Original article

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MOLECULAR EPIDEMIOLOGY OF CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII* COMPLEX ISOLATES FROM PATIENTS THAT WERE INJURED DURING THE EASTERN UKRAINIAN CONFLICT

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This study addressed carbapenem-resistant *Acinetobacter baumannii* complex (ABC) isolates from patients that were injured during the military conflict in the Eastern Ukraine and treated at German Armed Forces Hospitals in 2014 and 2015. Clonal diversity of the strains and potential ways of transmission were analyzed.

Patients with one or several isolation events of carbapenem-resistant ABC were included. Isolates were characterized by VITEK II-based identification and resistance testing, molecular screening for frequent carbapenemase genes, and DiversiLab rep-PCR-based typing. Available clinical information of the patients was assessed.

From 21 young male Ukrainian patients with battle injuries, 32 carbapenem- and fluoroquinolone-resistant ABC strains were isolated. Four major clonal clusters were detected. From four patients (19%), ABC isolates from more than one clonal cluster were isolated. The composition of the clusters suggested transmission events prior to the admission to the German hospitals.

The infection and colonization pressure in the conflict regions of the Eastern Ukraine with ABC of low clonal diversity is considerable. Respective infection risks have to be considered in case of battle-related injuries in these regions. The low number of local clones makes any molecular exclusion of transmission events difficult.

Keywords: *Acinetobacter baumannii* complex, rep-PCR, typing, clonal distribution, epidemiology, Ukraine, war, colonization, carbapenem resistance

Introduction

Systemic infections with *Acinetobacter baumannii* complex (ABC) are associated with a low but considerable mortality risk, particularly in the case of infections with multidrug-resistant isolates [1].

Systemic ABC infections with multidrug resistance were found to be frequent on intensive care units (ICU) of Southern Europe, Morocco, Turkey, and Iran [2, 3]. Application of morphine seems to be an independent risk factor for systemic ABC infections of injured patients on ICU with even more importance than the injury itself [4].

Skin and soft-tissue [5, 6], burn wounds [7–10], the lower respiratory tract [11], and combat wounds [12–22] are typical sites of ABC infections. Systemic infections with multidrug-resistant ABC like war-injury associated

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osteomyelitis [23, 24] or infected acute spinal cord injuries [25, 26] are particularly difficult to treat. Application of point-of-injury combat antimicrobials during the management of combat wounds neither increases nor decreases the acquisition risk of multidrug-resistant ABC [27].

The military conflicts of Afghanistan and Iraq were typical settings for combat-associated acquisition of multidrug-resistant ABC [28–35]. ABC infections of combat wounds are usually nosocomially transmitted by medical personnel and not associated with environmental contamination in the battlefield [36–39] or pre-existing skin colonization of the soldiers prior to their injuries [40]. Afghan and Iraqi patients were intensively colonized with multidrug-resistant bacteria, easily leading to nosocomial transmission of ABC if standard hygiene precautions were not adequately enforced [41, 42]. Asymptomatic colonization is also quite frequent in deployed military service members [40, 43, 44].

ABC strains are characterized by a remarkable ability to acquire or upregulate antibiotic drug resistance determinants [45]. This study, in particular, assessed the clonal distribution of carbapenem-resistant ABC isolates that were isolated from patients who were injured during the military conflict in the Eastern Ukraine and treated in German Armed Forces Hospitals. By doing so, potential ways of transmission were analyzed.

Methods

Strains and patients

Carbapenem-resistant *A. baumannii* complex (ABC) strains from patients that were injured in the military conflict in the Eastern Ukraine in 2014 and 2015 and medically treated in the German Armed Forces Hospitals of Berlin, Hamburg, Koblenz, and Ulm were included in the analysis. The strains comprised both clinical isolates like isolates from biopsy material and colonization flora like isolates from pharyngeal swabs. Next to the site of isolation, gender and year of birth of the injured patients were assessed.

The strains were isolated at the Central Institute of the German Armed Forces in Munich for patients that were treated in Ulm, at the Central Institute localized in Berlin for patients that were treated in Hamburg and Berlin, and at the Central Institute in Koblenz for patients that were treated in Koblenz. Accordingly, strains from patients that were treated in Hamburg and Berlin are assessed together in the following.

