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MOLECULAR ANALYSİS OF ANTİBİOTİC RESİSTANCE CHARACTERİSTİCS OF ACİNETOBACTER SPP. ISOLATED FROM FECAL AND CLİNİCAL SPECİMENS İN EASTERN TURKEY

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ABSTRACT

Aim: Acinetobacter baumannii has recently become a prominent source of nosocomial infections. *A. baumannii* can present as an opportunistic infection, especially in hospitalized individuals with weakened immune systems. *A. baumannii* can spread throughout the human body via multiple pathways and may lead to lethal infections. The aim of our study is to perform a molecular examination of the antibiotic resistance traits of Acinetobacter spp. isolated from stool and clinical specimens. **Material and Method:** From January 2018 to December 2020, clinical infection specimens (comprising sputum, puncture fluid, urine, blood, and device-associated samples) were gathered at Van Training and Research Hospital. Furthermore, 6000 fecal specimens were collected from August 2018 to December 2020. All isolated bacteria were identified as Acinetobacter species by recA gene analysis, biochemical procedures, and 16S ribosomal RNA (rRNA) sequencing, alongside conventional microbiological techniques. The genomic regions selected for investigation in the isolates were amplified utilizing polymerase chain reaction and ERIC-PCR techniques. **Findings:** A total of 285 carbapenem-resistant Acinetobacter isolates were collected during the study period, comprising 180 from clinical infection specimens and 105 from fecal research specimens. Subsequent identification indicated 201 *A. baumannii* and 84 non-baumannii Acinetobacter species. Among the non-baumannii Acinetobacter species, 12 isolates and 5 *A. baumannii* isolates exhibited resistance to colistin. All carbapenem-resistant Acinetobacter isolates were identified as multidrug-resistant (MDR). Twelve non-baumannii Acinetobacter and five *A. baumannii* isolates exhibited resistance to colistin, with seven non-baumannii Acinetobacter strains from fecal samples testing positive for the mcr-1 gene. **Discussion:** Colistin is regarded as a last-resort treatment for infections caused by carbapenem-resistant Gram-negative bacteria. The emergence of the mcr gene has resulted in more significant challenges in therapeutic treatment. The existence of two significant clinical resistance genes, namely blaNDM and mcr-1, in non-baumannii Acinetobacter gut colonization is notable. The variety and prevalence of antibiotic resistance genes found in Acinetobacter species within stool samples suggest that the gut may serve as a substantial reservoir for resistant opportunistic bacteria.

KEYWORDS: Acinetobacter spp., Carbapenem resistance, Colistin resistance, mcr-1.

INTRODUCTION

Acinetobacter species represent a diverse assembly of Gram-negative, aerobic, non-motile, non-fermentative, encapsulated coccobacilli, prevalent in various environmental settings.[1,2] Acinetobacter baumannii has recently emerged as a notable cause of hospital-acquired infections within this group. *A. baumannii* serves as an opportunistic pathogen, especially in hospitalized individuals with weakened immune systems. *A. baumannii* can spread throughout the human body through multiple pathways, potentially leading to lethal infections.[3,4] Moreover, infections attributed to Acinetobacter species beyond *A. baumannii* have emerged as a significant concern in recent years.[5] The resistance of Acinetobacter to various commonly used antimicrobials has resulted in antibiotic ineffectiveness and a rise in mortality associated with infections.[6] Carbapenems are commonly employed in the treatment of infections caused by Acinetobacter species; however, the rise of carbapenem-resistant Acinetobacter species poses challenges to treatment and may result in therapeutic failure.[7,8]

