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Antibiotic Resistance Profiles and Genetic Similarities Within a New Generation of Carbapenem-Resistant *Acinetobacter calcoaceticus–A. baumannii* Complex Resistotypes in Bosnia and Herzegovina

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Acinetobacter calcoaceticus–A. baumannii complex (ACB complex) is a nosocomial pathogen. Due to its high ability to develop antibiotic resistance, it has become a problematic challenge in the modern healthcare system. The molecular and genetic mechanisms of gaining multidrug resistance in ACB complex are well known. This study focuses on providing an overview of the antibiotic resistance profiles, genetic similarities and resistotypes, and general characteristics of carbapenem-resistant ACB complex (CRACB) in Bosnia and Herzegovina (BiH). In light of the data collected in this study, together with the already known information concerning antibiotic resistance of ACB complex, we intend to further elucidate the antibiotic therapy for CRACB strain resistotypes in BiH.

Introduction

Acinetobacter calcoaceticus–A. baumannii complex (ACB complex) is a gram-negative nonfermentative coccobacillus, first described in 1911 by Beijerinck.[1] It belongs to the Moraxellaceae family and 43 taxonomically distinct species of the genus *Acinetobacter* are currently identified.[2] Most *Acinetobacter* species are environmental organisms that cannot be related to human disease. However, this species is more commonly known as a nosocomial pathogen in recent years. Multidrug-resistant (MDR) strains of ACB complex are now a significant clinical problem throughout the world.[3] There are many different pathogens that can cause nosocomial infections. Nosocomial pathogens vary among different patients, populations, healthcare settings, facilities, and countries. The ACB complex is an opportunistic pathogen that can infect immunocompromised patients. This pathogen is emerging worldwide as a healthcare related to human pathogen most commonly associated with hospital outbreaks of nosocomial infections, especially those occurring in intensive care units.[4] This organism increasingly exhibits MDR to various groups of antibiotics, including carbapenems.[5] Moreover, ACB complex has shown a strong ability for developing antibiotic resistance.

Various identified types of antibiotic resistance are as follows: (1) ineffective antibiotic rendered by permeability barriers and reduced uptake, (2) active general or specific efflux pumps causing quick extrusion of antibiotics, (3) preventing or altering the target allowing for metabolic bypass, (4) antibiotic target modifications, (5) enzymes modifying the antibiotic, (6) enzymes inactivating the antibiotic, (7) ineffective antibiotic rendered by degrading and altering enzymes, and (8) an overproduction of target enzymes.

Antibiotic resistance is either intrinsic or acquired by horizontal gene transfer. Plasmids, transposons, or integrons can carry many of the antibiotic-resistant genes and function as vectors, transferring these antibiotic-resistant genes to bacteria in another genus or species. The three main mechanisms that can allow horizontal gene transfer are conjugation, transduction, and/or transformation.

Samples of the ACB complex can be isolated in the clinical setting from numerous sources, which include contaminated hospital personnel, medical equipment, and environmental surfaces. Also, both patient-to-patient and airborne transmissions are inevitable. Nosocomial infections occur worldwide and are a public health financial burden for health systems, patients, and their families in both developed and developing countries. In particular, these infections significantly contribute to increased morbidity, prolonged hospital stay, and unnecessary deaths, especially in individuals with compromised immunological defenses.[6]

These organisms are connected with a varying range of infections, including skin, soft tissue, respiratory tract, bloodstream as well as those related to prosthetic devices. Such

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antibiotic-resistant organisms pose a distinct threat in intensive care units where numerous outbreaks are especially challenging to control. As a major nosocomial pathogen, its rapid emergence and global dissemination are remarkable and an example of an organism's successful adaptation to the modern day hospital environment.

The data provided in the following report give an overview of the similarities and differences between carbapenem-resistant ACB complex (CRACB) in Bosnia and Herzegovina (BiH) focusing on antibiotic resistance profiles, genetics, and resistotypes of CRACB. This study provides a more in-depth understanding of antibiotic therapy for CRACB in BiH by combining current data with previously obtained information concerning the antibiotic resistance of ACB complex strains.

