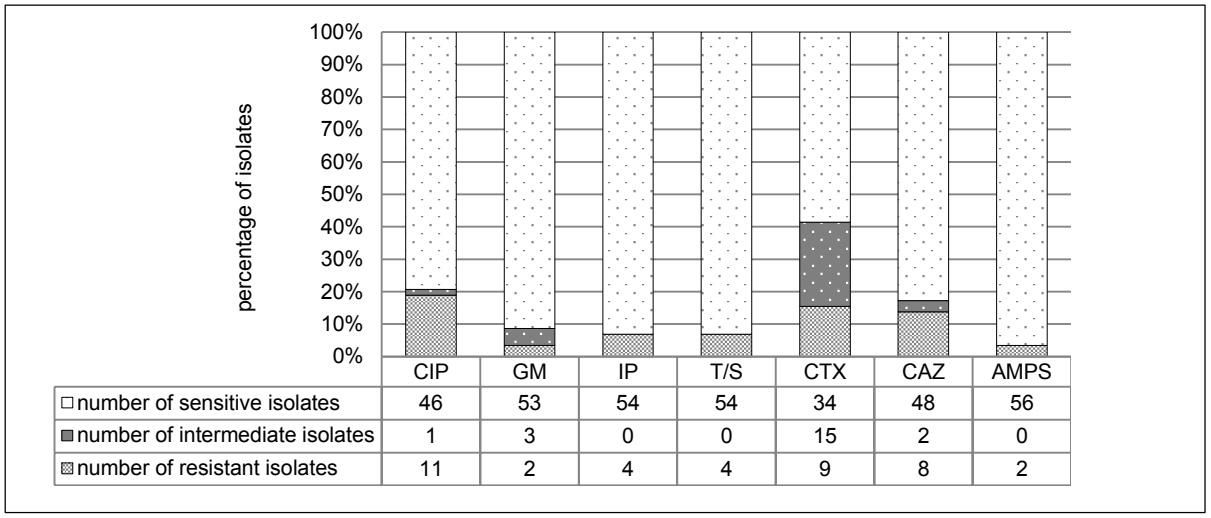
Figure 8: Antimicrobial resistances in clinical animal *A. baumannii* isolates using the VITEK®2 panel for veterinary antimicrobials




| | ENR | GM | IP | T/S | TE | PIP | CR | AMC | PB |
|---|---|---|---|---|---|---|---|---|---|
| □ number of sensitive isolates | 87 | 79 | 158 | 104 | 81 | 86 | 82 | 84 | 162 |
| ▩ number of intermediate isolates | 0 | 0 | 0 | 6 | 6 | 7 | 62 | 23 | 0 |
| ▨ number of resistant isolates | 76 | 84 | 5 | 53 | 76 | 70 | 19 | 56 | 1 |

Clinical animal *A. baumannii* isolates (n=163) derive from various host species and specimens and have been tested for their resistance pattern using the VITEK®2 system (BioMeriéux, France) by means of the VITEK®2 antimicrobial susceptibility panel for Gram-negative bacteria (AST-GN38, developed for veterinary use); assessment of resistance was made according to the breakpoints given in the CLSI guidelines M100-S26 and VET01S2 for *Acinetobacter* spp.; ENR: enrofloxacin, GM: gentamicin, IP: imipenem, T/S: trimethoprim/sulfamethoxazole, TE: tetracycline, CR: cefpirome, AMC: amoxicillin/clavulanic acid, PB: polymyxin B

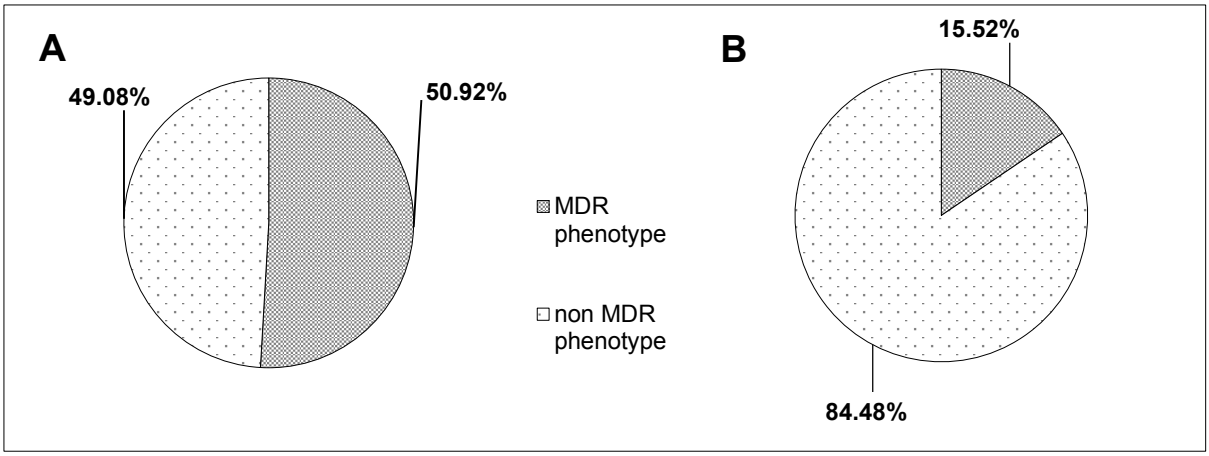
Figure 9: Antimicrobial resistances in clinical human *A. baumannii* isolates using the VITEK®2 AST-N263 panel for human antimicrobials




| | CIP | GM | IP | T/S | CTX | CAZ | AMPS |
|---|---|---|---|---|---|---|---|
| □ number of sensitive isolates | 46 | 53 | 54 | 54 | 34 | 48 | 56 |
| ▩ number of intermediate isolates | 1 | 3 | 0 | 0 | 15 | 2 | 0 |
| ▨ number of resistant isolates | 11 | 2 | 4 | 4 | 9 | 8 | 2 |

Clinical human *A. baumannii* isolates (n=58) derive from various specimens and have been tested for their resistance pattern using the VITEK®2 system (BioMeriéux, France) by means of the VITEK®2 antimicrobial susceptibility panel for Gram-negative bacteria (AST-N263, developed for use in human medicine); assessment of resistance was made according to the breakpoints given in the CLSI guidelines M100-S26 for *Acinetobacter* spp.; CIP: ciprofloxacin, GM: gentamicin, IP: imipenem, T/S. trimethoprim/Sulfamethoxazole, CTX: cefotaxime, CAZ: ceftazidime, AMPS: ampicillin/sulbactam

Figure 10: Proportion of animal (A) and human (B) *A. baumannii* isolates exhibiting a multi-drug resistant (MDR) and non multi-drug resistant (non MDR) phenotype



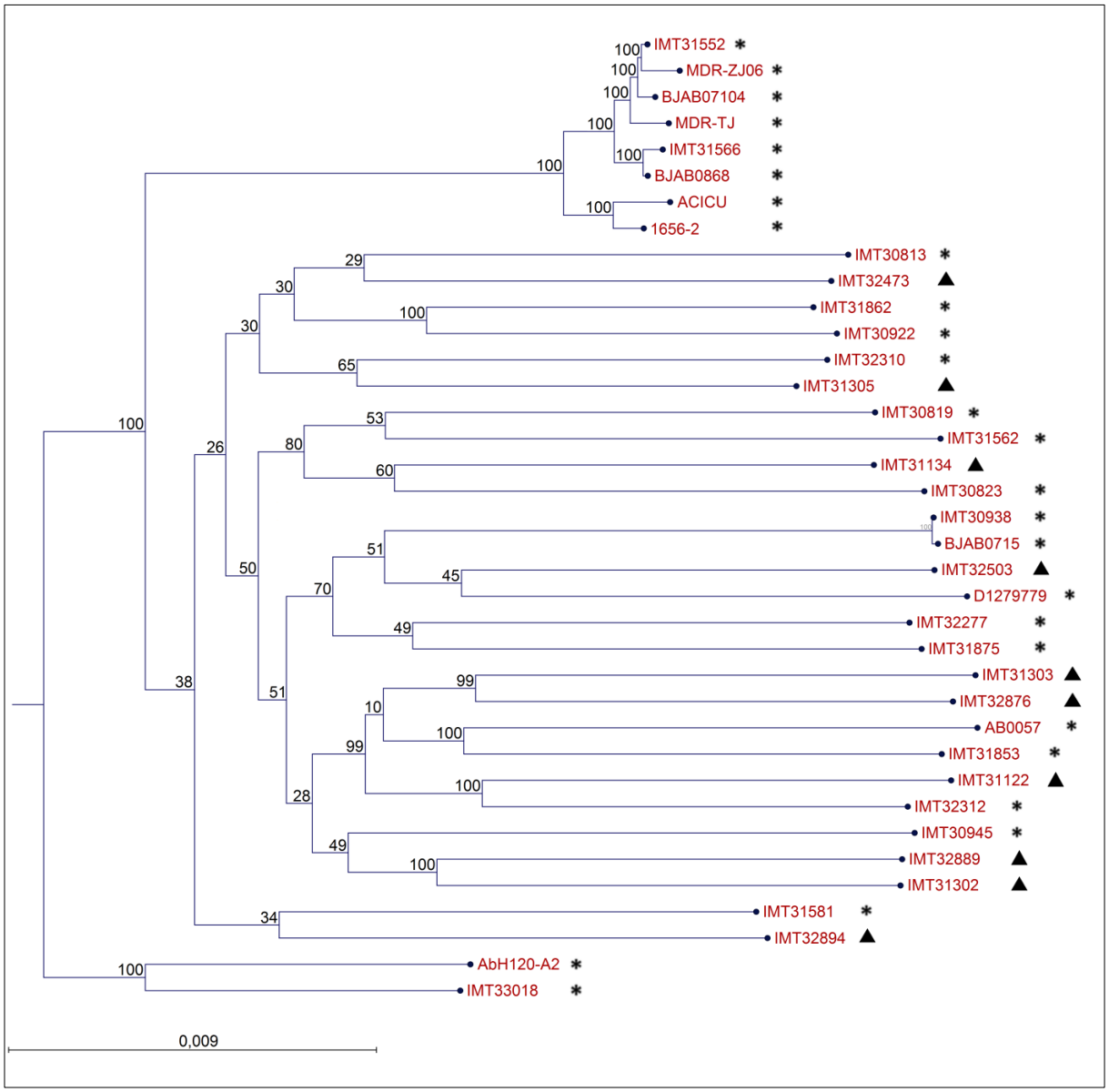

Antimicrobial susceptibility testing was performed for human (n=58) and animal (n=163) clinical *A. baumannii* isolates using the VITEK®2 system (BioMeriéux, France) by means of the VITEK®2 antimicrobial susceptibility panel for Gram-negative bacteria (AST-GN38); assessment of resistance was made according to the breakpoints of the CLSI guidelines M100-S26 and VET01S2 for *Acinetobacter* spp. for enrofloxacin, gentamicin, imipenem, trimethoprim/sulfamethoxazole, tetracycline, cefpirome, amoxicillin/clavulanic acid and polymyxin B; isolates were considered as being multi-drug resistant when they exhibited resistances against ≥ 3 tested antimicrobial classes

## 2 Genomic diversity of human and animal *A. baumannii* isolates

For analysis of the genomic diversity of human and animal *A. baumannii* isolates, 2506 orthologous genes were identified as being present in all investigated isolates (n=37). These genes represent the Maximum Common Genome (MCG) with a length of 2.065.761 bp. The diversity of the investigated isolates is illustrated in a maximum likelihood tree (figure 11). This tree shows a separate cluster for isolates belonging to the Pasteur MLST ST2, consisting of seven published *A. baumannii* genomes (NCGM 237, ACICU, 1656-2, BJAB0868, BJAB07104, MDR-Z J06 and MDR-TJ) and two clinical human *A. baumannii* isolates (IMT31552 and IMT31566). No clustering was observed for the other investigated isolates with an average distance to the next closest isolate of 29915 SNPs based on the calculated distance matrix. There were no separate clusters for human and animal isolates. Three human isolates showed the smallest number of SNPs and hence shortest distance to animal isolates (11.11%), whereas five of ten animal isolates (50.0%) showed the shortest distance to human isolates. The average number of SNPs (total number of SNPs to respective closest isolate divided by number of isolates) within the ST2 isolates was 1989. In contrast, the average number of SNPs within human non ST2 isolates was 28542 and within the animal isolates

33259. The lower average number of SNPs within the human isolates results from the close relatedness IMT30938 and BJAB0715 (410 SNPs). By excluding these two closely related isolates, the average SNP value within the human non ST2 isolates rises to 31852. The average number of SNPs between the non ST2 human isolates and their respective next closest animal isolate was 33546. Thus, the average SNP numbers within human non ST2 isolates, animal isolates and between human non ST2 and their next closest animal isolate are comparable. The distance matrix results and MLST sequence types are illustrated in table 36. Of the 28 investigated clinical *A. baumannii* isolates, 18 could not be assigned to any sequence type by means of the Pasteur MLST or Oxford MLST scheme and thus represent unknown sequence types.

Figure 11: Genomic diversity of *A. baumannii* isolates of human and animal origin (n=37) based on whole genome analysis, utilizing the Maximum Common Genome




Maximum likelihood tree based on the alignment of the Maximum Common Genome of *A. baumannii* isolates of human (n=27) and animal origin (n=10); number of orthologous genes: n=2506; number of aligned base pairs: 2.065.761; *: human *A. baumannii* isolates; ▲: animal *A. baumannii* isolates

## III Investigation of fluoroquinolone resistance in *A. baumannii*

### 1 Comparative functional analysis of enrofloxacin (ENR) sensitive wild-type and derived resistant mutant isolates

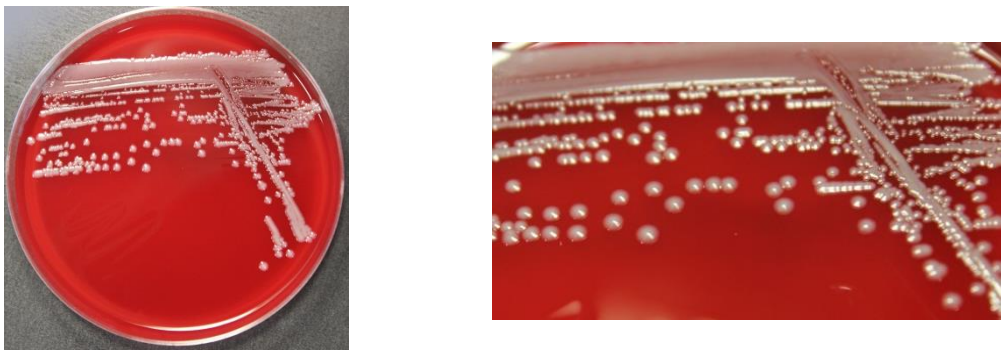
#### 1.1 Culture- and cell morphology

Cultivation of the ENR sensitive *A. baumannii* isolates IMT31302, IMT31303 and IMT31305 with subinhibitory ENR concentrations by gradient plates resulted in 26 spontaneous ENR resistant mutants. All obtained mutants and their respective wild-type isolates are listed in table 26. The three wild-type isolates grew as greyish, dampish colonies of medium size on COL S+ agar plates within 16h of incubation and showed Gram-negative coccoid cells. In comparison to the wild-type isolates, the 26 ENR resistant mutants grew more slowly (16h to 48h) and formed smaller colonies but preserved color and smell. The mutants ENRres2, ENRres13, ENRres14, ENRres15, ENRres20, ENRres22, ENRres25 and ENRres26 formed slightly pleomorphic colonies on COL S+ agar plates showing smaller and larger variants but had a homogeneous coccoid cell morphology and Gram-staining behavior (Gram-negative). The mutants ENRres4, ENRres7, ENRres10, ENRres11, ENRres12, ENRres16, ENRres17, ENRres18, ENRres19 and ENRres21 also showed pleomorphic colonies but the smaller and larger variants could be divided into two distinct lineages, showing the respective colony morphology in three consecutive subcultures. The designations of the distinct lineages were maintained but the additive I was added for the larger and the additive II for the smaller colony variants. The remaining eight ENR resistant mutants showed a homogeneous colony and cell morphology displaying typical *A. baumannii* features. Figure 12 shows the typical colony morphology by example of *A. baumannii* IMT31106.

Table 26: Obtained spontaneous enrofloxacin (ENR) resistant mutant isolates and their respective porcine *A. baumannii* wild-type isolates

| wild-type isolate | derived ENR resistant mutant isolates | |
|---|---|---|
| | selected for further analysis | not selected for further analysis |
| IMT31302 | ENRres1, ENRres2, ENRres3 | ENRres12, ENRres13 |
| IMT31303 | ENRres4, ENRres5, ENRres6, ENRres7 | ENRres14, ENRres15, ENRres16, ENRres17 |
| IMT31305 | ENRres8, ENRres9, ENRres10, ENRres11 | ENRres18, ENRres19, ENRres20, ENRres21, ENRres22, ENRres23, ENRres24, ENRres25, ENRres26 |

Figure 12: *A. baumannii* IMT31106 on a COL S+ agar plate displaying typical colony morphology



Incubation of clinical human *A. baumannii* isolate IMT31106 at 37°C for 16h

## 1.2 Antimicrobial susceptibility patterns of enrofloxacin (ENR) sensitive *A. baumannii* wild-type and resistant mutant isolates

Antimicrobial susceptibility testing was performed for the selected mutants ENRres1, ENRres2, ENRres3, ENRres4 I and II, ENRres5, ENRres6, ENRres7 I and II, ENRres8, ENRres9, ENRres10 I and II, and ENRres11 I and II (n=15) using the Epsilometer test method Etest (BioMérieux, France). The mean minimum inhibitory concentration (MIC) values, which have been calculated based on three biological replicates, are listed in table 37. All mutants but ENRres3 and ENRres11 I were tested resistant against enrofloxacin, while the MIC for the three wild-type isolates was < 0.1 µg/ml (susceptible). The enrofloxacin MIC values of nine

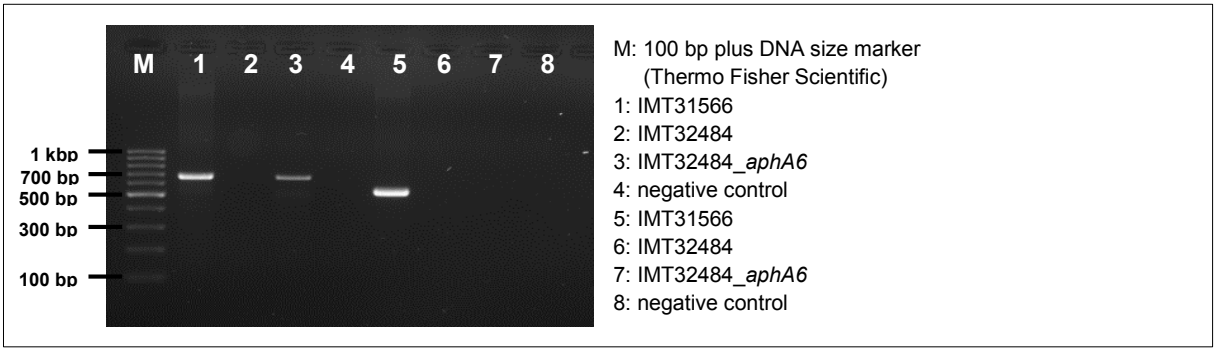
mutants were more than 4-fold higher than the CLSI breakpoint for resistance (cf. table 11). MIC values for ampicillin (AMP) increased more than 2-fold for seven of the mutants compared to their wild-type isolates (4-fold for ENRres4 I and ENRres7 I; 8-fold for ENRres4 II). Since there are no breakpoints given for AMP for *Acinetobacter* isolates, possible susceptibility changes cannot be assessed. Highest increases of piperacillin (PIP) MIC values were observed for ENRres4 I and II and ENRres7 I. Although the wild-type isolates were already resistant against cefpodoxime (PX), the MIC values of all mutants of IMT31303 increased from 8.00 µg/ml to 48.00 µg/ml for ENRres6, 144.00 µg/ml for ENRres7 II and to ≥ 256.00 µg/ml for ENRres4 I and II, ENRres5 and ENRres7 I and also for the IMT31305 derived mutant ENRres8. The MIC values for trimethoprim/sulfamethoxazole (T/S) also increased for six of the mutants, resulting in a change from susceptible to resistant. MIC values for cefpirome (CR) also increased but were within the range of susceptibility (≤ 8 µg/ml). MIC values for rifampicin (RI), tetracycline (TE), gentamicin (GM), imipenem (IP) and colistin (CO) remained almost unchanged or decreased for the mutants compared to their wild-type isolates. Due to the resistance against PX and the increased MIC values for ENR and T/S, the five mutants ENRres1, ENRres2, ENRres7 II, ENRres9 and ENRres11 II developed a multi-drug resistant phenotype (resistant ≥ 3 antimicrobial classes [361]). Since there are no breakpoints given for AMP, it remains unclear if the AMP MIC increase of ENRres4 I and II, ENRres5 and ENRres7 I would also account for a multi-drug resistant phenotype in these mutants.