Acinetobacter species employ three main mechanisms to acquire resistance to carbapenems. The mechanisms include: the production of enzymes that hydrolyze carbapenems (e.g., carbapenemases); modifications in the function of membrane-associated proteins like porins; and the activation of drug efflux pumps.[9] Acinetobacter species have been reported to produce various carbapenemases, including Ambler class B metallo-β-lactamases like IMP, VIM, and NDM; Ambler class D oxacillinases (OXAs); and Ambler class A β-lactamases, such as KPC.[10,11] The OXA carbapenemase genes in Acinetobacter species include several phylogenetic subgroups: blaOXA-23-like, blaOXA-51-like, blaOXA-24/40-like, blaOXA-58-like, blaOXA-143-like, and blaOXA-235-like. OXA carbapenemases exhibit weak hydrolytic activity against carbapenems; however, the blaOXA genes can confer increased resistance due to overexpression driven by a potent promoter alongside a mobile insertion element like ISBa1.[12,13] The reduction in outer membrane permeability, resulting from alterations in primary structure or the absence of the outer membrane protein CarO (25/29-kDa), which interacts with carbapenems, represents a well-established mechanism of intrinsic carbapenem resistance in Acinetobacter species.[14,15] Overexpression of drug efflux pumps is commonly linked to carbapenem resistance in Acinetobacter species, with the resistance-nodulation-division (RND) type being particularly clinically significant.[16] Additionally, five RND efflux pumps have been identified in Acinetobacter species: AdeABC, AdeDE, AdeFGH, AdeIJK, and AdeXYZ.[17]

Colistin has been utilized for more than 50 years in veterinary and human medicine. Colistin exhibits extensive therapeutic efficacy against Gram-negative bacteria. In human medicine, it is linked to nephrotoxicity and neurotoxicity, thereby restricting its clinical application. Colistin has been identified as an effective treatment for carbapenem-resistant bacteria, which presents a significant challenge. The overuse of colistin in treating these infections has resulted in the development of resistance to colistin.[18] Therefore, examining the mechanisms that contribute to resistance in Acinetobacter species is essential.

Mcr, initially reported in China in 2015, is recognized as a plasmid-mediated gene conferring resistance to colistin.[19] This discovery enhanced the understanding of colistin resistance mechanisms, which are distinct from chromosomal mechanisms. The plasmid-borne colistin resistance gene, mcr, has been shown to be transmissible and disseminated by various mobile genetic elements.[20]

Mobile genetic elements, including integrons, transposons, and insertion sequences, serve as significant sources of genetic variation in bacteria, thereby promoting the development of multidrug-resistant (MDR) strains. The transfer of mobile genetic elements between various bacterial species has been documented in Acinetobacter.[21,22]

Multiple bacterial species, including multidrug-resistant (MDR) bacteria, inhabit the gut microbiota, which functions as a substantial reservoir for resistance genes. Fecal material serves as an optimal specimen for the investigation and identification of antimicrobial resistance genes. Numerous studies indicate elevated rates of Acinetobacter carriage in stool, suggesting that the digestive system may serve as a potential source for nosocomial infections and outbreaks. [23] The aim of this study is to analyze resistance mechanisms and to perform the genotyping of carbapenem-resistant Acinetobacter from clinical infection and fecal research specimens in Eastern Turkey.

MATERIAL AND METHOD

Identification of bacterial isolates and species

From January 2018 to December 2020, 180 unique carbapenem-resistant Acinetobacter isolates were identified from clinical infection specimens, including sputum, puncture fluid, urine, blood, and device-associated samples, collected at Van Training and Research Hospital. Furthermore, 6000 fecal specimens were collected from August 2018 to December 2020 and inoculated onto MacConkey agar plates with 2 μg/mL meropenem, followed by incubation. A total of 616 isolates exhibiting Gram-negative and carbapenem resistance were recovered. The recA gene was identified in non-fermenting isolates and utilized as a marker for the Acinetobacter genus. Subsequently, 105 carbapenem-resistant Acinetobacter species were identified. The Medical Ethics Committee of Van Training and Research Hospital approved the study, which was conducted in accordance with the Declaration of Helsinki.