Materials and Methods

Sample collection

Purely 50 CRACB samples have been collected from different clinics of Clinical Center, University of Sarajevo in Bosnia and Herzegovina (USBiH) from October 2013 to June 2014 for this study. Isolates were detected from different clinical samples, including urine, wound swab, blood, bronchial aspirate, and other samples, which were collected from patients situated in various hospital wards, and CRACB was used in this study.

Culturing and species confirmation

All of the samples included in this study were inoculated onto blood agar, MacConkey agar, and incubated at 35°C (95°F). Typical colonies were further examined with standard microbiological methods. Final identification of ACB complex was determined by the VITEK 2 Compact system (bioMérieux, Marcy l'Étoile, France) using VITEK ID GN. ACB complex isolates were identified by using their cultural, morphological, and biochemical characteristics.

Antibiotic susceptibility testing

The antibiotic susceptibilities of CRACB isolates were determined by the Kirby–Bauer disk diffusion method on Mueller-Hinton agar, using the EUCAST standards.

In parallel, each isolate was tested for antibiotic susceptibility with the VITEK 2 Compact System (bioMérieux), using a VITEK AST card to determine the minimum inhibitory concentrations. Antibiotic susceptibility was determined for the following antibiotics: amikacin, amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, colistin, gentamycin, imipenem, levofloxacin, meropenem, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, and tobramycin. As a quality control strain *Pseudomonas aeruginosa* ATCC 27853 was used.

DNA extraction

Genomic DNA has been obtained from colony-purified CRACB. A few colonies (5–7) have been removed from fresh pure bacterial culture, suspended in 100 µl of sterile distilled water, and then heated at 96°C for 15 min. After centrifugation at 12,000 × *g* and a 5-min incubation at 4°C, the supernatant has been used as a source of template for PCR amplification. Prepared DNA extracts have been used immediately or stored at −20°C for further analysis.

DNA quantification

After the genomic DNA extraction, quantification of the DNA has been done with the BioSpec-nano Small-volume UV Spectrophotometer (Shimadzu, Columbia, MD). The system measures the quantity of double-stranded DNA (dsDNA) in ultrasmall volume (1–2 µl) of sample supernatant preparations. For rep-PCR, it was recommended to use 25–50 ng/µl of DNA. DiversiLab site software offers Dilution Calculator to calculate optimal dilution by adding the nuclease-free water to the 5 µl of DNA extract to get 35 ng/µl DNA for rep-PCR setup. Concentration below 25 ng/µl of DNA was not accepted for rep-PCR and the extraction procedure had to be repeated.

Genetic screening

Thirty-five nanograms per microliter diluted DNA was amplified using the DiversiLab Acinetobacter kit (bioMérieux). DNA fingerprinting of isolates was performed using noncoding repetitive extragenic palindromic (REP) sequence-based PCR (rep-PCR) technology. Highly conserved REP noncoding REP sequences have been described in ACB complex and the application is suitable for both strain characterization and subspecies identification.

PCR was performed on preheated thermal cycler (Eppendorf Mastercycler S) and DNA fingerprints were obtained according to the manufacturer's recommendations (bioMérieux). Rep-PCR fingerprinting products were compared to the manufacturer's preloaded library or a user-generated library to detect if an isolate clusters with a previously defined strain type by using the DiversiLab Microbial Genotyping System (bioMérieux). This system uses repetitive PCR (rep-PCR) technology with a semi-automatic method, high-throughput and rapid pathogen typing following the manufacturer's instructions. Rep-PCR product fragments were separated using microfluidics electrophoresis in a small volume of sample. Genetic screening results were analyzed using the DiversiLab software (v3.4) to determine the distance matrices and then dendrograms were generated.

