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Investigation of Colistin Resistance and Heteroresistance in *Acinetobacter* spp. Isolates from Various Clinical Specimens

Çeşitli Klinik Örneklerden İzole Edilen *Acinetobacter* spp. İzolatlarında Kolistine Direnç ve Heterodirencin Araştırılması

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Abstract

Introduction: Current studies draw attention to increased rates of resistance and heteroresistance of *Acinetobacter* spp. to colistin. This study aimed to investigate the colistin susceptibility of *Acinetobacter* spp. and detect heteroresistance in colistin-susceptible isolates.
Materials and Methods: *Acinetobacter* spp. isolated from clinical samples submitted to the medical microbiology laboratory were analyzed. Colistin susceptibility of the isolates was investigated by the broth microdilution technique according to European Committee on Antimicrobial Susceptibility Testing standards. In isolates within the susceptibility limits, heteroresistance was screened using Mueller-Hinton agar (MHA) plates containing 4 mg/L colistin. The *mcr-1* gene was amplified using polymerase chain reaction.
Results: Of the 110 isolates of *Acinetobacter* spp., 17 (15.5%) were resistant to colistin. After 24 h of incubation in the MHA medium containing 4 mg/L of colistin, growth was observed in 18 isolates, whereas growth was observed in eight isolates after 48 h. The minimum inhibitory concentration values of those isolates was ≥4 mg/L. Thus, of the 93 colistin-susceptible *Acinetobacter* spp. isolates, 26 (27.9%) were detected as heteroresistant. We did not observe *mcr-1* carriage in *Acinetobacter baumannii (A. baumannii)* isolates.
Conclusion: The high rates of colistin heteroresistance in *A. baumannii* isolates may result in the failure of treatment with colistin because of the selection of colistin-resistant subpopulations. The prediction of potential unresponsiveness before starting colistin therapy appears necessary, and the use of this method, which is more convenient in practice and can test several isolates, may contribute to clinical practice by allowing the clinician to consider various drug options and combinations for treatment.
Keywords: *Acinetobacter* spp., resistance, heteroresistance, colistin, mcr-1

Öz

Giriş: Güncel çalışmalar, *Acinetobacter* spp. izolatlarında kolistin için artan direnç oranlarına ve özellikle hetero-direnç oranlarına dikkat çekmektedir. Bu çalışmada *Acinetobacter* spp.'nin kolistin duyarlılığının saptanması ve duyarlı izolatlarda kolistin heterodirencinin araştırılması amaçlandı.
Gereç ve Yöntem: Hastanemiz tıbbi mikrobiyoloji laboratuvarına, gönderilen klinik örneklerden izole edilen 110 *Acinetobacter* spp. izolatı çalışmaya dahil edilmiştir. İzolatların kolistin duyarlılığı, sıvı mikrodilüsyon ile Avrupa Antimikrobiyal Duyarlılık Testi Komitesi standartlarına göre araştırılmıştır. Kolistin duyarlı izolatlarda heterodirenç varlığı, 4 mg/L kolistin içeren Mueller-Hinton agar (MHA) plaklarında tarama yöntemiyle tekrar değerlendirilmiştir. Polimeraz zincir reaksiyonu kullanılarak *mcr-1* gen varlığı araştırıldı.