All isolated bacteria were identified as Acinetobacter species through the use of the recA gene, biochemical analysis, and 16S ribosomal RNA (rRNA) sequencing, alongside standard microbiological techniques. The Acinetobacter calcoaceticus-*A. baumannii* complex was identified through the analysis of 16-23S rRNA and internal transcribed spacer sequences.[25]

Antimicrobial Susceptibility Test

The susceptibility analysis to antimicrobial drugs was conducted following the Clinical and Laboratory Standards Institute (CLSI) M100-S27 criteria and utilizing the automated Vitek II Microbiology System. The antibiotics evaluated were: ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam (4 mg/L tazobactam), ceftazidime, cefotaxime, cefazolin, cefepime, imipenem, meropenem, aztreonam, chloramphenicol, ciprofloxacin, levofloxacin, tetracycline, gentamicin, colistin, trimethoprim-sulfamethoxazole, and amikacin. Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 served as control strains for the quality assessment of antimicrobial susceptibility testing.

Molecular Analysis of Resistance Genes

DNA was extracted utilizing the AccuPrep® Genomic DNA Extraction Kit following the manufacturer's guidelines. Analysis of pertinent genes to ascertain the existence of antibiotic resistance was conducted utilizing PCR.

The study examined the following genes that encode carbapenemases: Class A β-lactamase genes include blaKPC[26]; Class B β-lactamase genes consist of blaIMP, blaVIM, blaSPM, and blaNDM[26]; and Class D oxacillinase genes are

represented by blaOXA-23, blaOXA-24/40, blaOXA-51, and blaOXA-58.[27] Colistin-associated resistance genes (*mcr-1, mcr-2, mcr-3, mcr-4, and mcr-5*) were also analyzed.[28] The role of the *A. baumannii* outer membrane protein in carbapenem resistance was investigated through the analysis of the protein-associated gene carO.[29] An evaluation of resistance-conferring genes linked to drug efflux pump components, specifically adeA[30], adeB, adeC, adeI, adeJ, and adeK[31], was performed.

DNA sequence similarity searches were conducted via BLAST (https://blast.ncbi.nlm.nih.gov). A phylogenetic tree for carO was constructed using MEGA through the neighbor-joining method. A p-distance model was developed to calculate the distances between nucleotide sequences. The importance of groupings was established using a 1000-replicate bootstrap analysis of the observed values on the generated trees.

Resistance genes linked to mobile genetic elements were examined, encompassing integrons [intI1[32,33], intI2[32,34], intI3[32]], transposons [tnpU[35] and tnp513[35]], variable sections of class 1 and class 2 integrons, and insertion sequences [IS26[36], ISAba1[37], and ISAba125[37]]. The PCR protocol included the following cycles: denaturation at 94°C for 5 minutes, succeeded by 35 cycles comprising 30 seconds at 94°C, 40 seconds of annealing at the optimal temperature for each gene, and extension at 72°C for 40 seconds for the genes and 4 minutes for the intI1 and intI2 variable regions. Sentebiolab Ltd. (Ankara, Turkey) conducted primer synthesis and sequencing of PCR products.

Eric-PCR

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was conducted on all isolates to evaluate species diversity.[38] Band comparisons were conducted utilizing Quantity One-v4.6.7, employing clustering analysis based on the unweighted pair group method with arithmetic mean (UPGMA) of the isolates.

RESULTS

Identification of Isolates

Throughout the study period, 285 carbapenem-resistant Acinetobacter isolates were obtained, comprising 180 from clinical infection samples and 105 from fecal research samples. The PCR amplification of the recA gene yielded positive results for all isolated and identified strains. Subsequent identification disclosed 201 A.baumannii and 84 non-baumannii Acinetobacter species. Significant variations were detected between species isolated from clinical infection cases and those from fecal research specimens. Acinetobacter baumannii had a significant prevalence among carbapenem-resistant isolates from clinical specimens, at 94.2%. Sixty-one carbapenem-resistant non-baumannii Acinetobacter isolates were acquired from fecal research specimens, with 74.6% classified as A. junii.