### 1.3 Comparison of plasmid acquisition of enrofloxacin (ENR) sensitive *A. baumannii* wild-type and resistant mutant isolates

Screening of clinical *A. baumannii* isolates for presence of aminoglycoside resistance genes, which are suitable as marker genes for conjugation, revealed that the human isolate IMT31566 is positive for *aphA6* and *armA*. Subsequently, the putative IncF plasmid pAB31566 was predicted. PAB31566 carries *aphA6*, has an approximate size of 71kbp and should be transferable between *Acinetobacter* species [367]. Genes located on pAB31566 are listed in table 38. In conjugation experiment A pAB31566 was supposed to be transferred from IMT31566 to *A. haemolyticus* IMT32484. After 24h of incubation haemolytic transconjugant colonies grew on selective COL S+ agar plates supplemented with 100 µg/ml kanamycin. An *aphA6* amplicon could be obtained by PCR for the transconjugants but not for IMT32484 (figure 13). Transconjugant colony 1 was afterwards named IMT32484_*aphA6*. *ArmA* could

not be amplified, neither for IMT32484 nor for IMT32484_*aphA6*, confirming that only *aphA6* had been transferred.

Figure 13: Electropherogram of PCR amplicons of *aphA6* and *armA* for *A. baumannii* IMT31566, *A. haemolyticus* IMT32484 and its transconjugant IMT32484_*aphA6*

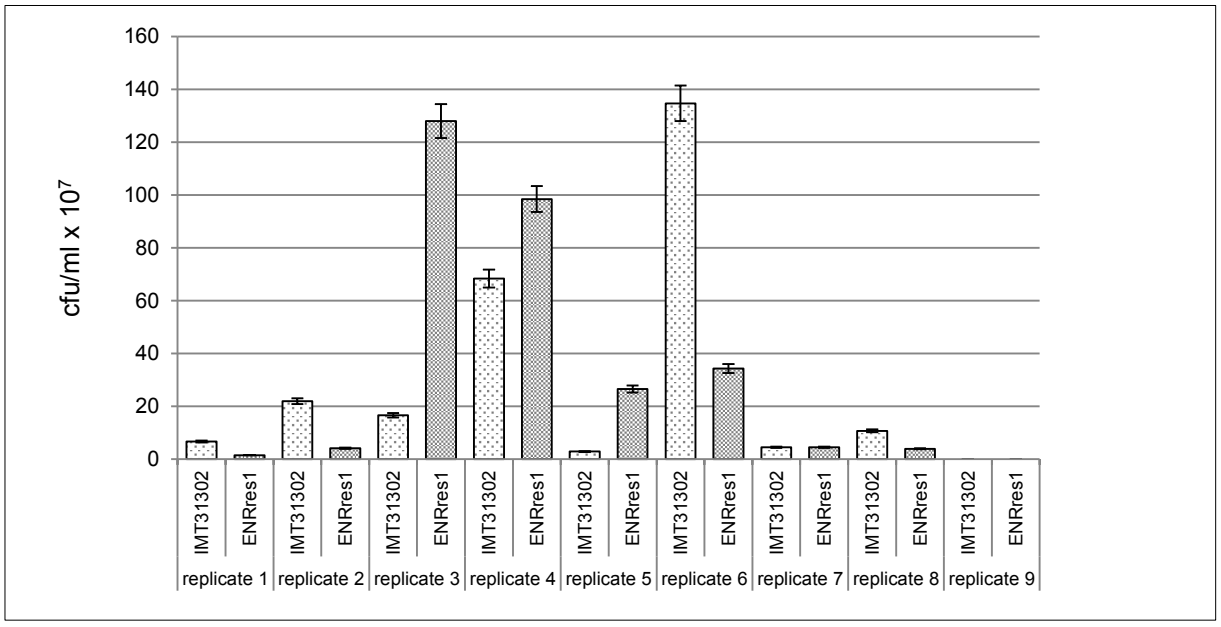



1-4: electropherogram of *aphA6* amplicons, 5-8: electropherogram of *armA* amplicons; transconjugant IMT32484_*aphA6* was achieved by transfer of putative plasmid pAB31566 from *A. baumannii* IMT31566 to *A. haemolyticus* IMT32484 by conjugation; running conditions for electropherogram: 1.5% agarose gel, 120 V, 45 min, 1x TBE buffer

IMT32484_*aphA6* was subsequently used as donor isolate in conjugation experiments B. For IMT31305 and its mutant ENRres9 no transconjugant colonies grew in any of the three biological replicates. Transconjugant colonies however were obtained for the two other wild-type/mutant pairs IMT31302/ENRres1 and IMT31303/ENRres6. For replicates 1-3 and for each of these four isolates, ten transconjugant colonies were randomly selected and subcultivated on COL S+ agar plates supplemented with 100 µg/ml kanamycin. Transconjugants showed the *A. baumannii* species-specific RFLP 16S-23S IGS restriction pattern (cf. figure 3), whereas donor colonies did not. Successful plasmid transfer was furthermore confirmed by means of *aphA6* PCR, which was also performed for the selected transconjugant colonies. *AphA6* amplicons were obtained for all samples. Electropherograms for RFLP and *aphA6* PCR of transconjugant colonies are given in figures 19 and 20. Additionally, the colony forming units cfu/ml were calculated and compared between the respective wild-type isolate and its mutant. The cfu/ml of IMT31302 were 4.41-fold and 5.32-fold higher than the cfu/ml of ENRres1 in the first two replicates. Accordingly, the cfu/ml of IMT31303 were 2.86-fold and 4.21-fold higher than the cfu/ml of ENRres6. In the third replicate IMT31303 still had a 2.42-fold higher cfu/ml value than its mutant but the relation changed for IMT31302 and ENRres1. The cfu/ml value of ENRres1 increased to 128.05 cfu/ml, which is

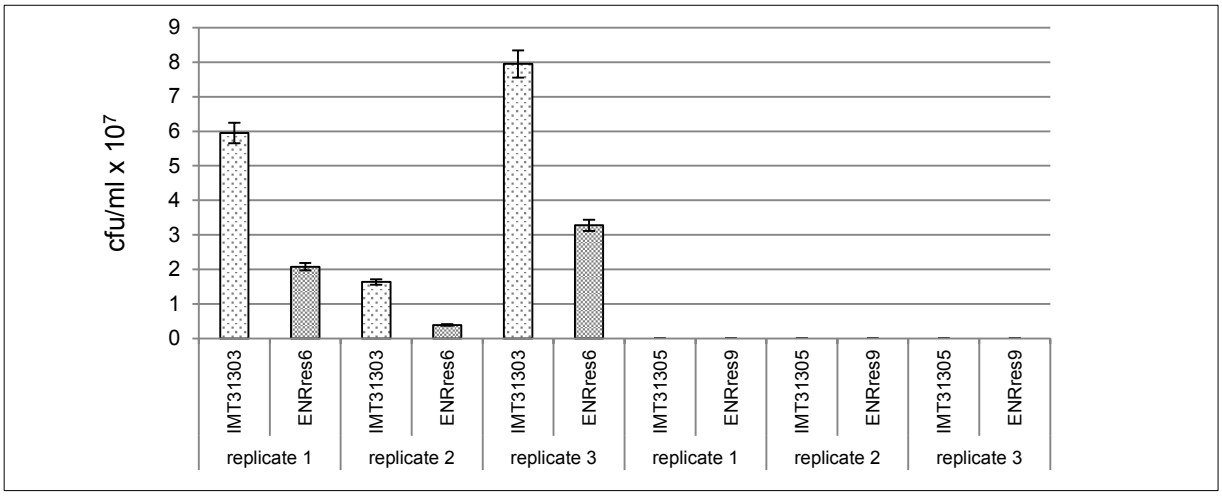
7.72-fold higher than the cfu/ml value of IMT31302. In order to assess the stability of the conjugation assay, six more replicates were performed for IMT31302/ ENRres1. The cfu/ml values varied also in these replicates. In replicates four and five, ENRres1 showed higher values than IMT31302, whereas IMT31302 had higher values in replicates six and eight. The cfu/ml values of both isolates were approximately the same in replicate seven. Moreover, no transconjugant colonies could be achieved in replicate nine. Figure 14 illustrates the cfu/ml values for IMT31302/ENRres1 and figure 15 for the other two wild-type/mutant pairs. Table 39 lists the calculated cfu/ml values.

Figure 14: Calculated colony forming units (cfu)/ml for transconjugants of *A. baumannii* IMT31302 and its enrofloxacin (ENR) resistant mutant ENRres1




Kanamycin resistant transconjugant colonies were achieved by conjugational transfer of the putative plasmid pAB31566 from the donor *A. haemolyticus* IMT32484_*aphA6* to the *A. baumannii* recipient isolates IMT31302 and its spontaneous enrofloxacin resistant mutant ENRres1; conjugation experiments were performed in nine biological replicates and cfu/ml were calculated for transconjugants of each recipient isolate and replicate

Figure 15: Calculated colony forming units (cfu)/ml for transconjugants of *A. baumannii* isolates IMT31303 and IMT31305 and their enrofloxacin (ENR) resistant mutants ENRres6 and ENRres9

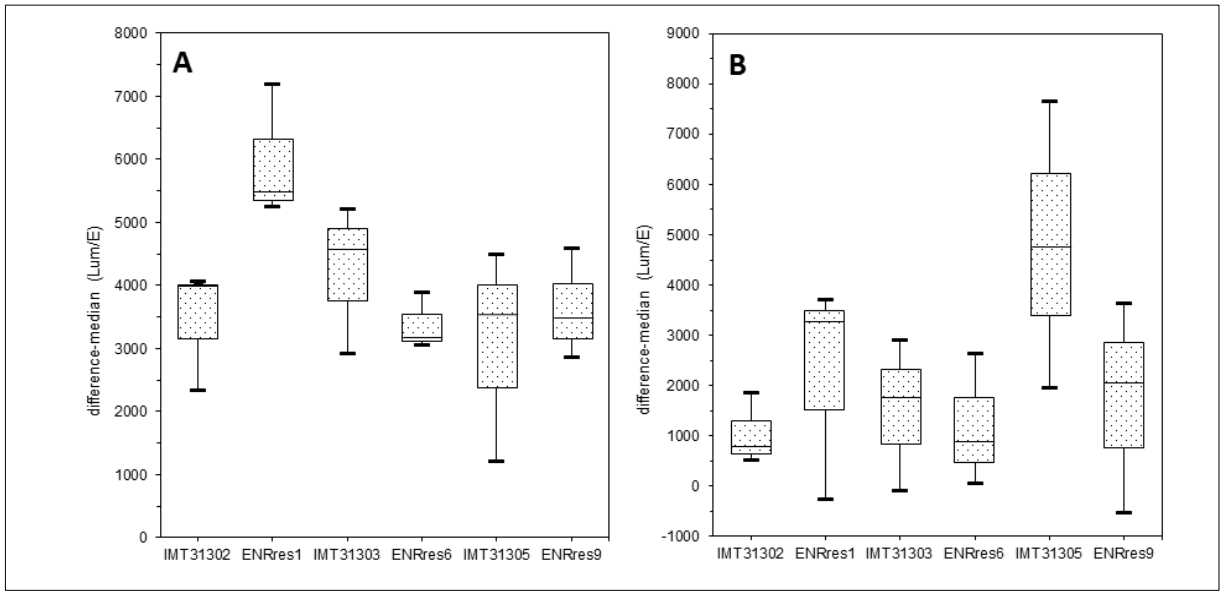



Kanamycin resistant transconjugant colonies were achieved by conjugational transfer of the putative plasmid pAB31566 from the donor isolate *A. haemolyticus* IMT32484_*aphA6* to the *A. baumannii* recipient isolates IMT31303 and IMT31305 and their respective spontaneous enrofloxacin resistant mutants ENRres6 and ENRres9; conjugation experiments were performed in three biological replicates and cfu/ml were calculated for transconjugants of each recipient isolate and replicate

## 1.4 NF-ĸB activation in 3D4/31 and THP-1 cells due to infection with enrofloxacin (ENR) sensitive *A. baumannii* wild-type and resistant mutant isolates

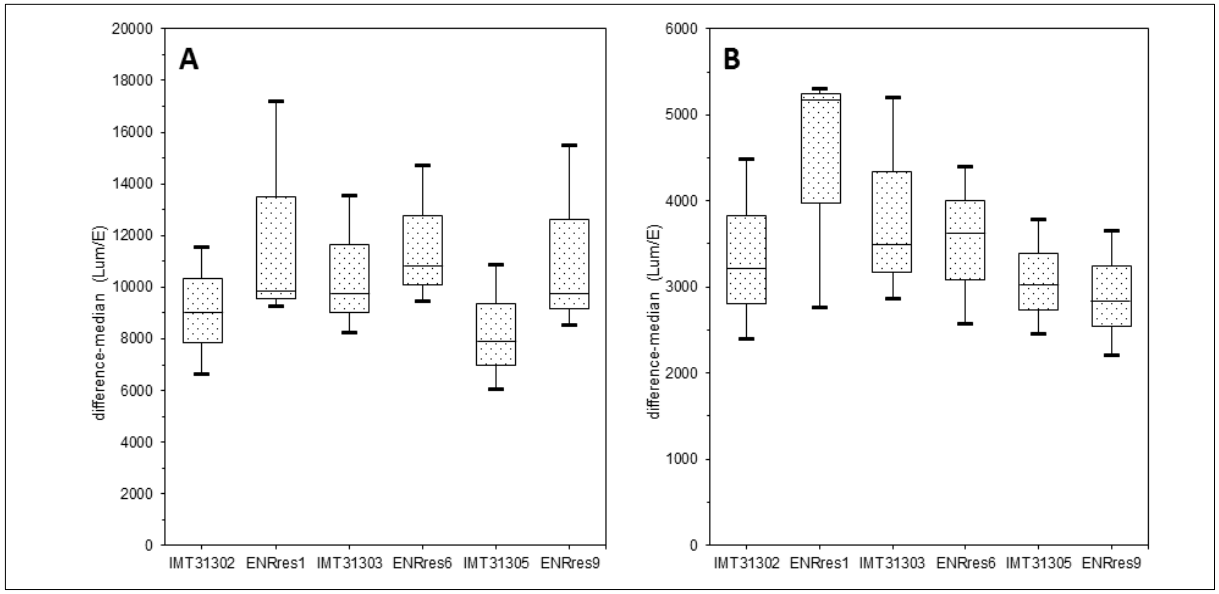
The porcine cell line 3D4/31 and the human cell line THP-1 were infected with IMT31302 and its mutant ENRres1, IMT31303 and its mutant ENRres6 as well as IMT31305 and its mutant ENRres9. The variable difference-median was generated, reflecting the level of induction of NF-ĸB expression. The pairwise comparison (Tukey test) displayed a significant difference (p-values ≤ 0.1) only for ENRres1 and IMT31305 (p=0.064) for 3D4/31 cells at 7 h p.i.. The p-value for the pairwise comparison of ENRres1 and its wild-type isolate IMT31302 for 3D4/31 cells at 7h p.i. was p=0.128, which is close to the level of significance. Comparison of the variable difference-median of ENRres1 to ENRres6 and ENRres9 resulted in p-values of p=0.108 and p=0.174. The generated boxplots for the investigated isolates showed furthermore no overlap for ENRres6 to IMT31302, IMT31305, ENRres1 and ENRres9 at 7 h p.i. after infection of 3D4/31cells. No significant differences could be detected in the pairwise comparison of the six investigated isolates for 3D4/31 cells at 19 h p.i., nor for THP-1 cells at 7 h p.i. and 19h p.i.. Calculated p-values are listed in table 40. Generated boxplots are shown in figures 16 and 17.

Figure 16: Results of NF-KB reporter assay for porcine 3D4/31 cells infected with *A. baumannii*




Boxplots were generated for the variable difference-median (Lum/E of infected cells minus Lum/E of uninfected cells) for porcine 3D4/31 cells measured 7h (A) and 19h (B) post infection with porcine *A. baumannii* isolates IMT31302, IMT31303, IMT31305 and their respective spontaneous enrofloxacin (ENR) resistant mutants ENRres1, ENRres6 and ENRres9; Lum/E values reflect measured luciferase activity of *luc* reporter cells, what is proportional to the level of NF-KB expression

Figure 17: Results of NF-KB reporter assay for human THP-1 cells infected with *A. baumannii*

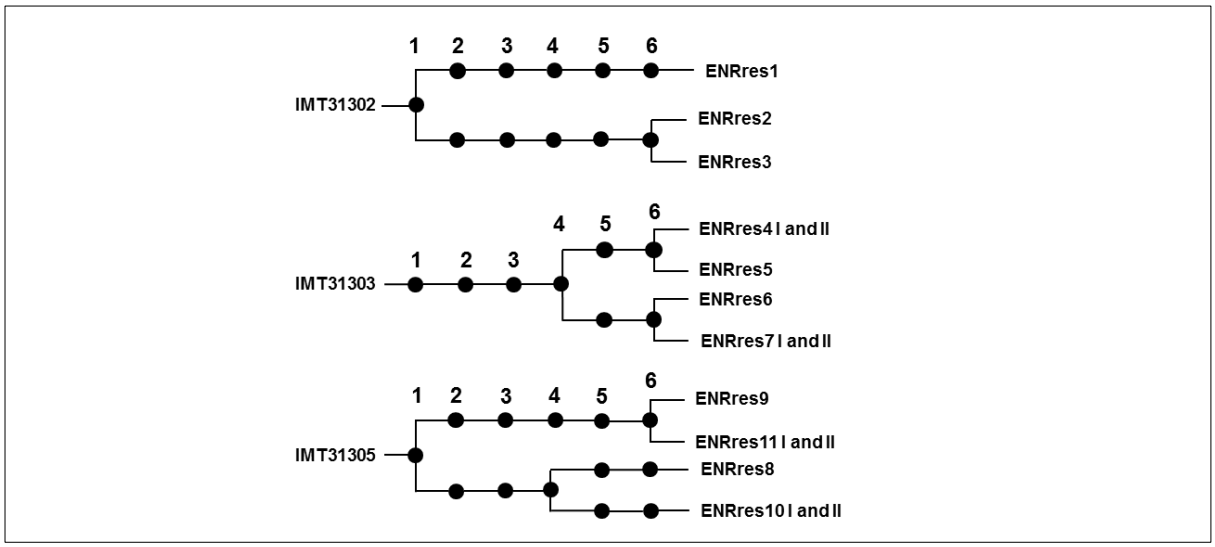



Boxplots were generated for the variable difference-median (Lum/E of infected cells minus Lum/E of uninfected cells) for human THP-1 cells measured 7h (A) and 19h (B) post infection with porcine *A. baumannii* isolates IMT31302, IMT31303, IMT31305 and their respective spontaneous enrofloxacin (ENR) resistant mutants ENRres1, ENRres6 and ENRres9; Lum/E values reflect measured luciferase activity of *luc* reporter cells, what is proportional to the level of NF-KB expression

## 2 Comparative molecular analysis of enrofloxacin (ENR) sensitive *A. baumannii* wild-type and derived resistant mutant isolates

The selected mutants (cf. table 30) ENRres1, ENRres2, ENRres3, ENRres4 I and II, ENRres5, ENRres6, ENRres7 I and II, ENRres8, ENRres9, ENRres10 I and II, and ENRres11 I and II (n=15) were analyzed for the occurrence of genomic mutations due to enrofloxacin (ENR) selective pressure. Based on the method for induction of enrofloxacin resistance, some ENR resistant mutants are more closely related than others. In order to obtain more than one mutant from a wild-type isolate, two colonies were picked after subcultivation on a gradient plate. This led to a separation of distinct mutant lineages, which are nevertheless related. A mutation occurring in two closely related mutants could thus be induced independently or, more likely, before the distinct mutant lineages emerged. For this reason, it is to expect that the closely related mutants, e.g. ENRres7 I and ENRres7 II, have only few unique mutations compared to each other. Figure 18 illustrates the relatedness of the selected ENR resistant mutants.