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Bulgular: Yüz on *Acinetobacter* spp. izolatının 17'si (%15,5) kolistine dirençli saptanmıştır. Duyarlı bulunan izolatların heterodirenç araştırılmasında; 4 mg/L kolistin içeren MHA besiyerinde 24 saatlik inkübasyon sonunda 18 izolatta üreme gözlenirken, 48. saatte, sekiz izolatta daha üreme gözlendi. Kolistinli MHA besiyerinde üreyen *Acinetobacter* spp. izolatlarına, tekrar sıvı mikrodilüsyon çalışılarak, tümünün minimum inhibitör konsantrasyon değerinin ≥4 mg/L olarak doğrulanmasıyla, heterodirenç tanımlaması yapıldı. Böylece 93 kolistin duyarlı *Acinetobacter* spp. izolatının, 26'sında (%27,9) heterodirenç varlığı tespit edildi. İzolatlarda *mcr-1* gen pozitifliği saptanmadı.
Sonuç: *Acinetobacter baumannii* izolatlarında kolistin heterodirenç oranının yüksek olması, kolistine dirençli alt popülasyonların seçilmesi nedeniyle kolistin ile tedavinin başarısız olmasına neden olabilir. Kolistin tedavisine başlamadan önce potansiyel yanıtsızlığın öngörülmesi gerekli görünmektedir ve pratikte kullanımı daha uygun ve çok sayıda izolatın test edilebileceği bu yöntemle, klinisyenin tedavi için çeşitli ilaç seçeneklerini ve kombinasyonlarını değerlendirmesine olanak tanıyarak klinik uygulamaya katkıda bulunabilir.
Anahtar Kelimeler: *Acinetobacter* spp., direnç, heterorezistans, kolistin, mcr-1

Introduction

Acinetobacter spp. are widely found in nature and hospital environment, are opportunistic pathogens, and are responsible for high mortality and morbidity in hospital infections, especially those seen in intensive care units (ICU) such as ventilator-associated pneumonia, bacteremia, urinary system infections, and secondary meningitis[1,2]. *Acinetobacter baumannii (A. baumannii)* can be considered one of the leading nosocomial multidrug resistant (MDR) bacteria because it develops resistance to many antimicrobial agents, including colistin[3].

Carbapenems are traditionally the preferred treatment for MDR *A. baumannii*; the rates of carbapenem-resistant *A. baumannii* infections have increased dramatically, developing resistance to all antimicrobial agents except polymyxin and tigecycline. However, colistin-resistant and tigecycline-resistant *A. baumannii* isolates have been reported at increasing rates (2-53%) worldwide[3-6].

Heteroresistance is defined as the presence of a resistant subpopulation in an isolate that was determined to be susceptible by standard test methods[7]. Heteroresistance creates difficulties in determining minimum inhibitory concentration (MIC) values and causes treatment failures in antibiotic therapies that are initiated based on the results of standard susceptibility tests with the potential to result in the selection of antibiotics to which only a resistant subpopulation is susceptible. Different methods are required to detect heteroresistance[7-11].

Increasing numbers of multidrug- or carbapenem-resistant *A. baumannii* infections have prompted the use of polymyxin antibiotics (colistin and polymyxin B) for their treatment. The re-boosted polymyxin use increases the selective pressure that favors resistant strains[9-14]. Nevertheless, the utility of polymyxins is currently facing a worldwide increasing resistance, particularly due to the plasmid-encoded mobilized colistin resistance (*mcr*) gene present in pathogens. Moreover, the emergence of mcr, associated with moderate colistin resistance, makes this scenario even more adverse[15].

This study aimed to determine the susceptibility of colistin in *Acinetobacter* spp. isolates by the broth microdilution method and investigate colistin heteroresistance in colistin-susceptible *Acinetobacter* spp. isolates.

Materials and Methods

Bacterial Isolates and Identification

A total of 110 *Acinetobacter* spp. isolates from the medical microbiology laboratory biobank were included in the study. Only one isolate per patient was included in the study. The distribution of the clinical ward isolates was as follows: ICUs, 76 (69.09%) [internal ICU, n=30 (27.3%); surgical ICU, n=23 (20.9%); anesthesiology ICU, n=23 (20.9%)]; surgical inpatient units, 16 (14.6%); and internal medicine inpatient units, n=15 (13.6%). The majority of the samples were isolated from inpatients and patients in the ICU. Only three isolates (2.7%) were collected from outpatients. The distribution of the 76 *Acinetobacter* spp. isolates from the ICU is presented in Table 1.