Antimicrobial Susceptibility Test

The minimum inhibitory concentration (MIC) values of the evaluated antibiotics indicated that colistin resistance breakpoints for the *A. baumannii* complex aligned with the CLSI standard, whereas MIC breakpoints for non-baumannii Acinetobacter adhered to the EUCAST standard for colistin, given that the CLSI standard was exclusively relevant to the *A. baumannii* complex. All isolates exhibited resistance to carbapenems and cephalosporins, including ceftazidime, cefazolin, and cefepime, while 30.8% and 98.1% of the isolates displayed susceptibility to amikacin and colistin, respectively. Nonetheless, non-baumannii Acinetobacter isolates exhibited greater susceptibility to amikacin compared to A. baumannii. Twelve non-baumannii Acinetobacter isolates and five *A. baumannii* isolates shown resistance to colistin. All carbapenem-resistant Acinetobacter isolates were identified as multidrug-resistant (MDR).

Molecular Detection of Resistance Genes

Carbapenemase-Encoding Genes

The prevalence rates of blaOXA-51-like, blaOXA-23-like, blaOXA-24/40-like, and blaOXA-58-like gene structures were 80%, 72.4%, 3%, and 13.6%, respectively. The co-occurrence of blaOXA-51 and blaOXA-23-like genes was detected in 180 strains. Significantly, six strains concurrently possessed blaOXA-58-like, blaOXA-51-like, and blaOXA-23-like genes. Among the 201 carbapenem-resistant *A. baumannii* isolates, 199 (98.9%) exhibited the blaOXA-51-like gene, a characteristic feature of A. baumannii. Among the several carbapenemase genes present in all strains, 3 (0.90%) harbored the blaIMP gene, 6 (2.64%) possessed the blaVIM gene, and 62 (25.6%) contained the blaNDM gene. All blaNDM-positive strains were derived from fecal research samples; 51 strains possessed blaNDM-1, 14 strains have blaNDM-5, and 7 strains possessed blaNDM-4. The blaKPC and blaSPM genes were absent in all strains.

Colistin-Associated Resistance Genes

While twelve non-baumannii Acinetobacter isolates and five *A. baumannii* isolates were identified as resistant to colistin, seven non-baumannii Acinetobacter strains from fecal samples tested positive for the mcr-1 gene.

Protein-Associated Genes

The carO gene was found in 226 carbapenem-resistant Acinetobacter strains, and the nucleotide sequence of the carO porin was analyzed. All isolates, except for *A. baumannii* 1124668A and 515,009, displayed amplified products of approximately 1200 bp; an amplified product of approximately 2000 bp was noted, with the distal segment of the additional structure (nucleotides 1080 to 2268 in the sequence) demonstrating 100% similarity to a fragment from *A. baumannii* BJAB0719. Acinetobacter sp. 1029086 contains a 2 bp insertion in its carO gene nucleotide sequence at locations 247-249, resulting in the formation of a stop codon (TAA).

Sequence analysis of the carO genes revealed the presence of 13 distinct variants; 11 novel sequences were identified, defined by the occurrence of at least one nucleotide difference. The dominant sequence showed 99-100% similarity to that of *A. baumannii* strain 3027STDY5784960, and the eleven novel sequences exhibited 92-99% identity with sequences in the database. The novel sequences arose as a result of deletions, insertions, or point mutations.

Genes Associated with Drug Efflux Pump Components

Analysis of all isolates revealed that 72.6% possessed efflux system genes. Furthermore, adeIJK and adeABC were observed in 73.16% and 78.24% of isolates, respectively.