Results

Antibiotic susceptibility testing

All of the isolates were susceptible to colistin and most of them (36/50), 72%, were susceptible to tobramycin, (3/50) 6% isolates were susceptible to trimethoprim/sulfamethoxazole, and all of the isolates were resistant to amikacin, amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamycin, imipenem, levofloxacin, meropenem, and piperacillin/tazobactam antibiotics. The majority of our CRACB isolates were resistant to the most of the tested antibiotics.

Furthermore, we have seen CRACB isolates as resistotypes and their sample rates in BiH using this study. These resistotypes were distinguished into four different groups (Table 1). The first resistotype CRACB was susceptible to only colistin and its rate was 12/50 (24%). The second resistotype was susceptible to colistin–tobramycin and its rate was (35/50) 70%. The third resistotype was susceptible to colistin–trimethoprim and its rate was 2/50 (4%). The fourth resistotype was susceptible to colistin–tobramycin–trimethoprim and its

TABLE 1. RESISTOTYPES OF CRACB ISOLATES AND THEIR OCCURRENCE RATES IN BIH

Resistotypes	*Amikacin*	*Amoxicillin*	*Cefepime*	*Cefotaxime*	*Ceftriaxone*	*Ciprofloxacin*	*Gentamicin*	*Imipenem*	*Levofloxacin*	*Meropenem*	*Piperacillin*	*Colistin*	*Tobramycin*	*Trimethoprim*	*Sample numbers*	*Rate of resistotype occurrence (%)*
1	R	R	R	R	R	R	R	R	R	R	R	S	R	R	12/50	24
2	R	R	R	R	R	R	R	R	R	R	R	S	S	R	35/50	70
3	R	R	R	R	R	R	R	R	R	R	R	S	R	S	2/50	4
4	R	R	R	R	R	R	R	R	R	R	R	S	S	S	1/50	2

CRACB, carbapenem-resistant ACB complex.

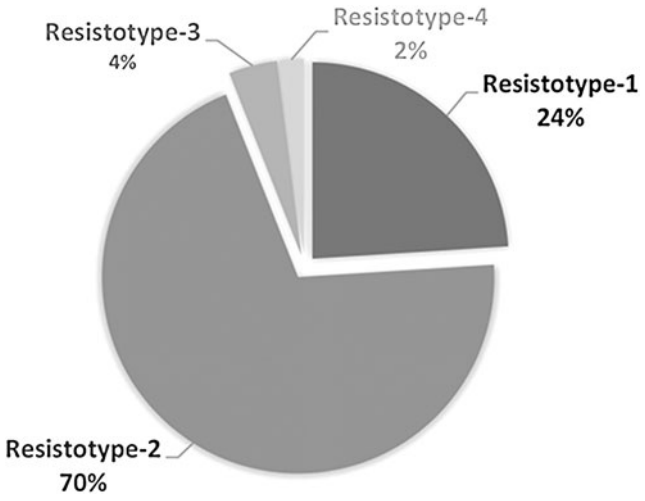

FIG. 1. Resistotype occurrence rates (%) of CRACB isolates in BiH. BiH, Bosnia and Herzegovina; CRACB, carbapenem-resistant ACB complex.

rate was 1/50 (2%). Percentage rates of CRACB resistotypes of isolates are shown in Fig. 1 as a pie chart.

Genetic screening

The DiversiLab Microbial Genotyping System was used for DNA fingerprinting and strain typing analysis. Results of DNA fingerprinting of all CRACB isolates using rep-PCR are shown in Fig. 2 and a threshold of ≥92% was used.

According to the results, more than half of the isolates (58%) were clustered into the first major distinct group that was 92% similar. Twenty-eight percent of the total strains were in the second largest group and the similarity was more than 92%. Samples numbered 30, 31, and 32 were clustered in the third group and the 33rd and 34th samples were clustered in the fourth group. Scatterplot indicating the results of DNA fingerprinting of CRACB isolates in BiH is shown in Fig. 3.

Samples numbered 18, 24, and 25 from the first major cluster are resistant to tobramycin but their close relatives are susceptible to tobramycin and their tobramycin resistance rate is almost (3/29) 10%. In the second major cluster, consisting of isolates 35–48, the tobramycin resistance rate is more than the first major group. Also, only (4/14) 29% of isolates from the second cluster are susceptible and the others (10/14), 71%, are resistant to tobramycin.