Figure 18: Relatedness of analyzed spontaneous enrofloxacin resistant (ENRres) mutants




Induction of enrofloxacin (ENR) resistance in porcine *A. baumannii* sensitive wild-type isolates IMT31302, IMT31303 and IMT31305 was achieved by means of gradient plates with increasing ENR concentrations in the course of six subcultures; selection of two mutant colonies from each subculture led to separation of mutant lineages; mutants are more closely related the later the separation was made; additives I and II correspond to subclones of the respective mutant which show larger (I) and smaller (II) colony variants; 1 – 6: respective number of gradient plate/ subculture

All genomic mutations which were identified in the analyzed ENR resistant mutant isolates are listed in table 41. The IMT31303 mutants ENRres4 I and II, ENRres5, ENRres6, ENRres7 I and II, as well as the IMT31305 mutants ENRres9 and ENRres11 I and II showed alterations in the DNA gyrase alpha-subunit (*gyrA*). Mutants of IMT31303 revealed a C → T SNP at bp242 leading to a S81L substitution, whereas mutants of IMT31305 had a novel CAC triplet insertion at bp1841, causing a proline insertion. Mutations in the DNA gyrase beta-subunit (*gyrB*) were detected in ENRres2, ENRres8 and ENRres10 I and II. Although these mutants derive from different wild-type isolates, they showed a very similar mutation: a GTA triplet insertion at bp1469 in ENRres2 and a GTG triplet insertion at the same position in ENRres8 and ENRres10 I and II. In both cases, the triplet insertions result in a serine insertion at amino acid (aa) 491. Interestingly, ENRres1 and ENRres3 did not have any mutation in *gyrA*, *gyrB* or *parC*, which are known to mediate fluoroquinolone resistance in *A. baumannii* [239, 248, 250]. However, both mutants showed alterations in genes involved in $Mg^{2+}$ metabolism. $Mg^{2+}$ is required for appropriate binding and interaction of fluoroquinolones to the DNA gyrase [242, 244]. Two different variants of the magnesium and cobalt transport protein CorA were present in all investigated *A. baumannii* isolates and mutations occurred in the *corA* genes of both variants. ENRres1 had a SNP only in CorA variant I, whereas ENRres3 had mutations in CorA variant I and II and additionally in *mgtA* encoding a $Mg^{2+}$ ATPase. Alterations in transcriptional regulator genes for multi-drug efflux pumps were detected in mutants of all three wild-type isolates. ENRres1, ENRres2, ENRres3, ENRres9 as well as ENRres11 I and II showed mutations in *adeL* (the LysR-type regulator of *A. baumannii* MDR efflux pump AdeFGH [255, 274]), which caused amino acid depletions. Mutations in the *adeN* gene, encoding the transcriptional regulator of MDR efflux pump AdeIJK [277], occurred in ENRres1, ENRres8, ENRres10 I and II as well as in all IMT31303 mutants. Mutations were moreover identified in genes involved in translational and transcriptional processes. For ENRres4 I and II, ENRres5 as well as ENRres7 I and II mutations in ribosomal proteins were identified. The affected proteins were L23p, S14p and S18p. Besides a R66C substitution in the transcription termination factor Rho (*rho*), ENRres2 showed an interesting 337bp deletion in a region coding for tRNA Asp – tRNA Val – tRNA Asp. The latter was also observed for ENRres3. ENRres9, ENRres10 I and II and ENRres11 I and II furthermore showed mutations in the citratesynthase si gene *gltA*. Although these mutants derive from the same wild-type isolate (IMT31305), they showed different *gltA* mutations. Comparison of the two lineages of ENRres4 did not reveal genomic differences between the two, as it was also the case for ENRres10 I and II.

# DISCUSSION

## I Genotypical and phenotypical species identification of *Acb*-complex isolates

Since phenotypical species identification methods are considered to be unreliable [13, 159], molecular techniques are required for species assignment. Several molecular typing methods have consequently been developed, such as 16S rDNA restriction analysis (ARDRA), 16S rDNA, and partial *rpoB* sequencing [356, 378, 379]. Furthermore, the intergenic spacer (IGS) sequence separating the 16S and 23S rRNA genes has been shown to be a suitable target gene for discrimination of *Acinetobacter* species [352, 380]. The 16S-23S IGS shows a low degree of variability within the same bacterial species, but a high degree of variability between different species [351, 352, 381-383]. Although differences in the 16S-23S IGS copy numbers among *Acinetobacter* spp. have been reported, variation within the same species is considered low [355, 384-386]. Moreover, Chang et al. reported that IGS lengths were highly conserved within isolates of the *Acb*-complex, with intraspecies similarities of 0.99 to 1.0 (corresponding to 99-100%) [351]. In 1995, Dolzani et al. described the suitability of restriction digestion of the 16S-23S IGS for species discrimination of *Acb*-complex species [352], while Chang et al. were able to identify several *Acb*-complex isolates based on 16S-23S IGS sequencing with an overall identification rate of 96.2% [351]. In general, sequencing methods are more time consuming than PCR-based methods due to the sequencing step, and since they require suitable laboratory equipment. Smaller laboratories that attach importance to reliable and fast species identification might thus favor methods excluding sequencing steps.

Restriction fragment length polymorphism (RFLP) of 16S-23S IGS amplicons is a molecular method that is meant to combine the time efficiency of a PCR-based method and the reliability of target gene sequencing, because it requires presence of specific restriction sites. While combined digestion with *Alu*I and *Nde*II is necessary for species discrimination in the RFLP method introduced by Dolzani et al. [352], *Acb*-complex species-specific restriction patterns could be obtained in the present study using only one digestion step with *Mbo*II. Within a large set of 642 clinical *Acb*-complex isolates, one species-specific restriction pattern could be obtained for *A. baumannii* and *A. pittii*, while three different patterns could be assigned to *A. calcoaceticus* due to single nucleotide polymorphisms at the *Mbo*II restriction site. Presence of different species-specific patterns could correspond to presence of different clonal lineages within *A. calcoaceticus*.

However, species assignment by RFLP of the 16S-23S IGS using *Mbo*I could be verified for 98.04% of the presumable *A. baumannii* isolates and for 98.18% of the presumable *A. pittii* isolates using 16S-23S IGS and partial *rpoB* sequencing (cf. table 21). This demonstrates a high discriminatory power of the presented method for these two *Acinetobacter* species, which are most frequently isolated from clinical specimens [13, 14, 108, 356, 387, 388]. Although the accordance of species assignment by the applied methods was lower for *A. calcoaceticus* (86.67%), the reliability still seems to be sufficient, since *A. calcoaceticus* is an environmental species that is not usually associated with disease. In contrast, RFLP of the 16S-23S IGS using *Mbo*I does not seem to be a suitable technique for identification of *A. nosocomialis*, because species assignment could not be confirmed for any of the investigated eleven isolates. 16S-23S IGS and partial *rpoB* sequencing, however, also did not produce consistent results (cf. table 32), highlighting the difficulties in typing *Acinetobacter* isolates.

Intraspecies similarities in the reference alignments were comparable for both sequenced target genes and ranged from 96.9-100.0 % for the 16S-23S IGS and from 96.5-100.0% for the partial *rpoB* sequences. This is slightly lower than the intraspecies similarities reported for the two genes by Chang et al. and Gundi et al. (99.0-100.0 % and 98.0-100.0%, respectively) [351, 356]. Of note, recent research questions the reliability of the 16S-23S IGS region. Maslunka et al. illustrated the presence of indels within the 16S-23S IGS of *Acinetobacter* species, probably due to horizontal gene transfer [355]. While indels have not been reported for *A. baumannii*, their presence might lead to mistyping of other isolates of other *Acinetobacter* spp.. Nevertheless, the reported indels show a length of up to 37bp, but more frequently of less than 20bp, and are randomly incorporated within the 16S-23S IGS [355]. Thus such indels only cause deviating restriction patterns if they are located at one of the very few *Mbo*I restriction sites. Given that species-specific *Mbo*I restriction fragments achieved by the method described in this work show sizes varying from 145-542bp, incorporation of small indels up to 20bp might not be recognized in the respective electropherogram. It nevertheless cannot be excluded that indels might be responsible for the divergent results using 16S-23S IGS and *rpoB* as target genes for *Acb*-complex species discrimination. Whole genome sequence analysis of isolates considered as being not typeable due to deviating sequencing results would allow further molecular typing and therefore assessment of reliability of 16S-23S IGS RFLP, 16S-23S IGS and partial *rpoB* sequencing. All three methods nevertheless showed a very good accordance for *A. baumannii* and *A. pittii* isolates and still a rather sufficient accordance for *A. calcoaceticus*. Because only *Acinetobacter* isolates that had

already been identified as *Acb*-complex isolates by phenotypic methods (VITEK2®, BioMeriéux, and MALDI Biotyper®, Bruker Daltonics) were included in the present study, the suitability of RFLP of the 16S-23S IGS by *Mbo*II for other *Acinetobacter* spp. not belonging to the *Acb*-complex could not be determined. However, *in silico* restriction of IGS sequences of several *Acinetobacter* spp. available at GenBank® with *Mbo*II did produce restriction patterns which differed from those reported for *A. baumannii*, *A. pittii,* and *A. calcoaceticus* (data not shown). All together, the method presented in this work is a simple, time and cost efficient molecular tool, which shows a high discriminatory power for *A. baumannii* and *A. pittii* isolates, which currently are the most relevant pathogenic *Acb*-complex species.

To date, automated species identification systems delineate bacteria according to their heterogeneous metabolic properties or, in case of MALDI-tof MS based systems, according to their respective protein profile. These systems have successfully been used for years for identification of the majority of bacterial species. Nevertheless, *Acb*-complex species are closely related and show a considerable variability of metabolic properties. Thus, phenotypic species identification is considered unreliable [46], although recent studies showed promising results for the suitability of MALDI-tof MS based systems [389-391]. Therefore, clinical *Acb*-complex isolates were tested for their ability to metabolize a selection of carbon sources. Although the reproducibility among the three biological replicates was given, the metabolic variability between the investigated clinical *Acb*-complex isolates was high in experiments utilizing the *Acinetobacter* test medium. All four clinically investigated *A. baumannii* isolates reacted conformingly for the selected carbon sources D-ribose, D-malic acid, citraconic acid, and L-ornithine, but not for L-hydroxyproline. Heterogeneous metabolization was also observed for the analyzed clinical isolates of *A. calcoaceticus* and *A. pittii*, reflecting the metabolic variability. Although these findings do not facilitate phenotypic species identification, they are in accordance with previous findings. Bouvet and Grimont developed a biotyping scheme which has been used in different studies, for example by Nemec et al., who could assign *Acb*-complex isolates to up to ten different biotypes within the same bacterial species [387, 392]. Dijkshoorn et al. moreover suggested using the metabolic heterogeneity for delineation of strains of the same clonal lineage during outbreak scenarios [12]. Substrates were considered suitable for *Acb*-complex species discrimination based on the Omnilog® Phenotypic MicroArray, which was performed for one reference isolate for each complex species. Bernards et al. already showed in 1995 that the Omnilog® Phenotypic MicroArray is suitable for investigation of metabolic properties of *Acinetobacter* species [393]. Generated

biotypes were compared to genomic species by DNA-DNA hybridization, showing that 84.5% of all isolates were correctly identified to genus level utilizing the Omnilog® system. Of note, 42 of the 51 incorrectly assigned isolates belonged to species of the *Acb*-complex [393], illustrating the high metabolic variability of *Acb*-complex isolates. In this regard it was not completely unexpected that Ominolog® Phenotypic MicroArray results of the reference isolates were not reproducible in the substrate test using the *Acinetobacter* test medium. *A. baumannii* isolate COL 20820 showed deviating results only for L-ornithine, whereas *A. calcoaceticus* DSMZ 1139 for two, *A. nosocomialis* ATCC 17903 for three, and *A. pittii* DSMZ 9308 for all investigated substrates (cf. table 34).

While the deviating results for the clinical isolates of one *Acb*-complex species can be traced back to metabolic variability, this seems to be less likely in case of the investigated reference isolates. All three biological replicates of the reference isolates produced the same results utilizing the respective test system (Omnilog® Phenotypic MicroArray and *Acinetobacter* test medium). As already mentioned by Bernards et al. [393], the Omnilog® system measures the color change of the redox indicator triphenyltetrazolium chloride (TTC) and thus the oxidation taking place in the presence of the respective carbon source. In contrast, phenol red of the *Acinetobacter* test medium changes its color due to acidification following substrate metabolization. Phenol red was chosen as indicator because TTC did not change its color in the *Acinetobacter* test medium for D-malic acid and citraconic acid, although isolates were previously tested positive in the Omnilog® Phenotypic MicroArray. This observation indicates that TTC might be less sensitive compared to phenol red. Different turnover points of the two indicators might be a possible explanation for the observed discrepancies. However, L-ornithine was tested negative for *A. baumannii* COL 20820 in the *Acinetobacter* test medium, but positive in the Omnilog® Phenotypic MicroArray, contradicting the possible influence of the utilized indicator. Further unknown factors, e.g. composition of the respective test medium, are thus likely to contribute to an isolate's ability to metabolize carbon sources. Suitably, Bernards et al. also observed significant differences in the assessment of metabolic properties of *Acb*-complex species in their study using the Omnilog® system compared to the results of Bouvet and Grimont, who used a different liquid medium [46, 393]. Overall, the Omnilog® Phenotypic MicroArray seems to generate results which are hardly reproducible in other test systems, at least in case of isolates belonging to the *Acb*-complex species.

## II Analysis of human and animal clinical *Acb*-complex isolates

### 1 *Acb*-complex species distribution and antimicrobial susceptibility of human and animal *A. baumannii* isolates

The highest proportion of collected animal clinical isolates belonged to *A. baumannii* (44.41%). Determination of the resistance profile of the *A. baumannii* isolates of animal origin revealed a multi-drug resistance rate of 50.92%, compared to the lower MDR rate of 15.52% in the human *A. baumannii* isolates. Similarly, animal isolates overall exhibited an extensively-drug resistant phenotype more often than human *A. baumannii* isolates (although the determined XDR rates were less deviating, 4.91% compared to 3.45%, respectively). Considering the different host species of animal *A. baumannii* isolates, it is striking that only 38 of the 163 isolates derive from cats but account for seven out of eight animal XDR isolates. This corresponds to a XDR rate of 18.42% for feline *A. baumannii* isolates while the XDR rate of canine isolates was only 0.94%. This finding raises a key question: to what extent cats might facilitate the development and spread of antimicrobial resistances in *A. baumannii*, especially regarding the zoonotic transmission of XDR resistant *A. baumannii* belonging to the major epidemic lineages. Notably, two recent publications by Pomba et al. [21] and Ewers et al. [20] report the detection of the OXA-23 carbapenemase in feline *A. baumannii* isolates belonging to IC II and IC I.

Various studies have addressed the contribution of the gastrointestinal microbiota composition to susceptibility to gastrointestinal diseases. While dysbiosis facilitates gastrointestinal infections; for example, with *Clostridium difficile* or *Mycobacterium avium* ssp. *paratuberculosis*, microbial substitution using probiotics and other bacteria has been shown to be beneficial in humans as well as in dogs and cats [394-402]. Taking these findings into account it seems imaginable that deviations in the microbiota might enable *A. baumannii* to survive and possibly also to persist within the host. In fact, it has been shown that the feline intestinal microbiota, beyond individual deviations in its composition, inherits a higher proportion of anaerobic bacteria (up to 50% belonging to the genus *Clostridium*) compared to the intestinal microbiota of humans and dogs [397, 403-405]. One might assume, that the prevailing conditions in the feline intestine might favor *A. baumannii* colonization. Furthermore, the physiological body temperature of cats ranges between 38.3°C and 39.0°C which corresponds to febrile temperatures for humans. In contrast to other *Acinetobacter* spp., *A. baumannii* is able to grow at temperatures up to 44°C which could be construed to an

adaption to febrile body temperatures during human infection, as an adaption to host species with higher physiological body temperatures, or as a combination thereof. Thus, colonization of the cat's intestine by *A. baumannii* may, on the one hand, be enabled by the host-specific microbiota and, on the other hand, be promoted by the higher body temperature, which might be beneficial for growth of *A. baumannii*. Future research should therefore investigate whether cats could serve as an infection source for multi-drug and extensively-drug resistant *A. baumannii* isolates.

Returning to the broader issue at hand, *A. baumannii* of animal origin in general showed a significantly higher multi-drug resistance rate compared to human isolates (50.92% vs. 15.52%). Besides the possibility that *A. baumannii* have their natural reservoir in animals, it is conceivable that 'host jumps' took place as it has been described for *Staphylococcus* (*S.*) *aureus*. Similar to CC398 methicillin resistant *S. aureus* (MRSA) isolates from animals which descend from human CC398 methicillin sensitive *S. aureus* (MSSA) [406-408], specific *A. baumannii* lineages like the IC I-III may have spilled over from humans to animals, and consequently acquired further resistance mechanisms. The fact that IC I-III and multi-drug resistant *A. baumannii* isolates have more frequently been reported in companion animals, which usually have closer contact to humans, than in livestock supports this assumption. Nevertheless, recent evidence reports the presence of IC I-III in livestock [163-165] possibly due to further transmission. It can be presumed that emergence of epidemic *A. baumannii* within food-producing animals will be accompanied with further enrichment of antimicrobial resistances and the establishment of new infections routes e.g. by food of animal origin. However, for *A. baumannii* isolates of human origin, reported antimicrobial resistance rates are higher within intensive care units (ICU) than in other clinical wards [140]. Because metadata of the present study does not contain information concerning the origin of isolates within the hospital setting, questions regarding varying resistance rates on ICU and non-ICU wards cannot be investigated. The structure of veterinary health care facilities moreover differs from the division of human hospitals into clearly separated wards, since few veterinary clinics display separated intensive care units. Studies addressing the antimicrobial resistance rates of *A. baumannii* isolates in different hospital wards are therefore difficult to conduct in veterinary medicine.

Besides *A. baumannii,* also *A. pittii* and *A. calcoaceticus* isolates could be collected during the one-year time-period from human and animal clinical specimens. While *A. baumannii* was the

predominant *Acb*-complex species among animal isolates (most commonly isolated from wound, respiratory and urinary tract specimens), *A. pittii* was most frequently isolated from human sources. This finding is not in accordance with the majority of previous studies, which identified *A. baumannii* as being the predominant *Acb*-complex species in human samples worldwide [213, 356, 387, 388, 409-411]. In contrast, according to the results obtained in this work, two studies investigating *Acb*-complex isolates from Germany also reported a predominance of *A. pittii* [92, 134]. It seems thus likely that the geographical origin of *Acb*-complex isolates has an impact on species distribution. One possible assumption could be the regional emergence of *A. pittii* as a human pathogen in Germany. Moreover, given that it is often hard to differentiate between colonization and infection, another explanation attempt might be that carrier rates of *A. pittii* are higher in some geographical areas than currently expected.