Resistance Genes Associated with Mobile Genetic Elements

Multidrug resistance to quinolones, tetracycline, aminoglycosides, and trimethoprim-sulfamethoxazole results from the presence and high prevalence of mobile genetic elements, notably IS26 (98.6%), ISAba1 (92.3%), and ISAba125 (95.7%). Furthermore, the genetic elements tnpU (63.52%) and tnp513 (68.03%) were identified, though at reduced frequencies. Class 1 and class 2 integrons were identified in 195 (75.9%) and 7 (3.3%) isolates, respectively; class 3 integrons were not detected. The analysis of amplicon sizes indicated a range between 1.7 and 2.8 kb. One band was identified in 186 isolates, while two bands were identified in five isolates. Sequencing results indicated that the variable region of class 1 integrons contained four distinct gene cassettes (aadA2-catB9-aacA5, aacC2-OrfA-OrfB-aadA2,

dfrA18-aadA6, aadA3-orfF-dfrA13), while the variable region of class 2 integrons included one gene cassette (dfrA2-sat3-aadA2-orfX).

Eric-PCR

Genotypic analysis using ERIC-PCR revealed high genetic diversity among non-baumannii Acinetobacter isolates from various microbiological specimens, and one representative of similar banding patterns was selected for dendrogram cluster analysis. In contrast, the similarity rate among *A. baumannii* isolates from clinical specimens was above 90%, suggesting that they may have originated from a single clone.

DISCUSSION

The analysis of carbapenem resistance genes indicated the presence of the blaNDM gene in 62 isolates, which were exclusively found in fecal research specimens. Additionally, Acinetobacter species have been documented to display a multidrug-resistant phenotype due to resistance to multiple antibiotics.[39] Previous research indicates a high prevalence of the blaNDM gene in Acinetobacter species in China.[40,41] Acinetobacter spp. significantly contributes to the rising prevalence of New Delhi metallo-β-lactamase-1 (NDM-1) carbapenemases. Reducing the prevalence rates of NDM-1 may become significantly more challenging and could facilitate its ongoing emergence. Our analysis revealed that 98.9% of the *A. baumannii* isolates exhibited an identical blaOXA-51 gene sequence. Lowings et al.[44] and Correa et al.[45] found that 99% and 97.5% of *A. baumannii* strains, respectively, possessed this gene. Zhao et al.[46] found that 91.7% of *A. baumannii* isolates possessed this gene. Zander et al.[47] identified three *A. baumannii* isolates lacking the blaOXA-51-like gene. A more detailed examination of the genomic context of these isolates revealed variations in the blaOXA-51-like genetic structures. Consequently, variants of blaOXA-78 or blaOXA-66 disrupted by insertion sequences were identified. A study performed in twenty-seven hospitals across fourteen Mediterranean and European countries from 2009 to 2011 indicated that 277 (67.4%) of Acinetobacter species possessed the blaOXA-23 gene.[48] Correa et al. found that 97.5% of isolates in Colombia tested positive for the blaOXA-23 gene from 2008 to 2010.[45] It has been shown that the presence of the blaOXA-23 gene was positive in 107 (91.5%) isolates collected from the Gulf Cooperation Council countries.[49] Carvalho and colleagues reported that 96 out of 110 individuals (87%) in Rio de Janeiro carried the blaOXA-23 gene.[10] It was reported that only 3.24% (6/185) of carbapenem-resistant *A. baumannii* strains in northern Croatia and Istria between 2009 and 2010 possessed the blaOXA-23 gene.[50] In Mexico, 152 strains (%100) tested negative for carrying the blaOXA-23 gene.[51]

This study identified twelve non-baumannii Acinetobacter isolates and five *A. baumannii* isolates exhibiting resistance to colistin. Seven isolates of A. junii, derived from fecal research specimens, were identified as carriers of the mcr-1 gene. Additionally, all four A. junii isolates were found to carry the blaOXA-58 gene. One isolate also contained blaVIM, blaNDM, blaOXA-58-like, and mcr-1, while the other strains included blaNDM, blaIMP, blaOXA-58-like, and mcr-1 genes. This observation aligns with reports indicating the co-occurrence of the mcr-1 gene alongside various carbapenem resistance genes in Acinetobacter species.