Clinical data and epidemiological data regarding the transport of patients to Germany and their management at the hospitals were collected from the hygiene departments of the German Armed Forces Hospitals.

Biochemical identification and resistance testing

The ABC strains were biochemically identified using GN-cards (charge ID: 241337740) of the Vitek II system (BioMérieux, Marcy-l'Étoile, France) at the Central Institute of the German Armed Forces in Koblenz. Resistance testing was performed with the same system, using AST-N214 cards (charge ID: 614351840, software version 07.01). Interpretation of the breakpoints was based upon the breakpoint tables for interpretation of MICs (minimum inhibitory concentrations) and zone diameters by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), version 6.0.

Molecular characterization of the carbanemase resistance mechanisms

A polymerase chain reaction (PCR)-based screening for frequent carbapenemase genes was based on a set of three multiplex PCRs that were performed as described [46] at the Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg. The three multiplex PCRs comprised the target genes bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC}, and bla_{OXA-48} coding for β-lactamases. Either well-characterized positive strains (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{DIM}, bla_{GIM}, bla_{KPC}, bla_{BIC}, and bla_{OXA-48}) or plasmids (bla_{SPM}, bla_{AIM}, and bla_{SIM}) were used as positive controls for the PCRs.

Rep-PCR-based typing of the strains

All strains were subjected to rep-PCR-based typing using the BioMérieux DiversiLab system at the Department of Medical Microbiology, Virology, and Hygiene of the University Medicine Rostock strictly following the DiversiLab protocol. In detail, all confirmed ABC strains were grown overnight on Columbia agar with 5% sheep blood (BD, Heidelberg, Germany). DNA was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Purified DNA samples were amplified using the DiversiLab *Acinetobacter* DNA fingerprinting kit (ref. no.: 410 946, BioMérieux) on a T-personal thermal cycler (Biometra, Göttingen, Germany). Rep-PCR products were detected by chip-based DNA separation on an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

Documentation and band-pattern analysis were performed using the DiversiLab software version 3.6.1. (BioMérieux) utilizing the Pearson correlation method. All library entries were analyzed in duplicate.

Ethics

Ethical clearance for the study was obtained from the ethics committee of the medical association of Hamburg (WF-029/15).

Results

Strains and patients

Altogether, 32 carbapenem-resistant *A. baumannii* complex (ABC) isolates from 21 Ukrainian patients could be included in the study. From six out of 21 patients, more than one ABC strain was isolated.

Eleven patients were treated at the German Armed Forces Hospitals Hamburg and Berlin, seven at the German Armed Forces Central Hospital in Koblenz, and three at the German Armed Forces Hospital of Ulm. The patients were males without exemptions. The average year of birth was 1985 (±7 years standard deviation, SD). The median of the years of birth was 1986.5 in a right-shifted distribution.

Isolation sites comprised the skin, the perianal, inguinal, nasal, and pharyngeal regions of the patients, superficial, and deep wounds as well as biopsy materials and catheters. Between one and five ABC isolates were detected per patient from identical or different isolation sites. Details are shown in *Table 1*.

The patients showed moderate to severe injuries at the time of admission to the German Armed Forces Hospi-

Table 1. Characterization of the assessed *Acinetobacter baumannii* complex isolates by sample number, patient number, hospital, and isolation site

Patient number	Year of birth	Sample number	Military hospital	Isolation site/material
01	1985	V86037	BER/HH	Inguinal swab
02	1993	V86038	BER/HH	Pharyngeal swab
03	1982	V86029	BER/HH	Swab of a surgical wound
03	1982	V86030	BER/HH	Swab of a superficial wound
04	1977	V86031	BER/HH	Inguinal swab
05	1978	V86032	BER/HH	Swab of a superficial wound
05	1978	V86033	BER/HH	Swab of a deep wound
05	1978	V86034	BER/HH	Swab of a deep wound
06	1980	V86039	BER/HH	Pharyngeal swab
07	1978	V86040	BER/HH	Inguinal swab
08	1991	V86035	BER/HH	Nasal swab
09	1995	V86041	BER/HH	Swab of a superficial wound
10	1991	V86042	BER/HH	Swab of a superficial wound
11	1994	V86036	BER/HH	Swab of a superficial wound
12	1991	V58144-4	KOB	Pharyngeal swab
12	1991	V58148-4	KOB	Perianal swab
12	1991	V58147-4	KOB	Inguinal swab
12	1991	V58144-2	KOB	Pharyngeal swab
12	1991	V58143-5	KOB	Nasal swab
13	1988	V58118-4	KOB	Swab of a superficial wound
13	1988	V58118-2	KOB	Swab of a superficial wound
13	1988	V58111-1	KOB	Nasal swab
14	1988	V66728-3	KOB	Swab of a deep wound
14	1988	V67479-2	KOB	Bioptic material
15	1982	V58812-2	KOB	Nasal swab
16	1982	V66706-1	KOB	Skin swab (hairline)
17	1983	V60248-1	KOB	Not further characterized sample material
18	1983	V77717-2	KOB	Perianal swab
19	1996	V3752-1	ULM	Intrusion site of a peridural catheter
20	1965	V3753-1	ULM	Inguinal swab
21	1977	V37581/1	ULM	Inguinal swab
21	1977	V3758-1	ULM	Inguinal swab