In general, the present study has limitations in terms of the variety of sources of *Acb*-complex isolates, because these do not derive from defined sample populations but from routine diagnostic laboratories. Therefore, results might be influenced by a selective sample receipt. For example, animal samples include only limited numbers of livestock samples because these are usually sent to other laboratories. Future studies concerning *A. baumannii* isolates of animal origin should pay attention to defined sample populations, ideally taking different clinic wards into account. The present study nevertheless clearly illustrates the importance of *A. baumannii* as a veterinary pathogen in different host species including reptiles and birds, with a high occurrence of antimicrobial resistances. Furthermore, the higher proportion of MDR *A. baumannii* among animal compared to human isolates suggests an animal contribution in the spread of multi-drug and extensively-drug resistant *A. baumannii*.

### 2 Genomic diversity of human and animal *A. baumannii* isolates

Data obtained from the maximum likelihood tree and the distance matrix based on the maximum common genome (MCG) suggests existence of two distinct populations of *A. baumannii* isolates. The first population comprises isolates which belong to the international clone II (IC II), corresponding to the determined Pasteur ST2, which are more closely related compared to the isolates of the second population. Isolates that did not cluster within the ST2 isolates were much more heterogeneous and could often not be assigned to any MLST

sequence type. Because isolates of this second population did not belong to the known outbreak lineages it might be assumed that their epidemic potential is rather low. The much lower average number of SNPs within the MCG of ST2 isolates substantiates the hypothesis that the ancestor of the IC II separated in the recent past from the overall heterogeneous population with subsequent adaption to a new ecological niche. Although descending clonal lineages show several SNPs they are still more closely related than isolates of the heterogeneous *A. baumannii* population, which, on average, shows more than 30.000 SNPs. In 1999 Nemec et al. also found that sporadic *A. baumannii* strains were more heterogeneous than those belonging to the IC I and II [387].

Of note, no separate clustering of human and animal *A. baumannii* isolates with an overall comparable diversity could be observed within the heterogeneous non ST2 isolates. The obtained data do not indicate host specificity within the investigated non ST2 isolates, but rather points towards their zoonotic potential. Detection of sequence types which have previously been reported in human *A. baumannii* in a canine isolate (ST241) and an isolate obtained from a rabbit (ST22) supports this assumption [412-414]. ST22 belongs to the clonal complex (CC) 22, which has been shown to account for 86.8% of carbapenem resistant isolates in a multicenter study from China due to presence of OXA-23 [412]. High resistance rates in ST22 isolates were also observed among others for aminoglycosides, fluoroquinolones, minocycline and piperacillin/tazobactam, revealing that all ST22 isolates were multi-drug resistant [412]. Furthermore, a study from South Korea reported an ST22 *A. baumannii* isolate that was resistant against all investigated antimicrobials, including tigecycline and polymyxins [415]. Since carbapenem resistant ST22 isolates have also been reported from Australia, some have suggested the emergence of a global epidemic carbapenem resistant *A. baumannii* ST22 clone [412, 416].

It is thus of particular interest that the analyzed animal isolates did not display any resistances against carbapenems, aminoglycosides, fluoroquinolones, tetracycline, trimethoprim/ sulfamethoxazole and polymyxin B. This finding supports the hypothesis that human multi-drug resistant *A. baumannii* lineages derive from a susceptible heterogeneous population from which they split and subsequently adapted to antimicrobial selective pressure [12, 15]. Further supporting this assumption, a very recent study by Klotz et al. reported isolation of IC II *A. baumannii* from cattle being susceptible to the investigated antimicrobials [417]. In general, high susceptibility to antimicrobials can be expected in isolates which derive from their natural

habitat, when no antimicrobial selective pressure necessitated development of resistance mechanisms. In this regard, analysis of the genomic diversity of susceptible IC I-III *A. baumannii* isolates compared to MDR IC I-III isolates could give further insights into the evolution of the outbreak lineages. If MDR clonal lineages were more closely related than non-MDR isolates, the hypothesis of partitioning of the special outbreak clones in the recent past would be substantiated. In that case, only a few clones of the same lineage (e.g. IC I-III) would have separated and adapted to the hospital setting followed by acquisition of a multi-drug resistant phenotype. This is in accordance to the much lower number of SNPs detected in the ST2 isolates within this study.

The clear distinction between ST2 isolates and non-ST2 isolates is also supported by bootstrap values of 100 in the maximum likelihood tree and can thus be considered reliable. Bootstrap values within the branches of the heterogeneous group of isolates are lower, thus reducing the reliability of arrangement of these isolates. This does however not disrupt the conclusion that a high diversity exists within the non-ST2 isolates. It has to be mentioned that the human *A. baumannii* isolate IMT30938 was very closely related to an isolate from China (*A. baumannii* BJAB0715; sequence published by Zhu et. al [413]). Both isolates belonged to ST23, demonstrating a global distribution of this sequence type, which to date has only rarely been detected. Because IMT30938 did not display significant antimicrobial resistances in difference to BJAB0715, it is conceivable that the German isolate belongs to a susceptible ancestral ST23 lineage.

## III Genomic and functional analysis of enrofloxacin resistant *A. baumannii* mutant isolates

### 1 Genomic analysis

Several mutations associated with the development of fluoroquinolone resistance could be detected in enrofloxacin (ENR) resistant *A. baumannii* mutant isolates by comparison of their whole genome sequence to their respective sensitive wild-type isolate. Functional analysis revealed that the acquisition of enrofloxacin resistance can be associated with a multi-drug resistant phenotype. Further functional analysis of three different wild-type/mutant pairs did not provide evidence for alterations associated with fluoroquinolone resistance in terms of plasmid

acquisition and its impact on host immune response. Decreased growth rates and smaller colony sizes of the mutants compared to their wild-type isolates suggests that fluoroquinolone and, in some cases, multi-drug resistance might be associated with a fitness loss. Genomic analysis moreover revealed the occurrence of novel DNA gyrase mutations in enrofloxacin resistant isolates. Besides mutants of IMT31303, which showed a C → T SNP at position 242 in the *gyrA* gene, leading to a S81L substitution, some mutants of IMT31305 had a CAC triplet insertion at position 1841, causing a proline insertion outside the quinolone resistance determining region of *gyrA*. Mutations in *gyrB* have, moreover, only rarely been reported in *A. baumannii* [239], but have also been detected in the present study in mutants of two wild-type isolates.

While the upregulation of the AdeABC efflux pump was most frequently reported to cause multi-drug resistance in *A. baumannii*, neither the genes encoding this pump nor its regulators were affected in the present study. Instead, mutations in the transcriptional regulator genes of the AdeFGH and AdeIJK RND-family efflux pumps, named *adeL* and *adeN* were present in all investigated ENR resistant mutants. Mutants of IMT31303 showed the same mutation in *adeN*. This finding is not surprising, given that these mutants are more closely related than mutants of the other wild-type isolates (cf. figure 18). In contrast to the other investigated ENR resistant *A. baumannii*, ENRres1 showed mutations in both genes, *adeN* and *adeL*. Of note, ENRres1 did not have any mutations in the DNA gyrase or topoisomerase IV genes. The phenotypic enrofloxacin resistance may thus be a result of synergistic effects of upregulation of AdeFGH and AdeIJK, both of which cause fluoroquinolone efflux. This assumption suggests a repressor function of the two regulator genes, which is enabled by the respective genomic mutations. Indeed, Coyne et al. already showed that mutations in *adeL* are associated with overexpression of AdeFGH, which can confer multi-drug resistance [255], as has already been reported to be the case for AdeIJK overexpression [418]. Besides ENRres1 the mutant ENRres3 also did not show topoisomerase mutations, but rather a deletion in *adeL*. Interestingly, the enrofloxacin MIC value of ENRres3 was only in intermediate susceptible ranges, while the MIC of ENRres1 was within the range of resistant, supporting the assumption of a synergistic effect of mutations in both, *adeL* and *adeN*. Nevertheless, the MIC value for enrofloxacin determined for ENRres1 was lower than the ENR MIC values of the mutants (except for ENRres11 I), which inherited DNA gyrase or topoisomerase IV mutations. It is therefore likely that the upregulation of multi-drug efflux pumps, for example by mutations in

regulator genes, is a general reaction of *A. baumannii* isolates to overcome antimicrobial selective pressure until specific mutations for the respective antimicrobial can be developed.

Although it has been reported that the overexpression of AdeFGH and AdeIJK can confer multi-drug resistance [255, 418] and a multi-drug resistant phenotype could be observed in some of the spontaneous ENR resistant mutants, no association between acquisition of specific mutations and overall resistance profile could be observed. This suggests additional factors influencing the regulation of resistance genes and efflux pumps. Actually, some ENR resistant mutants showed mutations in the small and large ribosomal proteins S14p, S18p and L23p, suggesting an association of fluoroquinolone selective pressure with development of mutations in ribosomal proteins in some *A. baumannii* strains. Changes in the ribosomal structure may have a direct impact on bacterial translational processes and thus on gene expression. Indeed, only recently, it was shown that mutations in *rpsJ* encoding the S10 ribosomal protein was associated with decreased tigecycline susceptibility in *A. baumannii* [419].

The mode of action of antimicrobials targeting protein biosynthesis by interaction with the bacterial ribosome has been studied extensively [420-425]. Many cellular mechanisms which are initiated by antimicrobial selective pressure nevertheless remain poorly understood, although transcription of various genes appears to be significantly influenced also by antimicrobials that do not target ribosomes [426]. In fact, enrofloxacin resistant mutants with ribosomal alteration exhibited higher MIC values for cefpodoxime than the other mutants, except ENRres8. This mutant also displayed a cefpodoxime MIC value of ≥ 256.00 µg/ml but showed mutations only in *gyrA*, *adeN*, *panB* and *metH* (the latter two genes being involved in pantothenate and methionine biosynthesis). It is therefore more likely that a factor which is not represented by specific mutations contributes to the resistance profile of the respective mutant isolates. Reactive oxygen species (ROS) like hydroxyl radicals are candidate molecules for this phenomenon. Such ROS are formed not only by host cells, but also in the bacterial cell during DNA damaging conditions when stressed by DNA damaging drugs like quinolones [427-430]. In reaction to increasing concentrations of ROS molecules, bacteria increase expression of error-prone DNA polymerases and therefore mutagenesis [27-29, 430]. In 2010, Kohanski et al. were able to show that sublethal antimicrobial concentrations can lead to a multi-drug resistant phenotype in *E. coli* isolates by increased mutation rates due to ROS [431]. Moreover, antimicrobials causing oxidative-stress induce complex redox alterations within the

bacterial cell in addition to their target-specific mode of action. This leads to ROS formation which, in turn, causes alterations in central metabolism, cellular respiration and iron metabolism [432]. Given that the ENR resistant mutants in this study were obtained by cultivation of ENR sensitive wild-type isolates in subinhibitory ENR concentrations, it can be expected that the amount of ROS increased within the bacterial cells, thus influencing the cell metabolism on different levels.

Besides ROS the regulation of gene expression can also be influenced by small RNAs (sRNAs), which are non-coding RNA molecules that show regulatory effects on protein biosynthesis on the post-transcriptional level [433-436]. These sRNA molecules are part of complex regulatory networks showing both activator and repressor functions on mRNA translation for a variety of genes, including those involved in bacterial drug resistance and virulence [434-438]. Thus, regulation of gene expression by sRNAs plays an important role not only in unstressed bacterial cells but also in bacterial response to altered environmental conditions and modulation of stress response [436]. Recently, *A. baumannii* was shown to possess several sRNAs, which seem to be unique to this species. Furthermore, the expression of the sRNA AbsR25 varied depending on environmental and internal stress conditions and was assumed to be involved in regulation of an efflux pump and drug resistance [439]. It can be assumed that ROS formed during oxidative stress response may have an additional influence on regulation of gene expression by sRNAs. Thus, antimicrobial selective pressure by DNA damaging drugs likely involves an interplay of oxidative stress by ROS, regulation of gene expression by small RNAs and mutagenesis.

In addition, cellular reactions to the oxidative stress are likely individual in each affected bacterial clone and thus may result in the observed unique resistance profiles. However, mutagenesis based on ROS production induced during sublethal antimicrobial concentrations can be beneficial for bacteria in low stress situations by initiating protective defense mechanisms [432, 440, 441]. Moreover, induced mutagenesis, which has been evidenced in *A. baumannii* isolates exposed to subinhibitory ciprofloxacin concentrations [27], can lead to novel clones armed with further advantages. In this regard, inadequate application of DNA damaging antimicrobials like fluoroquinolones can be assumed to reinforce the worrisome issue of antimicrobial resistances in *A. baumannii*. Besides the probable beneficial effect of ROS under low stress conditions, high antimicrobial concentrations lead to bacterial cell death, which is augmented by oxidative stress [432]. Therefore, beneficial mutations, e.g. in *gyrA*,

can be associated with decreased cell variability and thus decreased bacterial fitness, which can cause decreased growth rates. This actually has been observed in this work. Previous studies already showed that antimicrobial resistances can be associated with reduced bacterial fitness and virulence [442-444]. It can be assumed that with the disappearance of the antimicrobial selective pressure, ROS concentrations will decrease offsetting the negative effects on cell variability. In this case, acquired genomic mutations will be beneficial for bacterial cells, which survived the negative effects of the antimicrobial selective pressure. Fitness costs subsequent to sublethal antimicrobial concentrations might thus be only of temporary duration.

**2 Functional analysis**

In order to gain further information whether the development of quinolone resistance is associated with phenotypic alterations in host-pathogen interactions (besides the resistance phenotype in the ENR resistant mutants compared to their respective sensitive wild-type isolates), NF-ĸB reporter assays and conjugation experiments were performed. Previous studies showed that macrophages play a central role in recruitment of neutrophils during early stages of *A. baumannii* infection and therefore in an efficient immune response [336, 337]. In turn, decreased macrophage response results in lower neutrophil influx at the infection site, resulting in decreased bacterial killing and a better survival of the pathogen within the host. Usage of the NF-ĸB reporter assay allows assessment of cell signaling activities in various cell types by measurement of the chemo-luminescence intensity (Lum/E) via a luciferase reporter gene [445]. Furthermore, reporter vectors have previously successfully been used to study macrophage activation by monitoring NF-ĸB expression [446]. In this work, the response of porcine macrophages as well as human monocytes (cell lines 3D4/31 and THP-1) to infection with three different ENR resistant mutant and their respective sensitive *A. baumannii* wild-type isolates were investigated. Since the sensitive wild-type isolates were isolated from pigs, the NF-ĸB reporter assay for 3D4/31 cells reflects the host specific immune response, whereas the NF-ĸB reporter assay for THP-1 cells reflects a host un-specific immune response. Additionally, in order to take time-dependency of the immune response into account, Lum/E values were measured at 7h and 19 h post infection (p.i.) with the six investigated *A. baumannii* isolates.

For the human cell line THP-1 no significant differences could be detected with the ENR resistant mutants compared to their wild-type isolates at 7 h p.i. nor at 19 h p.i. using the Tukey Test for pairwise comparison of the isolates. In contrast, the mutant ENRres1 was significantly different to IMT31305 (p=0.064) at 7 h p.i.. Of note, IMT31302 is the wild-type isolate of ENRres1. A p-value of p=0.128 was calculated for the pairwise comparison of ENRres1 and IMT31302, which is close to the level of significance of p=0.1 (level of significance was chosen based on the number of samples). Analysis of the generated box plot graph for 3D4/31 cells at 7 h p.i. on the other hand reveals a significant difference of ENRres1 to all other investigated isolates (no overlap of box plots), although the calculated p-values indicated a significant difference only for ENRres1 and IMT31305. This can nevertheless be explained by the rather low sample size (three biological replicates and thus only three median values) what can influence statistical algorithms. However, the response of 3D4/31 cells to infection with ENRres1 clearly differs from response of 3D4/31 cells to infection with the other isolates, including its wild-type isolate. The finding that the measured Lum/E values were significantly higher for ENRres1 corresponds to an increased NF-KB expression and therefore increased macrophage activation. This raises the question if the other isolates were able to suppress macrophage response during early stages of infection (until 7 h p.i.), pointing towards an adaption of porcine *A. baumannii* isolates to the porcine host immune response. This is particularly supported by the fact that no differences could be detected in human THP-1 cells infected with porcine *A. baumannii*.

Since the macrophage response was lower in the other five isolates compared to ENRres1, it can be assumed that ENRres1 lost phenotypic properties by random mutations in genes, which enable host specific immune evasion. SNP analysis of the whole genome sequences of the investigated *A. baumannii* isolates revealed that ENRres1 developed unique genomic mutations in the outer membrane protein IMP, the domain of unknown function containing protein DUF1176 and a hypothetical protein. In fact, the outer membrane protein IMP (corresponds to *lptD*) has been described to be involved in envelope biogenesis/LPS synthesis [447] and mutations in the IMP encoding gene, thus influence the bacterial outer membrane PAMP profile and therefore macrophage response. The other two proteins besides IMP may also be involved in immune evasion mechanisms, but since no information about their functions exists, this needs further experimental assessment. A further indication for the existence of a host specific immune evasion mechanism is that the measured Lum/E values were, in general, considerably higher in THP-1 cells than in 3D4/31 cells at 7 h p.i. (maximum

Lum/E value for THP-1 cells: approximately 17.000; maximum Lum/E value for 3D4/31cells: approximately 7.500). Admittedly, however, this could also be due to cell line specific variations independently of the bacterial pathogen.

Since loss of immune evasion mechanisms is adverse for bacterial pathogenesis, one may presume that ENRres1 would not prevail under natural conditions, e.g. in hospital settings. In fact, ENRres1 did not reveal mutations in topoisomerase II genes, which have been described in clinical fluoroquinolone resistant *A. baumannii* isolates [239, 248, 250]. Instead of *gyrA*, *gyrB* or *parC* mutations, ENRres1 showed alterations in *adeL* and *adeN* suggesting a synergistic effect of the multi-drug efflux pumps which are regulated by these genes. Moreover, ENRres1 as well as ENRres2 and ENRres3 had mutations in genes which are involved in $Mg^{2+}$ metabolism. Because $Mg^{2+}$ is required for adequate binding of the fluoroquinolone to the cleavage complex [242-244], it is plausible that altered $Mg^{2+}$ concentrations within the bacterial cell can also contribute to the aforementioned synergistic effect

.

It is important to note that even though the MOI was adjusted for each bacterial isolate prior to infection of the respective cell line, differences in bacterial growth rates were not taken into account. There are two reasons why the assay is nevertheless meaningful for the present issue. Firstly, it was designed as an invasion/phagocytosis assay with application of 10 µg/ml gentamicin already at 1h p.i., so slight differences in growth rates do not significantly influence the number of extracellular bacteria within this first hour before bacterial killing. Secondly, no significant difference could be detected except for ENRres1 in 3D4/31 cells at 7h p.i. with enhanced macrophage response of this mutant compared to the other investigated isolates, although ENRres1 shows a slower growth rate than all three wild-type isolates and ENRres6. Because slower growth rates would result in decreased bacterial loads, it would be expected that the macrophage response would also decrease. This was however not the case. Taken together, the NF-ĸB reporter assays performed did not reveal any association of enrofloxacin-resistance in *A. baumannii* with altered macrophage response but indicate host-specific immune evasion mechanisms.

In addition to the consideration that fluoroquinolone resistance might be associated with alterations in the host immune response during infection, it was hypothesized that ENR resistant *A. baumannii* isolates may have a greater capacity to acquire foreign plasmids than ENR sensitive isolates. A greater capacity to incorporate foreign plasmids would entail

secondary selective advantages due to acquired novel plasmid encoded genes, e.g. resistance or virulence genes. To assess this possibility, conjugation experiments were performed with the three ENR sensitive porcine *A. baumannii* wild-type isolates and their respective ENR resistant mutants, which have also been investigated in the NF-ĸB reporter assays. All three wild-type/mutant pairs were initially tested in three biological replicates. In order to examine the steadiness of the conjugation assay, six further biological replicates were performed for IMT31302 and its mutant ENRres1.

Based on the obtained results, no association between the development of ENR resistance and the capacity of plasmid acquisition could be established. Moreover, results strongly suggest the existence of additional factors influencing plasmid transfer and acquisition what is indicated by the variable results received from IMT31302/ENRres1. This may be due to the relatively long intermediate subcultivation step in 50 µg/ml kanamycin containing liquid medium, which was necessary to obtain transconjugants in the ENR resistant mutants. This subcultivation step might facilitate alterations in the fragile donor (IMT32484_*aphA6*) – recipient (IMT31302/ENRres1) competition situation, resulting in differing outcomes depending on the respective successful isolate.