Sixty-two blaNDM genes were identified in fecal research specimens; the mcr-1 gene was found in seven isolates and was shown to co-occur with additional carbapenem resistance genes. The blaNDM and mcr-1 genes were absent in clinical infection specimens, and the co-occurrence of more than two carbapenemase genes was not observed. The comparison of results from clinical infection specimens and fecal research specimens revealed that carbapenem resistance and the mcr-1 gene were less prevalent in clinical specimens. Additionally, the co-occurrence of multiple

multidrug resistance (MDR) genes was less frequent among isolates from clinical specimens, whereas all isolates with resistance genes were derived from fecal samples. This indicates that the gut serves as a crucial location for the transfer of bacterial resistance genes, underscoring the necessity of managing the related risks for nosocomial infections.

This study's analysis indicated a significant mutation rate in the porin carO gene. Analysis of the carO genes revealed 13 distinct variants and 11 novel sequences. The homology of the carO gene in carbapenem-resistant Acinetobacter and the carbapenem-susceptible reference strain ATCC 17978 was analyzed. The identified amino acid and nucleotide sequences exhibited 94% and 92% similarity, respectively, to the carO sequence of ATCC 17978. Comparative analysis of our sequences with those in various databases, including the NCBI database, revealed a conserved N-terminal region (1-132) and two variable regions (133-163 and 201-239) based on amino acid sequences. Additionally, nucleotide sequence comparison identified two variable regions (398-481 and 589-678). We propose that the structural and functional properties of the CarO protein will exhibit significant differences related to the presence of the two identified variable regions. It has been reported that the carO structure may be associated with carbapenem influx; changes in the amino acid composition of CarO, which could lead to alterations in the porin, may result in carbapenem resistance[17], however, this will require further validation through new analyses.

Multiple studies have demonstrated the existence of the AdeABC efflux system in clinical strains of *A. baumannii*.[4] Numerous studies indicate the presence of these genes in MDR strains; however, some research has also identified them in both MDR and non-MDR isolates.[52,53] Our research identified the presence of adeIJK and adeABC in 76.16% and 78.24% of A. baumannii, respectively, and in 14.78% and 5.13% of A. junii, respectively. Sirawit et al.[53] reported that the multidrug-resistant phenotype in most *A. baumannii* isolates is linked to efflux pumps. Yoon et al.[54] identified the adeB gene in 92.86% of the 13 clinical isolates examined, and detected both adeG and intrinsic adeJ in all *clinical A. baumannii* strains. Our analyses align with other studies indicating that efflux systems are species-specific. [17] Research indicates that the overexpression of these pumps in reaction to antibiotic exposure leads to heightened antibiotic resistance.[55]

A. baumannii was first identified in clinical infection specimens, while non-baumannii Acinetobacter was initially detected in fecal research specimens. ERIC-PCR demonstrated significant genetic diversity among non-baumannii Acinetobacter derived from various specimens. The similarity among *A. baumannii* isolates from clinical infection specimens exceeded 90%, indicating that these isolates may have originated from a single clonal strain. The collection of all strains from samples gathered between 2018 and 2020 indicates the absence of a short-term outbreak.

The findings from this study indicate that carbapenem resistance genes are prevalent among Acinetobacter species in our region, with various systematic mechanisms contributing to this resistance. Colistin serves as a final therapeutic option for infections attributed to carbapenem-resistant Gram-negative bacteria. The emergence of the mcr gene has resulted in significant challenges in clinical treatment protocols. The existence of two significant clinical resistance genes, namely blaNDM and mcr-1, in non-baumannii Acinetobacter gut colonizers is noteworthy. The diversity and abundance of antibiotic resistance genes found in Acinetobacter species in stool samples suggest that the gut may serve as a significant reservoir for resistant opportunistic bacteria.

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