We have seen four different resistotypes of CRACB in BiH in the current study. The first resistotype of CRACB was the most resistant to the tested antibiotics and susceptible to only colistin. The first resistotype has members from every genetic cluster except the third cluster. The second resistotype was susceptible to both colistin and tobramycin. Most of its members (25/35), 71%, are from the first largest genetic cluster, (5/35) 14% are from the second largest genetic cluster, and five of them are from the others. The third resistotype was susceptible to both colistin and trimethoprim, and its members are from the second largest genetic cluster. The fourth resistotype is less resistant than the others and it was susceptible to colistin, tobramycin, and trimethoprim, and its members are from the third genetic cluster. We can state that there is no correlation between

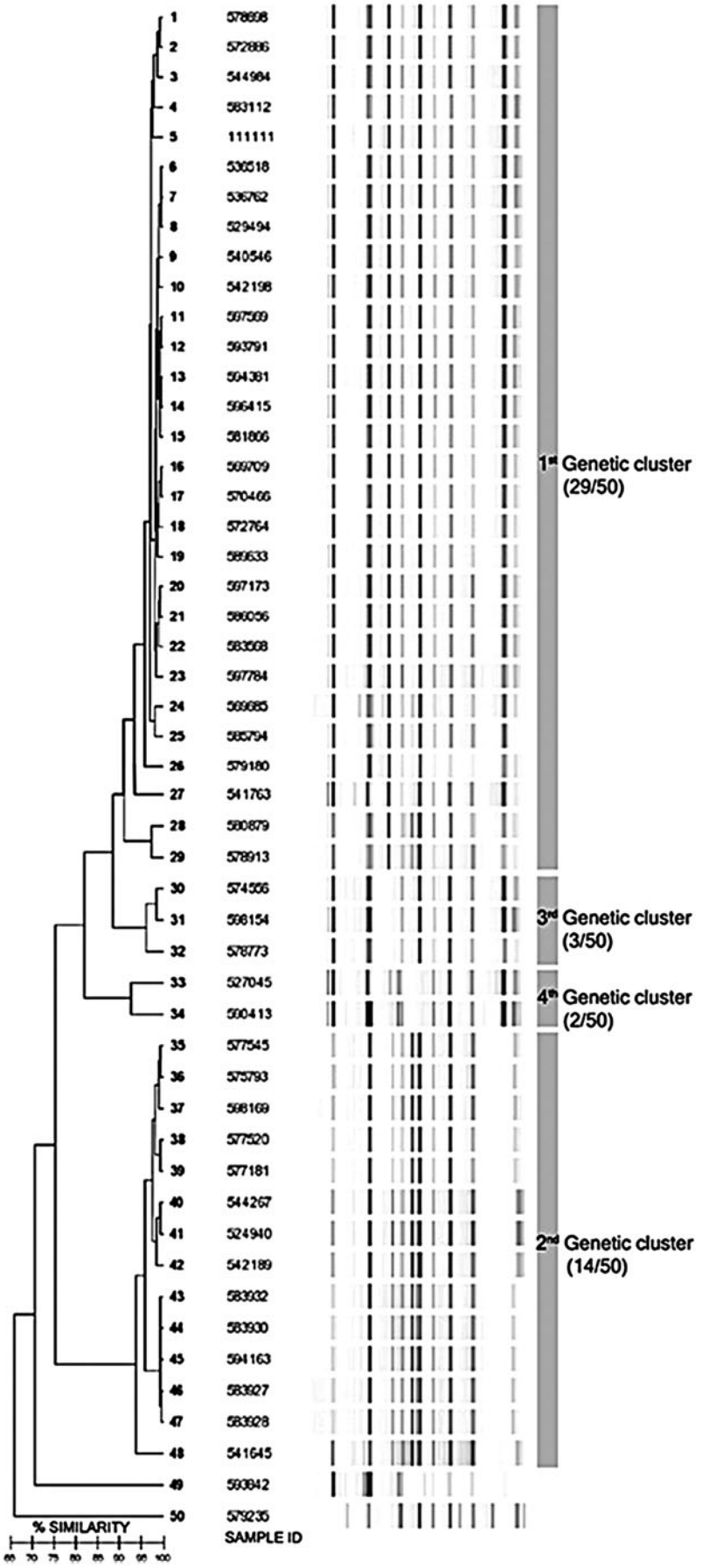

FIG. 2. Dendrogram representing the results of DNA fingerprinting of CRACB isolates in BiH.

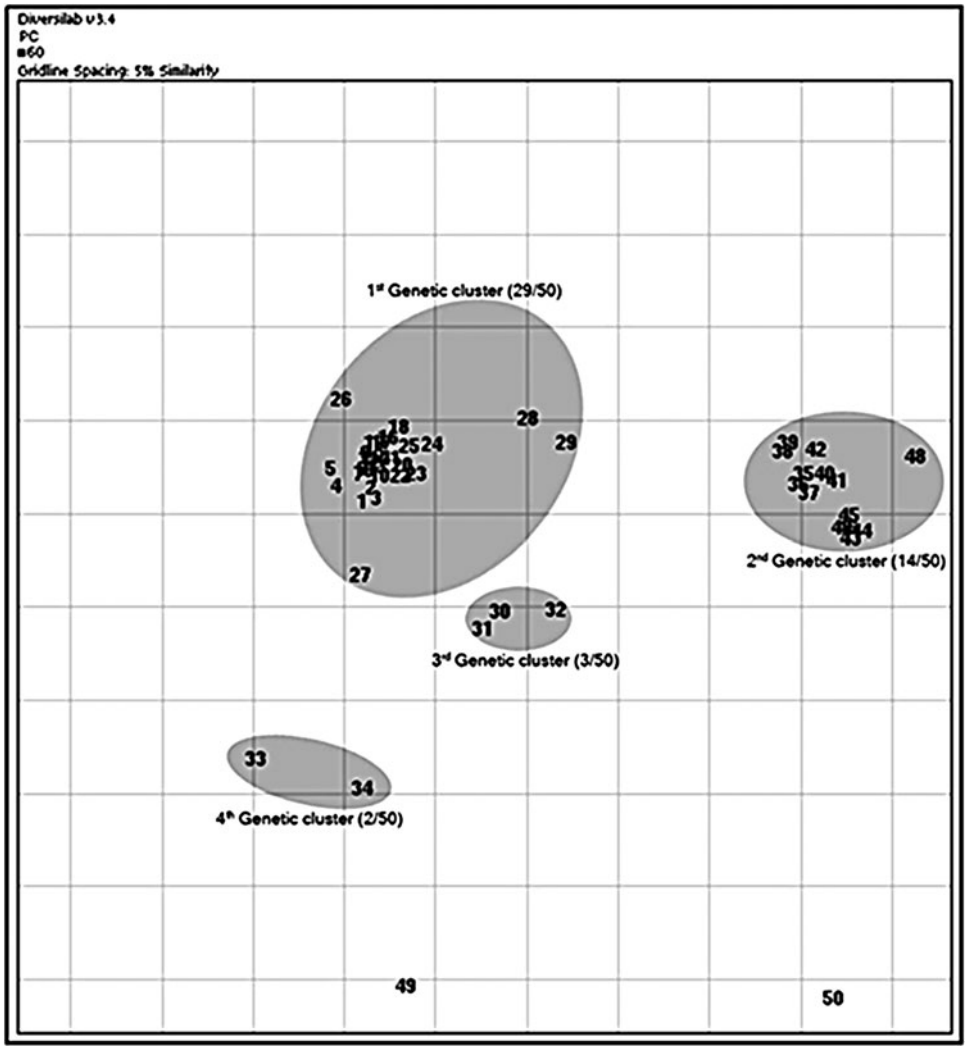

FIG. 3. Scatterplot indicating the results of DNA fingerprinting of CRACB isolates in BiH.

clusters and resistotypes even if the threshold level of similarities is more than 98%.