The factors contributing to these alterations or influencing plasmid uptake in *A. baumannii* cannot be elucidated in the present work. Nevertheless, this would be an interesting starting point for future research since it is not fully understood how the *A. baumannii* isolates were able to rapidly acquire a wide variety of antimicrobial resistant genes. The finding that no transconjugants could be obtained for IMT31305 and ENRres9 supports the assumption that there may be factors or genes which enable certain *A. baumannii* isolates to acquire plasmids more easily than others. Indeed, it has been hypothesized that differing tendencies to acquire foreign genetic material might be one of the reasons for the success of specific *A. baumannii* lineages [15]. It lends to reason that plasmid uptake in *A. baumannii* is influenced by both, competition of donor and recipient as well as further unknown factors. Previously, conjugation experiments for *A. baumannii* have been performed utilizing cloned Enterobacteriaceae spp. isolates as donors or recipients. Of note, the already mentioned intermediate cultivation step in 50 µg/ml kanamycin containing medium does not allow calculation of conjugation rates, because competition between donor and recipient as well as varying growth rates influence calculated colony forming units/ml. Ongoing mating within this additional cultivation step is unlikely since in pre-experiments plasmid transfer was only achieved by filter mating, which

creates very close cell contact under nutrient limiting conditions (that is not the case during cultivation in LB broth in Erlenmeyer flasks). The assay nevertheless allows one to assess whether transconjugants of one isolate are more successful than transconjugants of another isolate. Future studies that rely on this conjugation assay could reduce the duration of the intermediate cultivation step or forego entirely, if the isolates being investigated are more vital than the ENR resistant mutants were in this study. However, a kanamycin resistance plasmid originating from a clinical human *A. baumannii* isolate could successfully be transferred to an *A. haemolyticus* isolates as well as clinical porcine *A. baumannii* isolates, which expressed the *aphA6* gene and developed a kanamycin resistant phenotype. This clearly shows that genetic transfer can naturally occur between human and animal *A. baumannii* isolates as well as between different *Acinetobacter* species.

## CONCLUSION

Taken together, data obtained in this work clearly illustrate that *A. baumannii* is the predominant *Acb*-complex species in animal clinical specimens of various host species. The remarkably high proportion of multi-drug resistant animal *A. baumannii* of 50.92 % compared to 15.52 % in human *A. baumannii* isolates is especially worrisome since it points towards the considerable impact of animals in the emergence of antimicrobial resistance in *A. baumannii*. This raises the question as to whether specific animal species might serve not only as the infection source but also as the reservoir for multi-drug resistant *A. baumannii*. Along these lines, we suggest that cats play a key role in the dissemination of such isolates, because i) 18.42% of all obtained feline *A. baumannii* isolates were extensively-drug (XDR) resistant, whereas only 0.94% of canine and 3.45% of human isolates were XDR ii) cats show a unique intestinal microbiota, which differs from that of dogs and humans, possibly creating conditions favoring *A. baumannii* colonization [403-405] iii) the physiological body temperature of cats ranges from 38.3°C-39.0°C, which may facilitate growth of *A. baumannii* (which has adapted to temperatures up to 44°C [26]). Future research investigating the role of animals in enrichment of the resistome and dissemination of epidemic *A. baumannii* lineages are thus urgently needed.

While host restriction of *A. baumannii* has already been disproved in several previous studies (at least for the IC I-III [19-21]) it remains unclear if there was a single ‘host jump’ event of

specific *A. baumannii* lineages from animals to humans or vice versa, or if several 'host jumps' took place. In this regard, it can be hypothesized that several lineages deriving from a susceptible heterogeneous ancestral *A. baumannii* pool were able to adapt to human health care facilities and subsequently spilled over into animal populations. These lineages were then able to acquire further antimicrobial resistances and subsequently reinfect human hosts. Data obtained from analysis of the genomic diversity of clinical human and animal *A. baumannii* isolates performed in this study supports this hypothesis, because it was shown that i) ST2 isolates (corresponding to IC II) are closely related and cluster separately from the non-ST2 isolates ii) non-ST2 *A. baumannii* isolates comprise a heterogeneous group of isolates originating from humans and animals iii) a susceptible rabbit *A. baumannii* isolate could be assigned to ST22, which has been reported to be associated with carbapenem resistance in *A. baumannii* of human origin [412, 415, 448], suggesting the rabbit isolate may belong to an ancestral clonal lineage. The finding that host specific immune evasion mechanisms might exist among *A. baumannii* isolates belonging to the heterogeneous group is not in conflict with this hypothesis. Although such immune evasion probably gives bacteria an advantage to infections in specific hosts, it does not exclude the pathogen's ability to infect other host species.

However, a central question remains how specific *A. baumannii* lineages could acquire various antimicrobial resistances in a remarkably short period of time. Therefore, we hypothesized that the administration of fluoroquinolones would facilitate the development of antimicrobial resistances. We were able to demonstrate that development of enrofloxacin (ENR) resistance was also associated with phenotypic resistance against cefpodoxime and trimethoprim/sulfamethoxazole, leading to a multi-drug resistant phenotype in some of the ENR resistant *A. baumannii* mutants. Nevertheless, resistant phenotypes could not be assigned to specific genomic mutations, although there was an association of enrofloxacin selective pressure and mutations in the multi-drug efflux pump genes *adeL* and *adeN*.

Thus, there are presumably currently unknown regulatory processes in *A. baumannii* which play an important role under antibiotic stress conditions. In this regard, we suggest reactive oxygen species (ROS) besides sRNAs as a crucial factor triggering various metabolic alterations in sublethal antimicrobial concentrations, including antimicrobial resistance mechanisms. Since ROS also show negative effects on bacterial cell variability [432], development of resistance is likely associated with fitness costs for the bacteria due to

oxidative stress. This explains the herein observed smaller cell colonies and slower growth rates of ENR resistant *A. baumannii* mutants, although these could also be understood as persister cells, which are antimicrobial resistant dormant bacterial cell variants [449]. Performed NF-KB reporter assays and conjugation experiments could not evidence further selective advantages of the ENR resistant mutants beyond the resistant phenotype as has been shown to be the case in ESBL-plasmid carrying *E. coli* [450]. Investigations of ROS metabolism and regulation of gene expression by sRNAs during antimicrobial stress conditions should be investigated further in order to gain a deeper understanding of cellular mechanisms contributing to the rapid emergence of antimicrobial resistant *A. baumannii* lineages.

## SUMMARY

Antimicrobial resistance in bacteria is an ancient phenomenon that emerged as a serious threat to humans and animals within only the last few decades. Nowadays, multi-drug resistant bacteria cause severe diseases in humans as well as animals worldwide, leaving few therapeutic options. Among these, *Acinetobacter* (*A*.) *baumannii* is of increasing importance, especially with regard to the epidemic clonal lineages IC I-III, which are particularly associated with carbapenem and multi-drug resistance. Moreover, these clonal lineages could already be detected among *A. baumannii* of animal origin, indicating a zoonotic potential of this pathogen. The current work contributes to two aspects of current *A. baumannii* research: i) the occurrence of *A. baumannii* in animal clinical specimens, especially concerning the occurrence of antimicrobial resistance and ii) the identification of factors, e.g. specific antimicrobial compounds, which contribute to the enrichment of the resistome and clinical success of *A. baumannii*.

A total of 642 clinical human and animal *Acb*-complex isolates were collected for a one-year time-period starting in February 2013. Identification to species level was performed using restriction fragment length polymorphism (RFLP) of the 16S-23S IGS, as introduced in the present study and was verified by means of partial *rpoB* and 16S-23S IGS sequencing. *A. baumannii* was the predominant species among animal *Acb*-complex isolates, accounting for a proportion of 44.41% and originating from various host species, with a considerably high proportion of 50.92% of isolates being multi-drug resistant (compared to 15.52% of human *A. baumannii* isolates). This clearly points towards a role of animals in the reinforcement and

dissemination of antimicrobial resistances in *A. baumannii*. Subsequently, 27 clinical human and animal *A. baumannii* isolates were chosen for whole genome sequencing in order to gain insight in their genomic diversity and relatedness. Additionally, ten published complete genome sequences of human *A. baumannii* isolates were included in the analysis. Based on SNP analysis of the maximum common genome, a maximum likelihood tree and a distance matrix were generated, revealing a clear separation into a closely related cluster of human multi-drug resistant ST2 isolates and a heterogeneous group of human and animal antimicrobial susceptible non-ST2 isolates. In accordance with previous studies we therefore hypothesize that an ancestral ST2 isolate split from the heterogeneous group in the recent past followed by subsequent adaption to the hospital environment.

Moreover, we hypothesized that DNA damaging antimicrobials like fluoroquinolones may play a crucial role in adaption of *A. baumannii* to antimicrobial selective pressure and new ecological niches. Thus, spontaneous enrofloxacin (ENR) resistant mutant isolates were generated using sublethal ENR concentrations. Comparative genomic analysis of the whole genome sequences of the mutant and their respective wild-type isolates revealed novel mutations in the DNA gyrase encoding genes causing ENR resistance. Furthermore, an association of ENR selective pressure and mutations in the AdeFGH and AdeIJK efflux pump regulator genes *adeL* and *adeN* could be demonstrated.

Although the present work provides evidence that fluoroquinolone selective pressure can cause a multi-drug resistant phenotype in *A. baumannii*, no direct association of the resistance phenotype and genomic mutations could be proven. Future research should investigate the role of altered regulatory processes under antimicrobial stress conditions, e.g. due to ROS and sRNAs, in order to understand the mechanisms underlying the rapid evolution and clinical success of specific antimicrobial resistant *A. baumannii* lineages. Moreover, comprehensive epidemiological studies are urgently needed to assess the potential role of animals as reservoir for antimicrobial resistant *A. baumannii* and infection source for humans.

## ZUSAMMENFASSUNG

### Typisierung und funktionelle Charakterisierung von Isolaten des *Acinetobacter calcoaceticus- Acinetobacter baumannii* (*Acb*)-Komplexes unter besonderer Berücksichtigung multi-resistenter *Acinetobacter baumannii*

Bakterielle Resistenzen gegen antimikrobielle Wirkstoffe sind ein sehr altes Phänomen, welches sich in nur wenigen Jahrzehnten zu einem schwerwiegenden Gesundheitsrisiko entwickelt hat. Gegenwärtig verursachen multi-resistente Bakterien weltweit ernste Erkrankungen bei Menschen wie auch bei Tieren, zu deren Behandlung nur wenige therapeutische Möglichkeiten offenstehen. Zu diesen Bakterien gehört *Acinetobacter* (*A.*) *baumannii*, vor allem in Hinblick auf seine epidemischen klonalen Linien IC I-III, welche in besonderem Maße mit Carbapenem- und Multi-resistenz assoziiert sind. Darüber hinaus wurden diese klonalen Linien bereits bei *A. baumannii* Isolaten tierischer Herkunft nachgewiesen, was auf ein zoonotisches Potential dieses Krankheitserregers hinweist.

Die vorliegende Arbeit beinhaltet zwei Aspekte gegenwärtiger *A. baumannii* Forschung: i) das Vorkommen von *A. baumannii* in tierischen klinischen Proben, besonders in Hinblick auf das Auftreten von Antibiotikaresistenzen und ii) die Untersuchung von Faktoren, z. B. bestimmte antimikrobielle Wirkstoffe, die zu einer Vergrößerung des Resistoms und des klinischen Erfolgs von *A. baumannii* beitragen.

Insgesamt 642 klinische humane und tierische *Acb*-Komplex Isolate konnten innerhalb eines Jahres beginnend im Februar 2013 isoliert werden. Die Speziesbestimmung wurde anhand des Restriktionsfragmentlängen-Polymorphismus (RFLP) der 16S-23S IGS Region durchgeführt und durch partielle *rpoB* und 16S-23S IGS Sequenzierung verifiziert. *A. baumannii* war mit einem Anteil von 44.41% die vorherrschende Spezies bei tierischen *Acb*-Komplex Isolaten und konnte aus einer Vielzahl von Wirtsspezies gewonnen werden. Dabei war ein bemerkenswert hoher Anteil von 50.92% multi-resistent (verglichen mit 15.52% der humanen *A. baumannii* Isolate). Dies deutet auf eine Bedeutung von Tieren bei der Verstärkung und Verbreitung antimikrobieller Resistenzen von *A. baumannii* hin. Anschließend wurden 27 klinische humane und tierische *A. baumannii* Isolate für eine Gesamtgenom-Sequenzierung ausgewählt, um Einblicke in ihre genomische Diversität und Verwandtschaft

zu erlangen. Zusätzlich wurden zehn weitere publizierte humane Gesamtgenom-Sequenzen in die Analyse aufgenommen. Basierend auf der SNP Analyse des Maximum Common Genoms (MCG) wurden ein Maximum-Likelihood Baum sowie eine Abstands-Matrix erstellt, welche eine deutliche Trennung zwischen einem eng verwandten Cluster humaner multi-resistenter ST2 Isolate und einer heterogenen Gruppe Antibiotika empfindlicher humaner und tierischer nicht ST2 Isolate aufzeigten. Daher nehmen wir in Übereinstimmung mit vorangegangenen Studien an, dass sich in näherer Vergangenheit ein ursprüngliches ST2 Isolat von der heterogenen Gruppe abgespalten hat, gefolgt von der Adaptation an die Krankenhausumgebung.

Ferner haben wir angenommen, dass DNA-schädigende antimikrobielle Stoffe wie Fluorochinolone eine zentrale Rolle bei der Adaptation von *A. baumannii* an antimikrobiellen Selektionsdruck und neue ökologische Nischen spielen. Deshalb wurden spontane Enrofloxacin (ENR) resistente Mutanten durch subletale ENR Konzentrationen generiert. Die vergleichende genomische Analyse der Mutanten und ihrer jeweiligen Wildtyp-Isolate offenbarte neue Mutationen in den DNA-Gyrase kodierenden Genen. Des Weiteren konnte eine Assoziation zwischen ENR Selektionsdruck und Mutationen in den Regulatorgenen *adeL* und *adeN* der Effluxpumpen AdeFGH und AdeIJK demonstriert werden.

Obwohl die vorliegende Arbeit einen Nachweis dafür gibt, dass Fluorochinolon Selektionsdruck einen multi-resistenten (MDR) Phänotyp in *A. baumannii* verursachen kann, konnte kein direkter Zusammenhang zwischen MDR Phänotyp und genomischen Mutationen hergestellt werden. Zukünftige Studien sollten sich mit der Rolle veränderter regulatorischer Prozesse unter antimikrobiellen Stress Situationen befassen, beispielsweise durch reaktive Sauerstoffspezies oder kleine RNA, um die Mechanismen, welche der rapiden Evolution und dem klinischen Erfolg bestimmter resistenter *A. baumannii* Linien zu Grunde liegen, verstehen zu können. Darüber hinaus sind umfassende epidemiologische Studien zur Beurteilung der möglichen Rolle von Tieren als Reservoir für resistente *A. baumannii* und Infektionsquelle für den Menschen dringend nötig.

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# APPENDIX

## I Tables and Figures

Table 27: Clinical *Acb*-complex isolates selected for testing of their metabolic properties of selected substrates

| IMT number | *Acb*- complex species | host | specimen |
|---|---|---|---|
| IMT30818 | *A. baumannii* | human | tissue |
| IMT31128 | *A. baumannii* | dog | gall bladder |
| IMT31427 | *A. baumannii* | cat | urine |
| IMT31566 | *A. baumannii* | human | tracheal secretion |
| IMT31431 | *A. calcoaceticus* | dog | wound |
| IMT31731 | *A. calcoaceticus* | horse | trachea |
| IMT31407 | *A. pittii* | turtle | trachea |
| IMT31551 | *A. pittii* | human | venous catheter |
| IMT32901 | *A. pittii* | kangaroo | abscess |
| IMT31740 | *A. nosocomialis* | horse | wound |

Table 28: Human and animal clinical *A. baumannii* isolates selected for whole genome sequencing and their respective resistance profiles (MIC values in µg/ml)

| designation | host | specimen | CIP | ENR | GM | IP | T/S | N | AMPS | CAZ | PX |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IMT30813 | human | tissue | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT30819 | human | pharynx | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT30823 | human | tracheal secretion | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT30922 | human | wound | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT30938 | human | pharynx | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT30945 | human | skin swab | ≤ 0.25 | | ≤ 1.0 | ≤ 0.25 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31122 | dog | trachea | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT31134 | dog | wound | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMt31302 | pig | feces | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT31303 | pig | feces | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT31305 | pig | feces | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT31552 | human | nose | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31562 | human | wound | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31566 | human | tracheal secretion | ≥ 4.0 | | ≥ 16.0 | ≤ 1.0 | ≥ 320.0 | | ≤ 2.0 | 4.0 | |
| IMT31581 | human | abdominal cavity | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31853 | human | wound | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31862 | human | sputum | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT31875 | human | blood culture | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT32277 | human | urine | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT32310 | human | urine | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT32312 | human | sputum | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 4.0 | |
| IMT32473 | rabbit | nose | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |

Table 28: Continued

| designation | host | specimen | CIP | ENR | GM | IP | T/S | N | AMPS | CAZ | PX |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IMT32503 | dog | trachea | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT32876 | snake | trachea | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT32889 | dog | ear | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT32894 | rabbit | tissue | | ≤ 0.25 | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | | | ≥ 8.0 |
| IMT33018 | human | ulcer | ≤ 0.25 | | ≤ 1.0 | ≤ 1.0 | ≤ 20.0 | ≥ 512.0 | ≤ 2.0 | 8.0 | |

Number of clinical human *A. baumannii* isolates n=17, number of clinical animal *A. baumannii* isolates n=10; minimum inhibitory concentrations (MIC, in µg/ml) have been determined using the VITEK®2 system (BioMeriéux, France) by means of the VITEK®2 antimicrobial susceptibility panels for Gram-negative bacteria AST-N263 (developed for use in human medicine) and AST-GN38 (developed for veterinary use); CIP: ciprofloxacin, ENR: enrofloxacin, GM: gentamicin, IP: imipenem, T/S. trimethoprim/Sulfamethoxazole, N: nitrofurantion, AMPS: ampicillin/ sulbactam, CAZ: ceftazidime, PX: cefpodoxime

Table 29: *A. baumannii* published genomes used in the present study

| reference genome | designation | GenBank® accession number | size (bp) | sequence type (Pasteur MLST) |
|---|---|---|---|---|
| 1656-2 | *Acinetobacter baumannii* 1656-2<br>plasmid ABKp1<br>plasmid ABKp2 | NC_017162.1<br>CP001922.1<br>CP001923.1 | 3 940 614<br>74 451<br>8 041 | ST2 |
| AB0057 | *Acinetobacter baumannii* AB0057<br>plasmid pAB0057 | NC_011586.1<br>CP001183.1 | 4 050 513<br>8 729 | ST1 |
| AbH120-A2 | *Acinetobacter baumannii* strain AbH12O-A2 | CP009534.1 | 3 875 775 | ST79 |
| ACICU | *Acinetobacter baumannii* ACICU<br>plasmid pACICU1<br>plasmid pACICU2 | NC_010611.1<br>CP000864.1<br>CP000865.1 | 3 904 116<br>28 279<br>64 366 | ST2 |
| BJAB0715 | *Acinetobacter baumannii* BJAB0715<br>plasmid pBJAB0715 | CP003847.1<br>CP003848.1 | 4 001 621<br>52 268 | ST23 |
| BJAB0868 | *Acinetobacter baumannii* BJAB0868<br>plasmid p1BJAB0868<br>plasmid p2BJAB0868<br>plasmid p3BJAB0868 | NC_021729.1<br>CP003850.1<br>CP003888.1<br>CP003908.1 | 3 906 795<br>8 721<br>70 167<br>20 139 | ST2 |
| BJAB07104 | *Acinetobacter baumannii* BJAB07104<br>plasmid p1BJAB07104<br>plasmid p2BJAB07104 | CP003846.1<br>CP003887.1<br>CP003907.1 | 3 951 920<br>70 170<br>20 139 | ST2 |
| D1279779 | *Acinetobacter baumannii* D1279779<br>plasmid pD1279779 | CP003967.2<br>CP003968.1 | 3 704 284<br>7 416 | ST267 |