BER/HH = German Armed Forces Hospitals of Berlin and Hamburg, KOB = German Armed Forces Central Hospital of Koblenz, ULM = German Armed Forces Hospital of Ulm

tals. One patient at the German Armed Forces Hospital of Ulm died due to peritonitis as a consequence of a bullet hit of the liver and duodenum. Typical injury patterns comprised fragmentation bomb injuries, bullet injuries of the body and limbs, and grenade injuries. Prior to the transfer to Germany, previous therapeutic approaches in medical units in the Ukraine or Belarus (comprising Artemosk, Harkov, Kraramatursk, Kiev, and Minsk) had occurred. In part, the Ukrainian patients had been treated in identical medical units and transported together to Germany. Due to partially lacking documentation of the procedures outside Germany, no detailed reconstruction of the medical history prior to the transport to the German Armed Forces Hospitals was possible.

In the German Armed Forces Hospitals, the patients had been isolated due to suspected colonization with multidrug resistant bacteria. Medical personnel only entered the room in protective equipment to prevent further nosocomial spread of such pathogens.

Phenotypically detected resistance patterns

Lacking susceptibility against β-lactam antibiotic drugs including carbapenems was the prerequisite for the inclusion of the assessed ABC isolates in the study. Interpretation of the breakpoints was done in line with version 6 of the EUCAST guidelines. One isolate was tested susceptible to imipenem/cilastatin but already intermediately resistant against meropenem. Altogether, five isolates tested intermediately resistant against imipenem/cilastatin and two strains against meropenem, respectively. All other ones were clearly resistant. A total of 15/32 (46.9%) isolates were still susceptible to gentamicin; the rest was resistant. All isolates were resistant against ciprofloxacin, 25/32 (78.1%) also against trimethoprim/sulfamethoxazole, while the remaining seven isolates were still susceptible. The measured breakpoints including antibiotic drugs for which no clear definitions for susceptibility, intermediate resistance, and resistance are defined by the EUCAST guidelines are shown in the electronic *Supplementary material 1*.

Detected carbapenem resistance genes

In one isolate (v86039, patient 6), the $bla_{OXA\text{-}48}$ gene could be detected. The applied multiplex PCRs for frequent carbapenemase genes were negative for all other strains.

Clonal distribution as suggested by rep-PCR

Rep-PCR-based typing indicated four major clusters of ABC clones comprising five to nine strains *(Fig. 1)*. A fifth pseudo-cluster comprised two isolates that were subsequently grown from inguinal swabs of the same patients and, thus, have to be considered as copy strains.

Three out of four clusters comprised patients from Hamburg/Berlin, Koblenz, and Ulm; and one cluster patients from Hamburg/Berlin and Koblenz. Several patients from the same hospitals could be found in the distinct clusters *(Fig. 1)*.

A more detailed analysis of the six patients with more than 1 ABC isolate led to the following results. Only two out of six were colonized or infected with only one ABC clone. For one of these two patients, the clone was isolated from both a superficial and a surgical wound. For the other one, two isolates from the same inguinal swab with apparently different colony morphology but identical resistance patterns proofed to be only one clone.