Discussion

We have examined 50 CRACB samples collected between October 2013 and June 2014 in different clinics of Clinical Center of USBiH. Infections caused by ACB complex are associated with considerable morbidity and mortality.[7–9] Multidrug-resistant ACB complex (MDRACB) has become prominent in hospitals worldwide, such as in Taiwan,[10] Korea,[11] Iraq,[12] Israel,[13] Greece,[14] Italy,[15] Belgium,[16] Brazil,[17] and the United States.[18] Significantly increased resistance toward all antibiotics was reported in the Asia-Pacific Rim, Middle East, Africa, and Europe.[19] In addition, the rapidly spreading nosocomial infections caused by MDR isolates continue to cause worldwide concern.[20,21] For instance, from 2003 to 2010, there has been an increasing trend of antibiotic-resistant isolates in BiH.[22] At the beginning of 2003, there was a sustained outbreak of MDRACB nosocomial bloodstream infections in the Clinical Center of USBiH, as reported by Dedeić-Ljubović *et al.*[23] CRACB was isolated most frequently at the Clinical Center of USBiH during 2010.[22] Compatible with the previous general trend of resistance, we are reporting high resistance rates to antibiotics in our study, which is focused exclusively on CRACB.

Between 2005 and 2009, in a worldwide collection of ACB complex isolates, imipenem resistance rose to rates greater than 50% and many studies continue to report increasing rates of CRACB in clinical isolates.[24] In addition, there was no imipenem and meropenem resistance in 2003 but it was detected in 2005,[23] with an impressive increase from 0% to 53%.[22] We have detected a 28% resistance rate to tobramycin, while another study[25] reported a similar 32% rate of tobramycin resistance. There is a relatively low level of resistance to tobramycin, which suggests that it might be a useful alternative therapeutic agent to colistin.

Colistin resistance was not detected in BiH, but two South Korean hospitals have recently reported high rates of colistin resistance in ACB complex isolates.[26] This antibiotic remains effective in treating ACB complex and its resistance rates remain low when compared to other antibiotics. Furthermore, colistin has low reported toxicity and some studies suggest colistin could be used for the treatment of infections caused by carbapenem-resistant isolates.[27–29]

Four resistotypes of CRACB were detected in this study, as well as four distinct genetic clusters of CRACB isolates.

However, resistotypes of CRACB do not correspond to specific genetic clusters if the threshold of ≥65% is used for determining statistical significance. This might occur due to horizontal gene transfer of antibiotic resistance genes located on plasmid. Since DNA fingerprinting is performed only on bacterial chromosomal DNA, changes in resistotypes of the members of the same genetic cluster could not be detected.

This study serves as an overview of the general characteristics of CRACB in BiH by highlighting their antibiotic resistance profiles, including the resistance to polymyxins, quinolones, aminoglycosides, cephalosporins, a combination of piperacillin/tazobactam, amoxicillin/clavulanic, and trimethoprim/sulfamethoxazole, and genetic similarities. In light of the data collected from the current study, together with the already known information concerning antibiotic resistance of CRACB, we intent to elucidate antibiotic therapy for CRACB in BiH and define the characteristics of CRACB existing in daily life in BiH, especially in hospital institutions where individuals seek treatment. In this sense, we investigated chromosomal diversity and mismatches of gene clusters with resistotypes and showed that the main problem could be the plasmid resistance and its horizontal spread in hospitals. Other treatment options of common CRACB in BiH may be possible by developing and producing new effective types of antibiotics and by performing DNA fingerprinting of plasmid DNA. Furthermore, this study makes a contribution to the treatment attempts of the strains and may give some insights for further studies about ACB complex strains. Since there has not been a similar research focus within this area, this study, as the first of its kind, plays a significant role in characterization of CRACB resistotypes in the country.

Disclosure Statement

No competing financial interests exist.

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