Table 29: Continued

| reference genome | designation | GenBank® accession number | size (bp) | sequence type (Pasteur MLST) |
|---|---|---|---|---|
| MDR-TJ | *Acinetobacter baumannii* MDR-TJ<br>plasmid pABTJ1<br>plasmid pABTJ2 | CP003500.1<br>CP003501.1<br>CP004359.1 | 3 964 912<br>77 528<br>110 967 | ST2 |
| MDR-ZJ06 | *Acinetobacter baumannii* MDR-ZJ06<br>plasmid pMDR-ZJ06 | CP001937.1<br>CP001938.1 | 3 991 133<br>20 301 | ST2 |

Table 30: Enrofloxacin (ENR) sensitive *A. baumannii* wild-type and spontaneous resistant mutant isolates selected for whole genome sequencing

| wild-type isolate | ENR resistant mutant | number of lineages/ subclones |
|---|---|---|
| IMT31302 | ENRres1 | 1 |
| | ENRres2 | 1 |
| | ENRres3 | 1 |
| | ENRres4 | 2 |
| IMT31303 | ENRres5 | 1 |
| | ENRres6 | 1 |
| | ENRres7 | 2 |
| IMT31305 | ENRres8 | 1 |
| | ENRres9 | 1 |
| | ENRres10 | 1 |
| | ENRres11 | 2 |

Table 31: Reference plasmids used for sequence prediction of putative *A. baumannii* plasmid pAB31566

| reference plasmid | isolate | GeneBank® accession number | species |
|---|---|---|---|
| p2ABTCDC0715 | TCDC-AB0715 | CP002524.1 | *A. baumannii* |
| pAC29b | AC29 | CP008851.1 | *A. baumannii* |
| pAC30c | AC30 | CP007580.1 | *A. baumannii* |
| p1AB5075 | AB5075-UW | CP008707.1 | *A. baumannii* |
| pAB-G7-2 | G7 | KF669606.1 | *A. baumannii* |
| pC13-2 | C13 | KU549175.1 | *A. baumannii* |
| pA105-1 | A105 | KR535992.1 | *A. baumannii* |
| pD72-2 | D72 | KM051846.1 | *A. baumannii* |
| pD46-3 | D46 | KM977710.1 | *A. baumannii* |
| pACICU2 | ACICU | CP000865.1 | *A. baumannii* |
| pCR17A | CR17A | HG977527.1 | *A. baumannii* |
| pCS01A | CS01A | HG977523.1 | *A. baumannii* |
| pA85-3 | A85 | KJ493819.1 | *A. baumannii* |
| ABKp1 | 1656-2 | CP001922.1 | *A. baumannii* |
| unnamed1 | YU-R612 | CP014216.1 | *A. baumannii* |
| pAB_CC | TYTH-1 | KF889012.1 | *A. baumannii* |
| pAB04-2 | Ab04-mff | CP012008.1 | *A. baumannii* |
| pCR17B | CR17B | HG977528.1 | *A. baumannii* |
| pCS01B | CS01B | HG977524.1 | *A. baumannii* |

Table 32: Investigated clinical *Acb*-complex isolates considered non typeable

| designation | species based on 16S-23S IGS RFLP (*Mbo*II) | species displaying highest partial *rpoB* identity | species displaying highest 16S-23S IGS identity | final assessment |
|---|---|---|---|---|
| IMT30934 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT30950 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT31062 | *A. pittii* | *Acinetobacter* non *Acb* | *A. pittii* | not typeable |
| IMT31109 | *A. nosocomialis* | | *A. baumannii* | not typeable |
| IMT31115 | *A. nosocomialis* | *Acinetobacter* non *Acb* | *Acinetobacter* non *Acb* | not typeable |
| IMT31389 | *A. nosocomialis* | *A. calcoaceticus* | *A. calcoaceticus* | not typeable |
| IMT31414 | unknown | *A. baumannii* | *A. pittii* | not typeable |
| IMT31439 | *A. calcoaceticus* | *Acinetobacter* non *Acb* | *Acinetobacter* non *Acb* | not typeable |
| IMT31441 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT31450 | *A. nosocomialis* | *Acinetobacter* non *Acb* | *Acinetobacter* non *Acb* | not typeable |
| IMT31464 | *A. pittii* | *A. pittii* | *A. baumannii* | not typeable |
| IMT31561 | *A. pittii* | *A. pittii* | *Acinetobacter* non *Acb* | not typeable |
| IMT31587 | unknown | *A. pittii* | *A. pittii* | not typeable |
| IMT31740 | *A. nosocomialis* | *Acinetobacter sp.* | *Acinetobacter* non *Acb* | not typeable |
| IMT31749 | *A. baumannii* | *A. parvus* | *A. pittii* | not typeable |
| IMT31792 | *A. nosocomialis* | *Acinetobacter* non *Acb* | *A. baumannii* | not typeable |
| IMT31849 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT31866 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT31884 | *A. calcoaceticus* | *A. pittii* | *A. baumannii* | not typeable |
| IMT32275 | unknown | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT32276 | *A. pittii* | *A. pittii* | *Acinetobacter* non *Acb* | not typeable |
| IMT32328 | unknown | *A. pittii* | *A. pittii* | not typeable |
| IMT32329 | *A. nosocomialis* | *A. pittii* | *A. nosocomialis* | not typeable |
| IMT32467 | *A. nosocomialis* | *A. pittii* | *A. baumannii* | not typeable |
| IMT32469 | *A. calcoaceticus* | *A. pittii* | *A. baumannii* | not typeable |
| IMT33000 | unknown | *A. baumannii* | *A. baumannii* | not typeable |
| IMT33001 | *A. nosocomialis* | *A. nosocomialis* | *A. baumannii* | not typeable |
| IMT33003 | unknown | *A. nosocomialis* | *Acinetobacter* non *Acb* | not typeable |
| IMT33005 | *A. nosocomialis* | *A. pittii* | *A. baumannii* | not typeable |

Collected clinical *Acb*-complex isolates were identified to species level by restriction fragment length polymorphism (RFLP) of 16S-23S intergenic spacer (IGS) amplicons by *Mbo*II; species identification was verified by means of partial *rpoB* and 16S-23S IGS sequencing of a representative number of random samples; isolates were considered as being not typeable if species assignment by the applied methods did not produce consistent results

Table 33: Results of Omnilog® phenotypic MicroArray for the investigated *Acb*-complex reference isolates

| microtiter plate | metabolization in all reference isolates[1] | no metabolization in all reference isolates[1] | metabolization in all but one reference isolate[1] | no metbaolization in all but one reference isolate[1] | variable metabolization in more than one reference isolate[1] | different metabolic properties of reference isolates[1] |
|---|---|---|---|---|---|---|
| PM01 | A05, A07, B08, B09, B12, C05, D05, E01, E05, F02, G02, G05, G12, H08 | A03, A06, A10, A11, A12, B01, B02, B03, B04, B05, B06, B07, B10, B11, B12, C09, C01, C02, C06, C07, C10, C11, C12, D03, D04, D08, D09, D10, D11, D12, E02, E03, E04, E06, E08, E09, E10, E11, E12, F01, F03, F04, F10, F11, F12, G07, G08, G09, H02, H03, H04, H05, H06, H09, H10, H11, H12 | A02, A08, A09, C03, C08, D01, F05, F06, G10 | C09, G01 | D06, F08, G03, H01 | A04, C04, D02, D07, E07, F06, F07, F08, G04, G06, G11, H07 |
| PM2A | D10, E08, F01, F06, F08, G04, G06, H03, H08 | A02, A03, A04, A05, A06, A07, A08, A09, A10, A11, A12, B01, B02, B03, B04, B05, B06, B07, B08, B09, B10, B11, B12, C01, C02, C03, C04, C05, C06, C07, C08, C09, C10, C11, C12, D01, D02, D03, D04, D05, D06, D07, D08, D09, D11, E05, E06, E09, E10, E11, E12, F02, F03, F04, F05, F11, F12, G01, G02, G03, G05, G07, G11, G12, H04, H06, H07, H09, H11, H12 | G10, H02 | E01 | F09, F10, H10 | D12, E02, E03, E04, E07, E10, F07, G08, G09, H01, H05 |

[1]: given are designations of substrate containing wells according to the official layout of Omnilog® Phenotypic MicroArray microtiter plates PM01 and PM2A; assessment of metabolic properties based on 95% confidence interval (ci) plots generated for the respective bacterial isolate and for each substrate of microtiter plates PM01 and PM2A after 48h of incubation at 37°C; positive metabolization is reflected by 95% ci plots located in values larger than the threshold value of 100; no metabolization is reflected by 95% ci plots located in values smaller than the threshold value of 100; variable metabolization is reflected by 95% ci plots spanning values larger and smaller the threshold value of 100; threshold value of 100 was selected based on experiments utilizing the *Acinetobacter* test medium (data not shown); investigated reference isolates *A. baumannii* IMT30483, *A. calcoaceticus* IMT30485, *A. pittii* IMT30487 and *A. nosocomialis* IMT30488

Table 34: Results of testing of metabolic properties of clinical reference *Acb*-complex isolates utilizing the *Acinetobacter* test medium

| designation | species | host species | D-ribose | | | D-malic acid | | | citraconic acid | | | L-hydroxyproline | | | L-ornithine | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| IMT30483 (R) | *A. baumannii* | human | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |
| IMT30818 | *A. baumannii* | human | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |
| IMT31128 | *A. baumannii* | dog | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |
| IMT31427 | *A. baumannii* | cat | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |
| IMT31566 | *A. baumannii* | human | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - |
| IMT30485 (R) | *A. calcoaceticus* | human | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - |
| IMT31431 | *A. calcoaceticus* | dog | - | - | - | + | + | + | + | + | + | - | - | - | - | - | - |
| IMT31731 | *A. calcoaceticus* | horse | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| IMT30487 (R) | *A. pittii* | human | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| IMT31407 | *A. pittii* | turtle | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| IMT31551 | *A. pittii* | human | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - |
| IMT32901 | *A. pittii* | kangaroo | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| IMT30488 (R) | *A. nosocomialis* | human | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |

Substrates were selected based on results obtained from Omnilog® Phenotypic MicroArray for *Acb*-complex reference isolates IMT30483, IMT30485, IMT30488 and IMT30487 tested utilizing microtiter plates PM01 and PM2A; substrates were selected because the tested reference isolates showed different metabolic capabilities and thus substrates might be suitable for species discrimination; clinical isolates were randomly chosen; number of random samples reflects clinical relevance of the respective *Acb*-complex species (IMT30488 was the only investigated *A. nosocomialis* isolate since no clinical isolate could be collected); positive metabolization is reflected by color change of the indicator phenol red from red to yellow after 24h of incubation at 37°C; +: positive metabolization; -: no metabolization; 1: biological replicate 1; 2: biological replicate 2; 3: biological replicate 3; (R): reference isolate

Table 35: MIC values (µg/ml) of extensively-drug resistant (XDR) *A. baumannii* isolates of human and animal origin

| designation | host species | ENR | GM | TE | IP | T/S | AMC | PIP | CR | PB |
|---|---|---|---|---|---|---|---|---|---|---|
| IMT30947 | human | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | ≥ 64.0 | 0.5 |
| IMT31081 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | 16.0 | 1.0 |
| IMT31105 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | 16.0 | 2.0 |
| IMT31106 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | ≥ 64.0 | 2.0 |
| IMT31395 | dog | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | 16.0 | 1.0 |
| IMT31566 | human | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | ≥ 64.0 | 1.0 |
| IMT32487 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≤ 1.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | ≥ 64.0 | 1.0 |
| IMT32491 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | 2.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | 32.0 | 1.0 |
| IMT32875 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≥ 16.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | 16.0 | 1.0 |
| IMT32904 | cat | ≥ 4.0 | ≥ 16.0 | ≥ 16.0 | ≤ 1.0 | ≥ 4/ 76 | ≥ 32.0 | ≥ 128.0 | ≥ 64.0 | 1.0 |

Clinical human and animal *A. baumannii* isolates derive from various specimens and have been tested for their resistance pattern using the Vitek®2 system (BioMeriéux, France) by means of the Vitek®2 antimicrobial susceptibility panel for Gram-negative bacteria (AST-GN38, developed for veterinary use); assessment of resistance was made according to the breakpoints given in the CLSI guidelines M100-S26 and VET01S2 for *Acinetobacter* spp.; ENR: enrofloxacin, GM: gentamicin, TE: tetracycline, IP: imipenem, T/S: trimethoprim/sulfamethoxazole, AMC: amoxicillin/clavulanic acid, PIP: piperacillin, CR: cefpirome, PB: polymyxin B; the term extensively-drug resistant (XDR) was used according to Magiorakos et al. [109] for isolates that were susceptible against ≤ tested antimicrobial classes

Table 36: MLST sequence types and distance matrix results based on whole genome sequences of selected human and animal *A. baumannii* isolates (n=37)

| designation | host | specimen | sequence type (ST) | resistance genes | closest human isolate | closest animal isolate | SNPs to closest human isolate | SNPs to closest animal isolate |
|---|---|---|---|---|---|---|---|---|
| IMT30813 | human | tissue | unknown ST | *blaADC-2*, *blaOXA-91*, *blaTEM-116* | IMT31862[C] | IMT32473 | 32 444 | 33 231 |
| IMT30819 | human | pharynx | unknown ST | *blaADC-25*, *blaOXA-64* | IMT31862 | IMT31305[C] | 33 739 | 33 592 |
| IMT30823 | human | tracheal secretion | ST40 | *blaADC-25*, *blaOXA-69* | IMT32310[C] | IMT31302 | 35 089 | 35 141 |
| IMT30922 | human | wound | ST21 | *blaADC-25*, *blaOXA-51* | IMT31862[C] | IMT32473 | 28 406 | 31 941 |
| IMT30938 | human | pharynx | ST23 | *blaADC-25*, *blaOXA-68* | BJAB0715[C] | IMT31305 | 410 | 34 852 |
| IMT30945 | human | skin swab | unknown ST | *blaADC-25*, *blaOXA-51* | IMT31552[C] | IMT31305 | 34 333 | 34 379 |
| IMT31122 | dog | trachea | unknown ST | *blaADC-25*, *blaOXA-70* | IMT32312[C] | IMT32786 | 31 637 | 34 571 |
| IMT31134 | dog | wound | ST241 | *blaADC-25*, *blaOXA-91* | IMT33018 | IMT31305[C] | 33 801 | 33 720 |
| IMT31302 | pig | feces | unknown ST | *blaADC-25*, *blaOXA-65* | IMT31862 | IMT32889[C] | 32 893 | 32 575 |
| IMT31303 | pig | feces | ST465 | *blaADC-25*, *blaOXA-51* | IMT31312[C] | IMT31305 | 34 059 | 34 110 |
| IMT31305 | pig | feces | unknown ST | *blaADC-25*, *blaOXA-75* | IMT31862[C] | IMT32473 | 32 052 | 32 117 |
| IMT31552 | human | nose | ST2 | *blaADC-25*, *blaOXA-66*, *aacC1*, *strA*, *strB*, *aadA1*, *sul1* | BJAB07104[C] | IMT31305 | 1 237 | 32 744 |
| IMT31562 | human | wound | ST106 | *blaADC-25*, *bla OXA-91* | IMT31310 | IMT31305[C] | 35 630 | 35 247 |
| IMT31566 | human | tracheal secretion | ST2 | *blaOXA-23*, *blaOXA-66*, *armA*, *aphA6*, *strA*, *strB*, *sul1*, *msrE*, *mphE* | BJAB0868[C] | IMT31305 | 1 030 | 32 372 |
| IMT31581 | human | abdominal cavity | unknown ST | *blaADC-25*, *blaOXA-51* | IMT31862 | IMT31305[C] | 33 019 | 32 644 |
| IMT31853 | human | wound | unknown ST | *blaADC-25*, *blaOXA-106*, *aph(3')-IIa* | IMT32312[C] | IMT32889 | 32 797 | 33 463 |

Table 36: Continued

| designation | host | specimen | sequence type (ST) | resistance genes | closest human isolate | closest animal isolate | SNPs to closest human isolate | SNPs to closest animal isolate |
|---|---|---|---|---|---|---|---|---|
| IMT31862 | human | sputum | unknown ST | *blaADC-25*, *blaOXA-64* | IMT30922[C] | IMT32473 | 28 406 | 31 477 |
| IMT31875 | human | blood culture | unknown ST | *blaADC-25*, *blaOXA-51* | IMT31862[C] | IMT31305 | 34 012 | 34 158 |
| IMT32277 | human | urine | unknown ST | *blaADC-25*, *blaOXA-67* | IMT31682[C] | IMT31305 | 33 951 | 34 020 |
| IMT32310 | human | urine | unknown ST | *blaOXA-71* | IMT31862 | IMT31305[C] | 32 781 | 32 077 |
| IMT32312 | human | sputum | unknown ST | *blaADC-25*, *blaOXA-51*, | IMT31862 | IMT31122[C] | 32 790 | 31 637 |
| IMT32473 | rabbit | nose | unknown ST | *blaADC-25*, *blaOXA-93* | IMT31862[C] | IMT31305 | 31 477 | 32 117 |
| IMT32503 | dog | trachea | unknown ST | *blaADC-25*, *blaOXA-91* | IMT31862 | IMT31305[C] | 33 849 | 33 631 |
| IMT32876 | snake | trachea | unknown ST | *blaADC-25*, *blaOXA-66* | IMT32312[C] | IMT31305 | 33 215 | 34 129 |
| IMT32889 | dog | ear | unknown ST | *blaADC-25*, *blaOXA-70* | IMT33018 | IMT31302[C] | 32 840 | 32 575 |
| IMT32894 | rabbit | tissue | ST22 | *blaADC-25*, *blaOXA-69* | IMT31566 | IMT31305[C] | 33 561 | 33 045 |
| IMT33018 | human | ulcer | unknown ST | *blaADC-25*, *blaOXA-100* | AbH120-A2[C] | IMT31305 | 23 431 | 32 424 |
| 1656-2 | human | published genome[A] | ST2 | n.t. | ACICU[C] | IMT31305 | 3 565 | 32 534 |
| AB0057 | human | published genome[A] | ST1 | n.t. | IMT31312[C] | IMT31305 | 34 021 | 34 638 |
| AbH120-A2 | human | published genome[A] | ST79 | n.t. | IMT33018[C] | IMT31305 | 23 431 | 33 175 |
| ACICU | human | published genome[A] | ST2 | n.t. | 1656-2[C] | IMT31305 | 3 565 | 32 719 |
| BJAB0715 | human | published genome[A] | ST23 | n.t. | IMT30938[C] | IMT31305 | 410 | 35 039 |
| BJAB0868 | human | published genome[A] | ST2 | n.t. | IMT31566[C] | IMT31305 | 1 030 | 32 463 |

Table 36: Continued

| designation | host | specimen | sequence type (ST) | resistance genes | closest human isolate | closest animal isolate | SNPs to closest human isolate | SNPs to closest animal isolate |
|---|---|---|---|---|---|---|---|---|
| BJAB07104 | human | published genome[A] | ST2 | n.t. | IMT31552[C] | IMT31305 | 1 237 | 32 930 |
| D1279779 | human | published genome[A] | ST267 | n.t. | MDR-TJ[C] | IMT32503 | 33 204 | 34 245 |
| MDR-TJ | human | published genome[A] | ST2 | n.t. | IMT31552[C] | IMT31305 | 2 306 | 33 064 |
| MDR-ZJ06 | human | published genome[A] | ST2 | n.t. | IMT31552[C] | IMT31305 | 1 945 | 33 021 |