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Most of the samples (n=59, 53.6%) were isolated from the respiratory tract (tracheal aspirates, sputum, and bronchoalveolar lavage), followed by isolates from the blood/catheter (n=19, 17.3%), wounds (n=13, 11.8%), and urine (n=13, 11.8%). The

Table 1. Distribution of the 76 *Acinetobacter* spp. isolates from the intensive care units

Departments	Number (n)	Percent (%)
Anesthesiology ICU	23	30.3
Internal medicine ICU	16	21.1
Neurology ICU	12	15.8
Neurosurgery ICU	11	14.5
Cardiovascular surgery ICU	6	7.9
General surgery ICU	6	7.9
Coronary care ICU	2	2.6
Total	**76**	**100**

ICU: Intensive care unit

distribution of the isolates by the collection site is presented in Table 2.

Urine samples were inoculated onto sheep blood agar (SBA) and eosin methylene blue (EMB) agar. Other samples were inoculated onto SBA, EMB agar, and chocolate agar media. Consequently, the inoculated media were incubated at 37 °C. Blood culture specimens were incubated in the automated culture system (BD BACTEC™ FX blood culture system, USA). After the specimens' positive growth signal, they were sampled and inoculated onto SBA, EMB agar, and chocolate agar media. The inoculated media were incubated at 37 °C. Bacteria growth was identified by conventional methods and the BD Phoenix™ (BD Phoenix™ ID System) automated system. Antimicrobial susceptibility tests were performed using the BD Phoenix™ System (BD Phoenix™ AST System).

Colistin Susceptibility Testing

Colistin sulfate antibiotic powder (Carbosynth Limited, UK) was used to test colistin susceptibility. The broth microdilution test was performed, containing colistin in concentrations ranging from 32 to 0.03 mg/L. Colistin-susceptible *Escherichia coli (E. coli)* ATCC 25922 and colistin-resistant *E. coli* NCTC 13846 (mcr-1 positive) were used as quality control strains. Microplates were incubated in an aerobic environment at 35 °C for 18-20 h. MIC values obtained were evaluated in comparison to the breakpoint limits according to the European Committee on Antimicrobial Susceptibility Testing standards[16].

Investigation of Heteroresistance to Colistin

As described previously by Srinivas et al.[10], heteroresistance to colistin in *Acinetobacter* spp. isolates was investigated using Mueller-Hinton agar (MHA) plates containing 4 mg/L colistin. MHA medium plates with 4 mg/L colistin concentrations were prepared. Aliquots of 100 µL, from the 0.5 McFarland standard turbidity-adjusted suspensions of the isolates in saline, were transferred to MHA plates containing 4 mg/L colistin. A suspension of each isolate diluted to 500 colony forming unit (CFU)/ml was inoculated onto antibiotic-free MHA plates for growth control. The quality control strains, *E. coli* ATCC 25922 and *E. coli* NCTC 13846, were used at target bacterial counts of 1×10^4 CFU.

All plates were incubated in a 35 °C incubator for 48 h in an aerobic environment. The plates were examined with the naked eye on a black background. Bacterial growths were recorded as the number of colonies after 24 and 48 h of incubation. The cut-off value for growth was accepted as 20 CFU/ml. Growing colonies were identified as *Acinetobacter* spp. by conventional methods, and the results were confirmed by automated systems. Colonies grown on colistin-containing MHA plates were subcultured to SBA. MIC values of these populations were re-determined by the broth microdilution method. Isolates with MIC values confirmed as ≥4 mg/L were defined as heteroresistant[9].

Table 2. Distribution of the Acinetobacter spp. isolates by the site of collection

Clinical sample	Number (n)	Percent (%)
Tracheal aspirate	51	46.4
Blood/catheter blood	19	17.3
Wound	13	11.8
Urine	13	11.8
Sputum	7	6.4
CSF	4	3.6
Other (cyst, synovial fluid, BAL)	3	2.7
Total	**110**	**100**

BAL: Bronchoalveolar lavage, CSF: Cerebrospinal fluid

Determination of the *Mcr-1* Gene Using Polymerase Chain Reaction (PCR)

The *mcr-1* gene was amplified using gene-specific primers with conventional PCR[15].

Statistical Analysis

Descriptive statistics were used. Results were presented as frequencies and percentages.