From the remaining four patients, strains from two different clonal clusters were isolated. In detail, one patient had the same clone in the nostrils and in a superficial wound, while another clone was isolated from the same swab of the superficial wound. Another patient showed distinct clones in a deep wound and bioptic material. A third patient had an identical clone in a superficial and a deep wound, while another deep wound was infected with a further clone. A fourth patient, finally, was colonized with the same clone in the pharynx, the inguinal region, and the perianal region. Clonal identity *(Fig. 1)* was confirmed for two strains from the pharynx with different colony morphology and even different resistance patterns *(Supplementary material 1)*. The nostrils of this patient, however, were colonized with a strain that could not be assigned to any of the four clusters.

Discussion

The study was performed to assess the clonal diversity and potential transmission routes of carbapenem-resistant *Acintobacter baumannii* complex (ABC) strains of Ukrainian patients that were injured in the Eastern Ukrainian conflict and treated at German Armed Forces Hospitals.

Indeed, only four distinguishable clonal clusters were observed among the isolates from the Ukrainian patients. This observation is not self-evident, as high numbers of different ABC clones have been described from military hospitals from other parts of the world [47]. All clusters contained strains from various patients of the same hospital, not excluding nosocomial transmissions within the hospitals in spite of strict isolation of the patients. The fact that only a comparably small number of clonal clusters was identified makes molecular exclusion of transmission events particularly difficult.

The observed clonal complexes also comprised isolates from patients that were treated at different German Armed Forces Hospitals. This fact suggests that infection or colonization events must have also occurred prior to hospital admission in Germany. Potential transmission events could have occurred during transport flights in cohorts to Germany or during the medical management in the Ukraine or Belarus. The risk of importation of multidrug resistant bacteria by country-to-country transfer of