A total of 2506 orthologous genes were present in all investigated isolates (human isolates: n=27, animal isolates n=10) and thus represent the maximum common genome (MCG) with a length of 2.065.761 bp; a distance matrix displaying the number of single nucleotide polymorphisms (SNPs) in the pairwise alignment of the MCG of the investigated isolates was calculated; the determined number of SNPs in the pairwise alignments of the MCG correlates with the distance of the respective isolates; Pasteur sequence type (ST) and resistance genes have been identified using the Centre of Genomic Epidemiology Server (CGE); [C:] closest isolate (isolate with smallest number of SNPs in the pairwise alignment) according to distance matrix; [A:] for GeneBank® accession number please see table 29

Table 37: Mean MIC values (µg/ml) of enrofloxacin (ENR) sensitive *A. baumannii* wild-type isolates and spontaneous resistant mutant isolates (ENRres)

| wild-type isolate | mutant | ENR | AMP | PIP | PX | CR | RI | TE | GM | IP | CO | T/S | MDR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IMT31302 | wild-type | 0.06 S | 9.33 X | 8.00 S | 13.33 R | 1.33 S | 6.00 X | 1.50 S | 0.75 S | 0.19 S | 0.09 S | 0.17/3.23 S | no |
| IMT31302 | ENRres1 | 5.33 R | 6.67 X | 21.33 S | 24.00 R | 1.67 S | 2.67 X | 1.00 S | 0.04 S | 0.06 S | 0.02 S | 14.67/278.73 R | yes |
| IMT31302 | ENRres2 | 16.00 R | 14.67 X | 1.33 S | 10.67 R | 0.46 S | 0.58 X | 0.21 S | 0.02 S | 0.04 S | 0.02 S | ≥32.00/608.00 R | yes |
| IMT31302 | ENRres3 | 1.83 i | 21.33 X | 10.67 S | 6.00 S | 0.34 S | 1.67 X | 0.42 S | 0.10 S | 0.13 S | 0.03 S | 25.33/481.27 R | no |
| IMT31303 | wild-type | 0.07 S | 3.67 X | 8.00 S | 8.00 R | 1.33 S | 7.33 X | 0.92 S | 0.67 S | 0.13 S | 0.09 S | 0.23/4.37 S | no |
| IMT31303 | ENRres4 I | ≥32.00 R | 16.00 X | 42.67 S | ≥256.00 R | 3.67 S | 5.33 X | 2.00 S | 0.04 S | 0.07 S | 0.02 S | 0.92/17.48 S | no |
| IMT31303 | ENRres4 II | ≥32.00 R | 29.33 X | 48.00 S | ≥256.00 R | 5.33 S | 5.33 X | 1.50 S | 0.02 S | 0.07 S | 0.03 S | 1.83/34.77 S | no |
| IMT31303 | ENRres5 | ≥32.00 R | 12.00 X | 14.67 S | ≥256.00 R | 4.00 S | 1.83 X | 2.00 S | 0.03 S | 0.06 S | 0.02 S | 1.83/34.77 S | no |
| IMT31303 | ENRres6 | 8.67 R | 8.67 X | 17.33 S | 48.00 R | 2.00 S | 6.00 X | 1.17 S | 0.06 S | 0.08 S | 0.05 S | 0.42/7.98 S | no |
| IMT31303 | ENRres7 I | ≥32.00 R | 16.00 X | 29.33 S | ≥256.00 R | 4.00 S | 4.00 X | 1.33 S | 0.04 S | 0.06 S | 0.03 S | 0.58/11.02 S | no |
| IMT31303 | ENRres7 II | ≥32.00 R | 7.33 X | 10.00 S | 144.00 R | 1.83 S | 6.67 X | 0.54 S | 0.02 S | 0.02 S | 0.02 S | 6.00/114.00 R | yes |
| IMT31305 | wild-type | 0.06 S | 9.33 X | 9.33 S | 16.00 R | 1.50 S | 6.00 X | 1.83 S | 0.75 S | 0.19 S | 0.38 S | 0.19/3.61 S | no |
| IMT31305 | ENRres8 | 13.33 R | 8.00 X | 21.33 S | ≥256.00 R | 3.83 S | 8.00 X | 1.00 S | 0.30 S | 0.07 S | 0.04 S | 0.92/17.48 S | no |
| IMT31305 | ENRres9 | 5.33 R | 24.0 X | 17.33 S | 18.67 R | 0.58 S | 1.83 X | 0.38 S | 0.25 S | 0.07 S | 0.03 S | 4.67/88.73 R | yes |
| IMT31305 | ENRres10 I | 21.33 R | 6.67 X | 6.67 S | 32.00 R | 2.33 S | 7.33 X | 0.75 S | 0.42 S | 0.07 S | 0.04 S | 1.67/31.73 S | no |
| IMT31305 | ENRres10 II | ≥32.00 R | 4.00 X | 3.67 S | 10.67 R | 0.50 S | 6.67 X | 0.67 S | 0.46 S | 0.04 S | 0.05 S | 0.67/12.73 S | no |
| IMT31305 | ENRres11 I | 2.33 i | 18.67 X | 2.67 S | 16.00 R | 0.75 S | 2.00 X | 0.21 S | 0.25 S | 0.10 S | 0.03 S | 1.83/34.77 S | no |
| IMT31305 | ENRres11 II | 18.67 R | 18.67 X | 2.33 S | 18.67 R | 1.17 S | 2.33 X | 0.25 S | 0.07 S | 0.09 S | 0.02 S | 22.67/430.73 R | yes |

Isolates were tested for their antimicrobial susceptibility using Etest® (BioMeriéux, France); resistance was assessed according to the CLSI guidelines M100-S26 and VET01S2 for *Acinetobacter* spp.; the term multi-drug resistant was used for isolates resistant against ≥ 3 tested antimicrobials; S:susceptible; R: resistant, i: intermediate; X: no breakpoints given in the utilized guidelines

Table 38: Gene products encoded on putative *A. baumannii* plasmid pAB31566

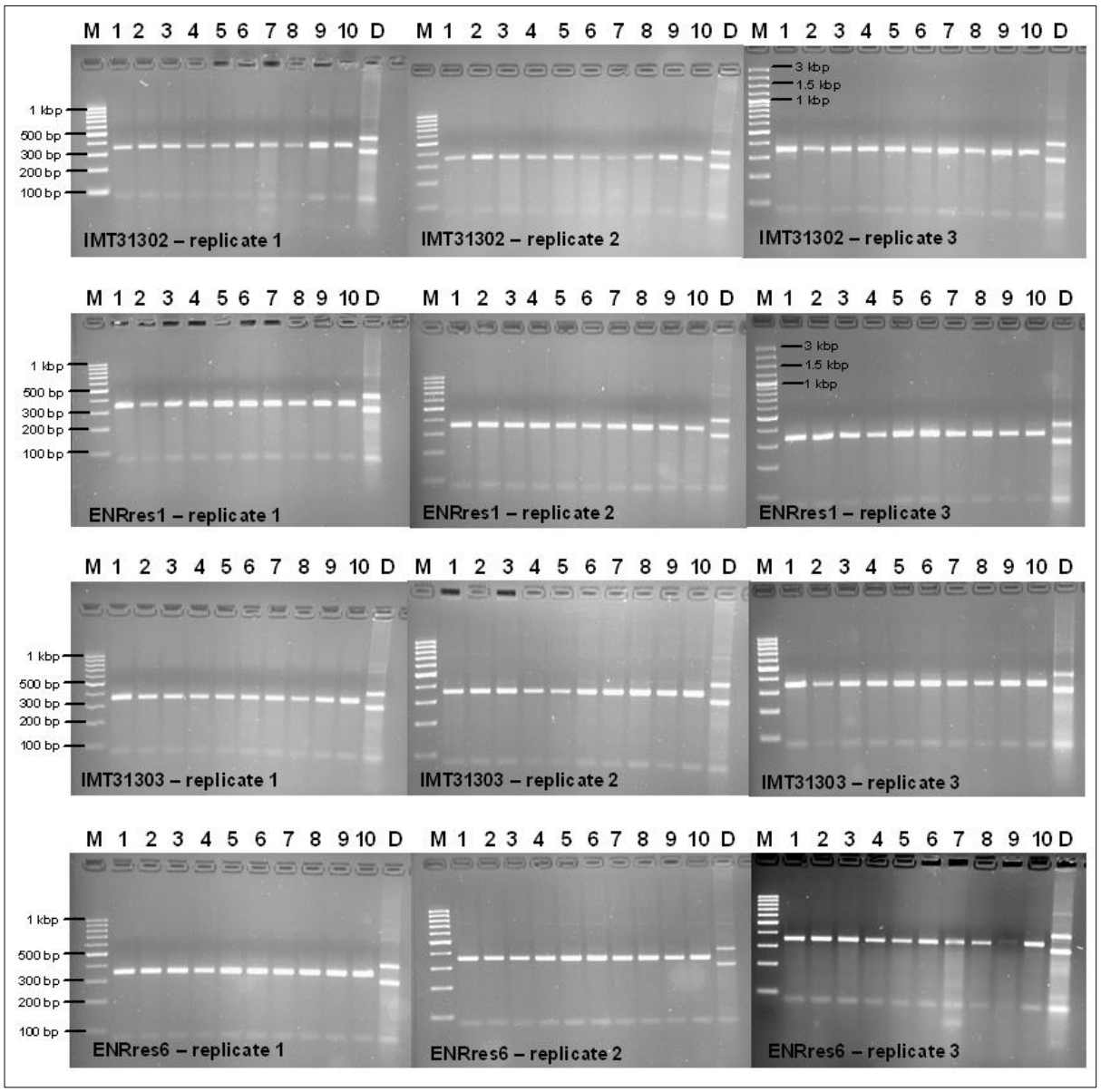
| gene products | length of gene (bp) |
|---|---|
| aminoglycoside phosphotransferase (AphA6) | 780 |
| ATP-dependent protease subunit | 309 |
| beta-lactamase (OXA-23) | 822 |
| cement precursor protein 3B variant 2 | 360 |
| error-prone, lesion bypass DNA polymerase V (UmuC) | 1293 |
| chromosome (plasmid) partitioning protein ParA | 774 |
| chromosome (plasmid) partitioning protein ParB | 1257 |
| conjugative transfer transglycosylase/ murein transglycosylase | 498 |
| cro-like protein/ DNA-binding protein | 300 |
| diaminopimelate decarboxylase/ addiction module toxin RelE | 360 |
| DnaJ-class molecular chaperone | 498 |
| hypothetical protein (n=45 ) | 129 – 1281 |
| micrococcal nuclease precursor | 477 |
| mobile element protein (n=3) | 156 – 687 |
| ornithine cyclodeaminase | 381 |
| probable resolvase | 639 |
| protein of unknown function DUF1173 | 1266 |
| putative DNA-binding protein | 327 |
| replicase RepA | 1164 |
| tellurite resistance protein/ toxic anion resistance protein TelA | 1104 |
| thiol:disulfide interchange protein DsbC | 723 |
| TraB | 1317 |
| TraC | 2727 |
| TraD | 2160 |
| TraE | 579 |
| TraF | 813 |
| TraG | 3003 |
| TraH | 1428 |
| TraK | 717 |
| TraL | 294 |
| TraN | 2058 |
| TraU | 1044 |
| TraV | 654 |
| TraW | 639 |
| TrbC | 708 |
| TrhF | 417 |
| TrwC (TraI homolog) | 3276 |

Table 38: Continued

| gene products | length of gene (bp) |
|---|---|
| type II restriction enzyme, methylase subunit YeeA | 333 |
| DNA helicase, restriction/modification system component YeeB | 555 |
| zeta toxin family protein | 1083 |

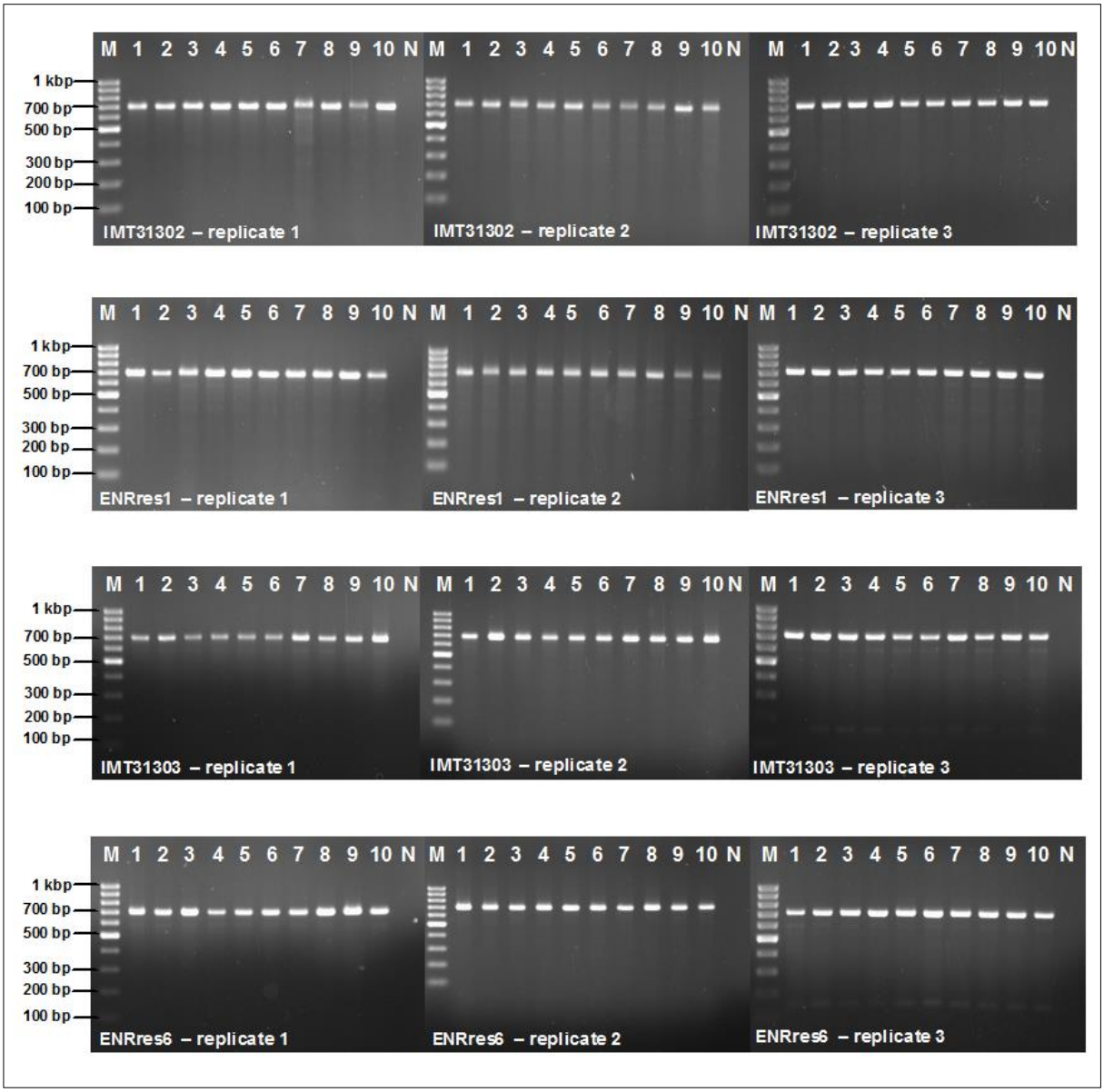
Sequence prediction of putative plasmid pAB31566 was performed using Geneious®6 and Basic Local Alignment Search Tool (Blast®), reference plasmids used for sequence prediction are listed in table 31; gene annotation was done using the RAST server

Figure 19: Electropherogram of 16S-23S IGS RFLP restriction patterns for transconjugant colonies of IMT31302/ENRres1 and IMT31303/ENRres6 after transfer of putative plasmid pAB31566




Transconjugant colonies were achieved by subcultivation on COL S+ agar plates supplemented with 100 µg/ml kanamycin after transfer of the putative plasmid pAB31566 from *A. haemolyticus* IMT32484_*aphA6* to the *A. baumannii* enrofloxacin (ENR) sensitive wild-type isolates IMT31302 and IMT31303 as well as to their respective spontaneous ENR resistant mutants ENRres1 and ENRres6; restriction of 16S-23S intergenic spacer (IGS) amplicons was achieved by *Mbo*II; conjugation experiments were performed in several biological replicates; 1-10 number of transconjugant colony (restriction pattern corresponds to species-specific restriction pattern for *A. baumannii*); D: Donor isolate IMT32484_*aphA*; M: 100 bp DNA size marker (for IMT31302 replicate 3 and ENRres1 replicate 3: M = 100 bp plus DNA size marker (Thermo Fisher Scientific)); running conditions: 1.5% agarose gel, 120 V, 45 min, 1xTBE buffer

Figure 20: Electropherogram of a*phA6* PCR for transconjugant colonies of IMT31302/ ENRres1 and IMT31303/ ENRres6




Transconjugant colonies were achieved by subcultivation on COL S+ agar plates supplemented with 100 µg/ml kanamycin after transfer of the putative plasmid pAB31566 from *A. haemolyticus* IMT32484_*aphA6* to the *A. baumannii* enrofloxacin (ENR) sensitive wild-type isolates IMT31302 and IMT31303 as well as to their respective spontaneous ENR resistant mutants ENRres1 and ENRres6; conjugation experiments were performed in several biological replicates; 1-10 number of transconjugant colony; N: negative control; M: 100 bp DNA size marker (Thermo Fisher Scientific); running conditions: 1.5% agarose gel, 120 V, 45 min, 1xTBE buffer

Table 39: Calculated colony forming units (cfu)/ml) for transconjugants of the enrofloxacin (ENR) sensitive wild-type isolates and their respective spontaneous resistant mutants

| designation | cfu/ml x $10^7$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | replicate 1 | replicate 2 | replicate 3 | replicate 4 | replicate 5 | replicate 6 | replicate 7 | replicate 8 | replicate 9 |
| IMT31302 | 6.65 | 21.98 | 16.56 | 68.35 | 2.85 | 134.75 | 4.46 | 10.68 | 0.00 |
| ENRres1 | 1.51 | 4.13 | 128.05 | 98.50 | 26.58 | 34.28 | 4.52 | 3.89 | 0.00 |
| IMT31303 | 5.95 | 1.64 | 7.95 | not tested | not tested | not tested | not tested | not tested | not tested |
| ENRres6 | 2.08 | 0.39 | 3.28 | not tested | not tested | not tested | not tested | not tested | not tested |
| IMT31305 | 0.00 | 0.00 | 0.00 | not tested | not tested | not tested | not tested | not tested | not tested |
| ENRres9 | 0.00 | 0.00 | 0.00 | not tested | not tested | not tested | not tested | not tested | not tested |

Colony forming units (cfu)/ml were calculated based on plating of three dilutions of the respective donor/ recipient solutions on COL S+ agar plates supplemented with 100 µg/ml kanamycin; *A. baumannii* colonies grown on COL S+ agar plates +100 µg/ml kanamycin are transconjugant colonies achieved by transfer of putative plasmid pAB31566 from *A. haemolyticus* IMT32484_*aphA6* to the *A. baumannii* enrofloxacin (ENR) sensitive wild-type isolates IMT31302, IMT31303 and IMT31305 as well as to their respective spontaneous ENR resistant mutants ENRres1, ENRres6 and ENRres9; cfu/ml were calculated for three biological replicates for IMT31303 and IMT31305 and their mutants ENRres6 and ENRres9; in order to test the stability of the conjugation assay nine biological replicates were done for IMT31302 and its mutant ENRres1