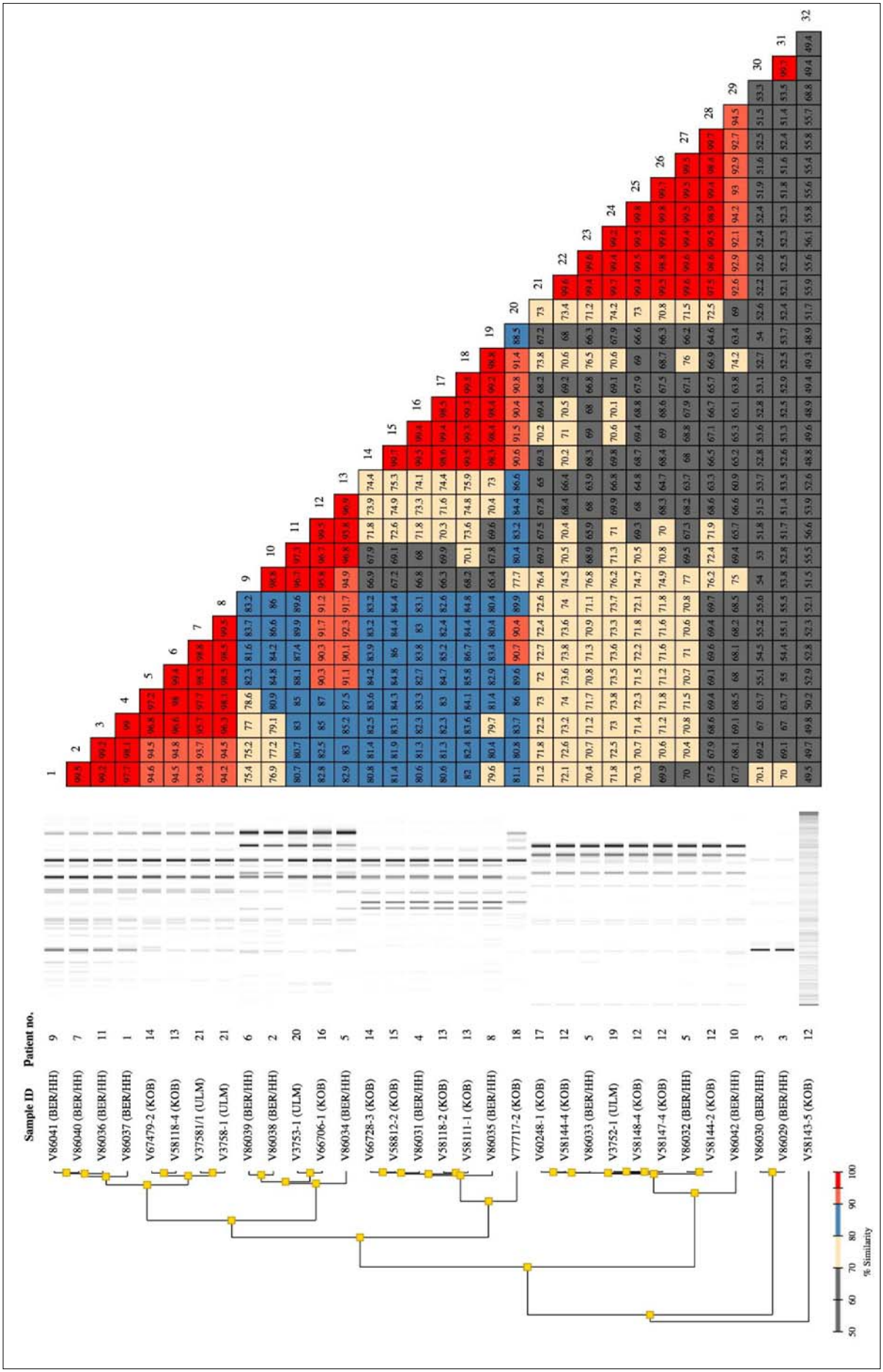

Fig. 1. DiversiLab rep-PCR-based typing of the Ukrainian carbapenem and fluoroquinolone resistant *Acinetobacter baumannii* complex isolates. Patient no. = patient number, BER/HH = German Armed Forces Hospitals of Berlin and Hamburg, KOB = German Armed Forces Central Hospital of Koblenz, ULM = German Armed Forces Hospital of Ulm

patients is a well-known problem of modern infectious disease management [48–50]. Transmissions of ABC by returned soldiers to medical personnel has been well documented [51], resulting in potential onsets of transmission chains. Infection control networks were suggested to adequately face this problem [52].

The observed fact that different clones of the same species complex can be isolated from the same patient is a previously described phenomenon [53]. However, the fact that different clones of carbapenem-resistant ABC were isolated of as many as 4/21 (19.0%) patients suggests a considerable colonization pressure.

Of note, the applied multiplex PCRs for frequent carbapenemase genes [46] identified the resistance mechanism for the ABC strain of only one patient. The identified gene of the bla_{OXA} group is typical of carbapenem-resistant ABC isolates [54]. This result suggests the presence of less frequent or simply other mechanisms [55–57]. In the Persian Gulf region, e.g., $bla_{OXA\text{-}23}$ dominates [58]. Future diagnostic approaches with alternative PCR schemes [59] or even modern next generation sequence technology might be of use to further assess the genetic background of carbapenem resistance of the Ukrainian strains. Of note, all strains were resistant against fluoroquinolones and high percentages of resistance against gentamicin and trimethoprim/sulfamethoxazole were observed. No instance of colistin resistance was detected (data not shown).

Multidrug resistance of ABC strains is an independent risk factor for mortality in case of systemic infections [1]. As most of the patients in this assessment were only colonized or showed superficial wound infections with ABC strains, no relevant attributable mortality was observed. This finding is well in line with previous observations by the Walter Reed Army Medical Center of a 30-day-mortality as low as 2% even in patients with ABC bacteremia associated with war-related trauma despite a high prevalence of multidrug-resistant strains [60]. Further, no relevant impact of infections with multidrug-resistant ABC on the mortality of burn patients could be shown [61].

As demonstrated by a Serbian study group, war or war-like situations even decrease the risk of detecting multidrug-resistant ABC on surgical wards while the total number of detected ABC infections or colonization is increased [62]. Of note, even in the early stages of the Iraq conflict, carbapenem resistance was still uncommon in ABC strains [63]. Susceptibility was further frequent regarding antimicrobial substances of third choice like colistin, polymyxin B, and minocycline [64].

As the situation has changed towards a higher frequency of carbapenem resistance in war-associated ABC isolates as shown for the Ukraine by the data presented here, soldiers engaged in endemic crisis settings are at increased risk and require adequate diagnostic approaches. Chromogenic agars for carbapenem-resistant ABC have been evaluated with acceptable sensitivity and specificity >95% [65] and should be considered for initial screening and diagnostic purposes in endemic military deployment settings.

If next generation sequencing (NGS) is not available in the diagnostic routine, automated rep-PCR is an acceptable typing approach for ABC [59, 66–72]. Alternative typing approaches like multiple locus variable number of tandem repeats analysis [73] can be considered but are usually more work-intensive due to lacking standardization and automation of such in-house procedures.

Limitations of the study comprise the lacking discrimination of the isolates on species level beyond the *A. baumannii* complex, the failed identification of the majority of molecular mechanisms of carbapenem resistance, and the scarce available data of the Ukrainian patients' medical history prior to their admission to the German Armed Forces Hospitals. Application of NGS or other further molecular approaches could allow for a more detailed discrimination of the strains and an identification of the molecular resistance mechanisms but could not be performed in this study for economic reasons.

Conclusions

In summary, the presented data indicate a high colonization pressure with carbapenem-resistant *A. baumannii* complex (ABC) in patients with battle-associated injuries from Eastern Ukraine. Further, they suggest that a small number of clonal complexes dominates. Last but not the least, the occurrence of identical clones at different German Armed Forces Hospitals indicates that transmission events occurred already in local medical facilities or during the transport of patients for further treatment to Western Europe. Military forces or humanitarian helpers that operate in the Eastern Ukraine have to consider infections with multidrug-resistant ABC in case of battle injuries.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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Supplemental Material

Sample number	Ampicillin	Piperacillin/ tazobactam	Imipenem	Meropenem	Gentamicin	Ciprofloxacin	Tigecycline	Trimethoprim/ sulfamethoxazole
V86041	8	≥128	≥16	≥16	≥16	≥4	1	≤20
V86040	16	≥128	≥16	≥16	≤1	≥4	1	160
V86036	16	≥128	≥16	≥16	≥16	≥4	≤0.5	≤20
V86037	16	≥128	≥16	≥16	≤1	≥4	2	≤20
V67479-2	16	≥128	≥16	≥16	≥16	≥4	≤0.5	≤20
V58118-4	≥32	≥128	≥16	≥16	≥16	≥4	4	160
V37581/1	16	≥128	≥16	≥16	≤1	≥4	2	≤20
V3758-1	16	≥128	≥16	≥16	≤1	≥4	2	≤20
V86039	8	≥128	≥16	≥16	8	≥4	≤0.5	≥320
V86038	16	≥128	8	≥16	≥16	≥4	≤0.5	≥320
V3753-1	16	≥128	≥16	≥16	≤1	≥4	1	160
V66706-1	≥32	≥128	≥16	≥16	≤1	≥4	1	160
V86034	16	≥128	≥16	≥16	≤1	≥4	≤0.5	≥320
V66728-3	≥32	≥128	2	8	4	≥4	2	≥320
V58812-2	≥32	≥128	≥16	≥16	≥16	≥4	4	≥320
V86031	≥32	≥128	≥16	≥16	8	≥4	2	≥320
V58118-2	≥32	≥128	≥16	≥16	≥16	≥4	4	160
V58111-1	≥32	≥128	≥16	≥16	≥16	≥4	2	320
V86035	≥32	≥128	≥16	≥16	8	≥4	2	≥320
V77717-2	≥32	≥128	4	≥16	≥16	≥4	≤0.5	≥320
V60248-1	≤2	≥128	8	≥16	≥16	≥4	≤0.5	≥320
V58144-4	≤2	≥128	4	8	≤1	≥4	≤0.5	≥320
V86033	≥32	≥128	≥16	≥16	8	≥4	≤0.5	≤20
V3752-1	≥32	≥128	≥16	≥16	≥16	≥4	≤0.5	160
V58148-4	≥32	≥128	8	≥16	4	≥4	≤0.5	≥320
V58147-4	≥32	≥128	≥16	≥16	4	≥4	≤0.5	≥320
V86032	≥32	≥128	≥16	≥16	8	≥4	≤0.5	160
V58144-2	16	≥128	≥16	≥16	4	≥4	≤0.5	≥320
V86042	≥32	≥128	≥16	≥16	≥16	≥4	≤0.5	160
V86030	16	≥128	≥16	≥16	≤1	≥4	≤0.5	160
V86029	16	≥128	≥16	≥16	≤1	≥4	≤0.5	160
V58143-5	≥32	≥128	≥16	≥16	4	≥4	≤0.5	≥320

Fig. S1. Detected minimum inhibitory concentrations (MIC) of the isolates for the assessed antibiotic drugs