Table 40: Calculated p-values for NF-KB reporter assays performed for enrofloxacin (ENR) sensitive *A. baumannii* wild-type isolates and respective resistant mutants utilizing cell lines 3D4/31 and THP-1

| | | 3D4/31 cells | | THP-1 cells | |
|---|---|---|---|---|---|
| designation | compared to | p – value 7h p.i. | p – value 19h p.i. | p – value 7 h p.i. | p – value 19h p.i. |
| IMT31302 | IMT31303 | 0.950 | 1.000 | 0.992 | 0.990 |
| | IMT31305 | 0.998 | 0.222 | 1.000 | 0.999 |
| | ENRres1 | 0.128 | 1.000 | 0.906 | 1.000 |
| | ENRres6 | 1.000 | 0.969 | 0.841 | 0.809 |
| | ENRres9 | 1.000 | 0.998 | 0.952 | 0.992 |
| IMT31303 | IMT31302 | 0.950 | 1.000 | 0.992 | 0.990 |
| | IMT31305 | 0.790 | 0.339 | 0.947 | 0.936 |
| | ENRres1 | 0.921 | 1.000 | 0.997 | 0.999 |
| | ENRres6 | 0.431 | 0.997 | 0.988 | 0.983 |
| | ENRres9 | 0.983 | 1.000 | 1.000 | 0.858 |
| IMT31305 | IMT31302 | 0.998 | 0.222 | 1.000 | 0.999 |
| | IMT31303 | 0.790 | 0.339 | 0.947 | 0.936 |
| | ENRres1 | 0.064 | 0.259 | 0.770 | 0.994 |
| | ENRres6 | 0.999 | 0.577 | 0.681 | 0.630 |
| | ENRres9 | 0.987 | 0.390 | 0.848 | 1.000 |
| ENRres1 | IMT31302 | 0.128 | 0.969 | 0.841 | 0.809 |
| | IMT31303 | 0.431 | 0.997 | 0.988 | 0.983 |
| | IMT31305 | 0.064 | 0.577 | 0.681 | 0.630 |
| | ENRres6 | 0.108 | 0.984 | 1.000 | 0.896 |
| | ENRres9 | 0.174 | 0.999 | 0.999 | 0.504 |
| ENRres6 | IMT31302 | 1.000 | 1.000 | 0.906 | 1.000 |
| | IMT31303 | 0.921 | 1.000 | 0.997 | 0.999 |
| | IMT31305 | 0.999 | 0.259 | 0.770 | 0.994 |
| | ENRres1 | 0.108 | 0.984 | 1.000 | 0.896 |
| | ENRres9 | 1.000 | 1.000 | 1.000 | 0.970 |
| ENRres9 | IMT31302 | 1.000 | 0.998 | 0.952 | 0.992 |
| | IMT31303 | 0.983 | 1.000 | 1.000 | 0.858 |
| | IMT31305 | 0.987 | 0.390 | 0.848 | 1.000 |
| | ENRres1 | 1.000 | 1.000 | 1.000 | 0.970 |
| | ENRres6 | 0.174 | 0.999 | 0.999 | 0.504 |

NF-KB reporter assays were performed for the porcine macrophage cell line 3D4/31 and the human monocytic cell line THP-1; cell lines were infected with enrofloxacin (ENR) sensitive *A. baumannii* wild-type isolates IMT31302, IMT31303 and IMT31305 and their respective spontaneous resistant mutants ENRres1, ENRres6 and ENRres9; p-values were calculated for the pairwise comparison of the investigated isolates (Tukey test) based on the measured Lum/E values at 7h and 19h post infection of the cell lines with the respective *A. baumannii* isolate; measured Lum/E values are proportional to NF-KB expression

Table 41: Genomic mutations identified in enrofloxacin (ENR) resistant *A. baumannii* mutant isolates by SNP analysis of their whole genome sequences

| | IMT31302 (wt) | | | IMT31303 (wt) | | | | | | IMT31305 (wt) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| gene/ protein | ENRres 1 | ENRres 2 | ENRres 3 | ENRres 4 I | ENRres 4 II | ENRres 5 | ENRres 6 | ENRres 7 I | ENRres 7 II | ENRres 8 | ENRres 9 | ENRres 10 | ENRres 10 II | ENRres 11 I | ENRres 11 II |
| *gyrA* | | | | bp242: C→T | bp242: C→T | bp242: C→T | bp242: C→T | bp242: C→T | bp242: C→T | | bp1841: CAC I. | | | bp1841: CAC I. | bp1841: CAC I. |
| *gyrB* | | bp1469: GTA I. | | | | | | | | bp1469: GTG I. | | bp1469: GTG I. | bp1469: GTG I. | | |
| *adeL* | bp911: 4 bp I. | bp994: 21 bp D | bp994: 21 bp D | | | | | | | | bp994: C→T | | | bp994: C→T | bp994: C→T |
| *adeN* | bp61: G→A | | | bp61: 26 bp D | bp61: 26 bp D | bp61: 26 bp D | bp61: 26 bp D | bp61: 26 bp D | bp61: 26 bp D | bp205: GGC I. | | bp205: GGC I. | bp205: GGC I. | | |
| L23p | | | | bp250: GT I. | bp250: GT I. | | | | | | | | | | |
| S14p | | | | | | bp31: 15 bp D | | | | | | | | | |
| S18p | | | | | | | | bp121: CTT I. | bp121: CTT I. | | | | | | |
| *panB* | | | | | | | | | | bp347: 4 bp I. | | | | | |
| *metH* | | | | | | | | | | bp3356: AGC I. | | bp3356: AGC I. | bp3356: AGC I. | | |
| *corA* variant I | bp131: T→G | bp597: 10 bp D | bp597: 10 bp D | | | | | | | | bp873: G→A | | | bp873: G→A | bp873: G→A |
| *corA* variant II | | | bp510: 12bp I. | | | | | | | | | | | | |
| $Mg^{2+}$ ATPase | | bp2040: 12 bp D | bp2040: 12 bp D | | | | | | | | | | | | |
| sensor histidine kinase | | bp555: CCT I. | bp555: CCT I. | | | | | | | | | | | | |

Table 41: Continued

| gene/ protein | IMT31302 (wt) | | | IMT31303 (wt) | | | | | | IMT31305 (wt) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ENRres 1 | ENRres 2 | ENRres 3 | ENRres 4 I | ENRres 4 II | ENRres 5 | ENRres 6 | ENRres 7 I | ENRres 7 II | ENRres 8 | ENRres 9 | ENRres 10 | ENRres 10 II | ENRres 11 I | ENRres 11 II |
| putative sensory trans-duction histidine kinase | | | | bp683: C→A | bp683: C→A | bp683: C→A | bp683: C→A | bp683: C→A | bp683: C→A | | | | | | |
| *gltA* | | | | | | | | | | | bp1259: C I. | bp159: 4 bp I. | bp159: 4 bp I. | bp1259: C I. | bp1259: C I. |
| *atpI* | | | | bp213: GC I. | bp213: GC I. | bp213: GC I. | bp213: GC I. | bp213: GC I. | bp213: GC I. | | | | | | |
| *fabI* | | | | | | | | | bp380: C→T | | | | | | |
| *hisB* | | | | | | | | | bp203: G→A | | | | | | |
| *rpoA* | | | | | | | bp1008: A→T | | | | | | | | |
| *pnp* | | | | | | | | | | | | | | bp1282: C→T | |
| *rho* | | bp196: C→T | | | | | | | | | | | | | |
| IMP | bp1364: CTG I. | | | | | | | | | | | | | | |
| hypotheti-cal protein | bp833: T→G | | | | | | | | | | | | | | |
| DUF1176 | bp1031: 10 bp D | | | | | | | | | | | | | | |

Table 41: Continued

| gene/ protein | IMT31302 (wt) | | | IMT31303 (wt) | | | | | | IMT31305 (wt) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ENRres 1 | ENRres 2 | ENRres 3 | ENRres 4 I | ENRres 4 II | ENRres 5 | ENRres 6 | ENRres 7 I | ENRres 7 II | ENRres 8 | ENRres 9 | ENRres 10 | ENRres 10 II | ENRres 11 I | ENRres 11 II |
| NAD(P) transhydrogenase alpha subunit | | bp132: C I. | | | | | | | | | | | | | |
| tRNA Asp, tRNA Val, tRNA Asp | | 337bp D | 337bp D | | | | | | | | | | | | |

Genomic mutations were identified by single nucleotide polymorphism (SNP) analysis of the whole genome sequences of the investigated spontaneous enrofloxacin (ENR) resistant *A. baumannii* mutant isolates (ENRres) in comparison to the whole genome sequences of their respective ENR sensitive wild-type isolate (wt); I: insertion, D: deletion; →: indicates base substitution (e.g. T→G means substitution of T by G); A: adenosine, C: cytosine, G: guanine, T: thymin; bp: base pair, numbers behind abbreviation 'bp' give location of SNP within the respective gene; additives 'I' and 'II' in mutant names indicate presence of two stable phenotypic lineages displaying large and small colony variants

## II Buffers and solutions

### 1 Species identification based on selected carbon sources

*Acinetobacter* test medium

| components | volume |
|---|---|
| 5x M9 Minimum salts | 16.0 ml |
| $CaCl_2$ (1M) | 8 µl |
| $MgSO_4$ (1M) | 160 µl |
| casein peptone (10%) | 800 µl |
| indicator (TTC or phenolred) | 800 µl |
| respective carbon source solution (20%) | 4.0 ml |
| Luria Bertani broth | 1. ml |
| $ddH_2O$ | ad 80.0 ml |

### 2 Plasmidpreparation

500 mM EDTA pH 8.00

| components | amount | |
|---|---|---|
| EDTA | 186.15 g | solve in 800 ml $ddH_2O$ and adjust pH to 8.00 |
| $ddH_2O$ | ad 1.00 l | |

10 % SDS solution

| components | amount |
|---|---|
| SDS | 5.00 g |
| $ddH_2O$ | ad 50.00 ml |

250 mM Tris

| components | amount |
|---|---|
| Tris | 15.14 g |
| $ddH_2O$ | ad 500.00 ml |

1 M Tris-Cl (pH 8.00)

| components | amount | |
|---|---|---|
| Tris | 60.57 g | solve in 30 ml $ddH_2O$ and adjust pH to 8.00 |
| $ddH_2O$ | ad 500.00 ml | |

TE Buffer (sterile autoclaved)

| components | amount |
|---|---|
| Tris-Cl (pH 8.00) | 5.00 ml |
| 500 mM EDTA | 1.00 ml |
| $ddH_2O$ | ad 500.00 ml |

Lysis Buffer

| components | amount |
|---|---|
| Millipore $H_2O$ | 950.00 µl |
| 10 % SDS solution | 600.00 µl |
| 250 mM Tris | 400.00 µl |
| 5 N NaOH | 30.00 µl |

## III Consumables and media for bacterial cultivation

Table 42: Consumables and media for bacterial cultivation

| item | catalog number | supplier |
|---|---|---|
| Biolog Redox Dye Mix A (100X), 20 ml | 74221 | Biolog, USA |
| Brain-Heart Infusion broth | CM1135B | Oxoid, Germany |
| Cell culture flask 25 $cm^2$ | CLS430639-20EA | Sigma-Aldrich, Germany |
| COL S+ agar plates | PB5039A | Oxoid, Germany |
| COL S+ agar plates | 254071 | Becton Dickinson, Germany |
| Cryo-pure 1.6 ml tube | 72.380 | Sarstedt, Germany |
| Etest ampicillin | 412253 | BioMeriéux, France |
| Etest cefpirome | 506400 | BioMeriéux, France |
| Etest cefpodoxime | 412289 | BioMeriéux, France |
| Etest colistin | 537300 | BioMeriéux, France |
| Etest enrofloxacin | 528900 | BioMeriéux, France |
| Etest gentamicin | 412368 | BioMeriéux, France |

Table 42: Continued

| item | catalog number | supplier |
|---|---|---|
| Etest imipenem | 412374 | BioMeriéux, France |
| Etest piperacillin | 412436 | BioMeriéux, France |
| Etest rifampicin | 412450 | BioMeriéux, France |
| Etest tetracycline | 412471 | BioMeriéux, France |
| Etest trimethoprim/ sulfamethoxazole | 412481 | BioMeriéux, France |
| Eppendorf tube 0.2 ml | 72.737.002 | Sarstedt, Germany |
| Eppendorf tube 0.5 ml | 72.735.992 | Sarstedt, Germany |
| Eppendorf tube 1.5 ml | 72.690.550 | Sarstedt, Germany |
| Eppendorf tube 1.5 ml safe seal | 72.706 | Sarstedt, Germany |
| Eppendorf tube 2.0 ml safe seal | 72.695.500 | Sarstedt, Germany |
| Falcon tube 15 ml | 62.554.502 | Sarstedt, Germany |
| Falcon tube 50 ml | 62.559.001 | Sarstedt, Germany |
| Inoculation loop, 1 µl | 86.1567.010 | Sarstedt, Germany |
| Iscove's Basal Medium with stable glutamin | FG 0465 | Biochrom, Germany |
| Luria Bertani broth | 6673.1 | Roth, Germany |
| Luria Bertani agar | 6675.1 | Roth, Germany |
| MasterPure DNA Purification Kit for Blood II | MB711740 | Epicentre Biotechnologies, USA |
| Microtiter plate (96-well flat bottom) | CLS3599-100EA | Sigma-Aldrich, Germany |
| Midori Green Advance | MG 04 | Nippon Genetics, Europe |
| Mueller-Hinton agar | X926.1 | Roth, Germany |
| Parafilm M | H951.1 | Roth, Germany |
| Pasteur pipette | 4522.1 | Roth, Germany |
| 10x PBS Dulbecco | L1835 | Biochrom, Germany |
| Petri dish empty (sterile), 92x16mm | 82.1473 | Sarstedt, Germany |
| Photometer cuvettes 1.5 ml | 759015 | Brand, Germany |
| Pipette tip 10.0 µl | 70.1130 | Sarstedt, Germany |
| Pipette tip 2 - 200.0 µl | 70.760.002 | Sarstedt, Germany |
| Pipette tip 50-1000 µl | 70.762 | Sarstedt, Germany |
| Pipette tip1250 µl | 3201 | Biolog, USA |
| PM01 plate for carbon sources | 12111 | Biolog, USA |
| PM2A plate for carbon sources | 12112 | Biolog, USA |
| Spreader, plastic | 86.1569.005 | Sarstedt, Germany |
| VITEK®2 AST card AST-GN38 | 22331 | BioMeriéux, France |
| VITEK®2 AST card AST-N263 | 413 755 | BioMeriéux, France |
| Wooden cotton swab | 80.628 | Sarstedt, Germany |

## IV Chemicals and enzymes

Table 43: Chemicals and enzymes

| reagents | catalog number | supplier |
|---|---|---|
| Acetic acid, 100%, p.a. | 3738.4 | Roth, Germany |
| Ammonium chloride | A0171-100G | Sigma-Aldrich, Germany |
| Agarose | CH1001.0500 | Biodeal, New Zealand |
| Boric acid | 6943.1 | Roth, Germany |
| Bright-Glo luciferase Assay substrate | E263A | Promega, Germany |
| Buffer B (10x) | BB5 | Thermo Fisher Scientific, Germany |
| 10x Green Buffer (with 20 mM MgCl2) | EP0702 | Thermo Fisher Scientific, Germany |
| Calcium chloride-dihydrat | 2382 | Merck Millipore, Germany |
| Citraconic acid | C0363 | TCI, Germany |
| di-Sodium phosphate | 6346 | Merck Millipore, Germany |
| 100 bp DNA Ladder | SM 1441 | Thermo Fisher Scientific, Germany |
| 1 kb DNA Ladder | SM0313 | Thermo Fisher Scientific, Germany |
| dNTPs (2,5 mM each) | 4030 | TaKaRa |
| Dream Taq Green DNA polymerase (85 U/µl) | EP0711 | Thermo Fisher Scientific, Germany |
| EDTA | 8043.2 | Roth, Germany |
| Enrofloxacin | 17849-5G-F | Sigma-Aldrich, Germany |
| Ethanol Rotipuran > = 99.8% | 9065.4 | Roth, Germany |
| Fetal bovine serum | S0113 | Biochrom, Germany |
| Gentamicin | A2712 | Biochrom, Germany |
| Glycerine Rotipuran ≥99,5 % | 3783.1 | Roth, Germany |
| L-Hydroxyproline | H0296 | TCI, Germany |
| Isopropanol | 9866.1 | Roth, Germany |
| Magnesium sulfate | 0261.1 | Roth, Germany |
| DL-Malic acid | M0020 | TCI, Germany |
| L-ornithine | 1.06906 | Merck Millipore, Germany |
| *Mbo*I endonuclease (5 U/µl) | ER0821 | Thermo Fisher Scientific, Germany |
| Tris Pufferan >= 99.9%, ultra quality | 5429.2 | Roth, Germany |
| Potassium dihydrogen orthophosphate | 3904 | Roth, Germany |
| Primer 100 pmol (target specific) | individual | Sigma-Aldrich, Germany or MWG Operon, Germany |
| Proteinase K | 7528.4 | Roth, Germany |
| Puromycin dihydrochloride | P8833-25MG | Sigma-Aldrich, Germany |
| D-Ribose | 1.07605 | Merck Millipore, Germany |
| SDS (Dodecyl sodium sulfat) | 20763.01 | Serva, Germany |
| Sodiumchloride | 3957.2 | Roth, Germany |
| Trypsin/ ETDA solution (10x) | P10-024100 | PAN Biotech, Germany |

## V Devices

Table 44: Devices

| device | type | supplier |
|---|---|---|
| Autoclav | DX-150 | Systec, Germany |
| Benchtop centrifuge for Falcon tubes | 3K30 | Sigma Laborzentrifugen, Germany |
| Benchtop centrifuge for microtiter plates | Rotina 46 R | Andreas Hettich, Germany |
| Biolog OmniLog® | | Biolog, USA |
| Bio Photometer (λ: 600 nm) | 6131 02928 | Eppendorf, Germany |
| Electrophoresis chamber | Compact M | Biometra, Germany |
| Electrophoresis photo documentation | HeroDoc Plus | Herolab, Germany |
| Electrophoresis power supplier | PowerPac Basic | Bio-rad, Germany |
| ELISA reader | Synergy HT | Bio-TEK, Germany |
| Freezer | | Liebherr, Germany |
| Ice machine | AF200 | Scotsman |
| Sequencing Machine | Illumina MiSeq | Illumina, USA |
| Incubator (37°C with 5% CO2) | 700-0029 | Binder, Germany |
| Incubator (37°C) | | |
| Lamina Flow | ScanLaf Mars Safety Class 2 | Labogene, Denmark |
| Millipore water dispenser | Simplicity, SIMS00000 | Merck Millipore, Germany |
| NanoDrop 1000, Spectralphotometer | G029 | Thermo Fisher Scientific, Germany |
| Ovation Electronic Pipettor | 3711 | Biolog, USA |
| Pump for agar preparation | 505DZ | Watson Marlow |
| Refrigerator | | Liebherr, Germany |
| Shaking incubator | 3031 | GFL Gesellschaft für Labortechnik, Germany |
| Tabletop centrifuge for Eppendorf tubes | 5415D | Eppendorf, Germany |
| Thermo Shaker | Thermomixer compact | Eppendorf, Germany |
| Thermocycler | T300 | Biometra, Germany |
| Turbidimeter | 3587 | Biolog, USA |
| Vacuum pump | N735 AN18 | KNF Neuberger, Germany |
| Vortex Mixer | Vortex 3 | IKA, Germany |

## LIST OF PUBLICATIONS

Article:

**Müller S, Janssen T, Wieler LH**. Multidrug resistant *Acinetobacter baumannii* in veterinary medicine--emergence of an underestimated pathogen? Berl Munch Tierarztl Wochenschr. 2014;127(11-12):435-46. PubMed PMID: 25872253.

Poster presentation 1:

**Stefanie Müller, Traute Janßen, Ivonne Stamm, Torsten Schmidt-Wieland, Martina Böhringer, Christa Ewers, Lothar Heinz Wieler:**
A molecular typing method for identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (*Acb*)- complex of human and animal origin. Fachgruppentagung der Deutschen Veterinärmedizinischen Gesellschaft Bakteriologie/Mykologie, Freising, Germany, 2014

Poster presentation 2:

**Stefanie Müller, Traute Janßen, Ivonne Stamm, Martina Böhringer, Torsten Schmidt-Wieland, Lothar Heinz Wieler:**
Characterization of clinical *Acb*- (*Acinetobacter calcoaceticus*- *Acinetobacter baumannii*-) complex isolates of human and animal origin collected during a one year time-period. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Münster, Germany, 2015

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**SELBSTSTÄNDIGKEITSERKLÄRUNG**

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 16. Dezember 2016

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