Results

Bacterial Isolates

As for the distribution of bacteria at the species level, 62 of the 110 isolates were identified as *A. baumannii-calcoaceticus* complex and 48 as *A. baumannii* by the Phoenix automated system (BD Phoenix™ ID & AST System). However, due to problems in discriminating the isolates of the *A. baumannii-calcoaceticus* complex via phenotypic testing, all isolates were classified as a general group of *Acinetobacter* spp.

Colistin Susceptibility Results

In this study, 17 (15.5%) isolates were detected to be resistant to colistin and 93 (84.5%) were susceptible to colistin. The distribution of resistant isolates by clinics revealed the ratios of resistant isolates as 2/30 in internal ICUs, 5/23 in surgical ICUs, 3/23 in anesthesiology ICUs, 3/16 in surgical wards, and 4/15 in internal medicine wards. The MIC_{50} and MIC_{90} values for colistin for all isolates were 0.5 and 8 mg/L, respectively.

Results of Heteroresistance Screening

After incubating 93 colistin-susceptible *Acinetobacter* spp. isolates inoculated onto MHA media-containing colistin, growth was observed in 18 after 24 h of incubation. After 48 h of incubation, growth was observed in eight more isolates

in addition to the former 18 isolates. The number of colonies is shown in Table 3, and growth was detected in 26 isolates in total. Table 3 shows the colony counts of each isolate in detail. Growth occurred in a total of 26 isolates.

Colonies grown on colistin-containing MHA media were subcultured onto SBA and EMB agar media. After 24 h of incubation at 35 °C, colonies were identified and confirmed as *Acinetobacter* spp. isolates. The MIC values of the subpopulations were determined again by the broth microdilution test performed on the subcultured colonies on the SBA medium. The MIC values of all isolates were ≥8 mg/L.

As a result, of the 93 *Acinetobacter* spp. isolates identified as susceptible to colistin by the standard test method, 26 grew on the MHA media containing 4 mg/L colistin. Because the MIC values of these growing colonies indicated resistance according to the results of the broth microdilution test, these were defined as heteroresistant isolates. Thus, of the 93 colistin-susceptible *Acinetobacter* spp. isolates, 26 (27.96%) were identified as having resistant subpopulations.

Determination of the *Mcr-1* Gene Using PCR

We did not observe mcr-1 carriage in *A. baumannii* isolates.

Discussion

Acinetobacter spp. are regarded as responsible for many healthcare-associated infections, including predominantly bacteremia, pneumonia, meningitis, and urinary tract infections. *Acinetobacter* spp. are opportunistic pathogens causing significant morbidity and mortality. The increasing rates of multiple antibiotic resistance, including carbapenems, and the potential of emerging outbreaks in the ICUs keep *A. baumannii* as a major pathogen[17].

Table 3. Colony counts of heteroresistant isolates

Isolate no	Colony count after 24 h of incubation (CFU/ml)	Colony count after 48 h of incubation (CFU/ml)	MIC values for the heteroresistant subpopulation (mg/L)	MIC value of the whole isolate at baseline (mg/L)
6	190	210	8	0.25
11	>1000	>1000	16	2
22	90	100	16	1
25	60	60	>32	0.5
27	0	90	8	0.25
30	0	60	32	<0.03
32	10	350	32	0.25
34	210	>1000	32	<0.03
43	20	20	32	0.25
47	0	130	32	0.5
49	0	40	8	0.12
67	20	20	16	2
69	20	20	32	0.25
71	150	150	16	0.5
74	0	100	32	2
77	20	70	>32	2
82	260	380	8	1
87	0	60	8	0.25
89	420	500	>32	2
93	130	250	>32	0.25
96	0	20	16	2
97	0	70	32	0.5
98	320	390	>32	1
101	40	80	>32	1
107	160	230	>32	1
108	60	110	32	0.5

MIC: Minimum inhibitory concentration, CFU: Colony forming unit

When the distribution of the samples was examined according to the submitting clinics, most of the isolates (76 isolates, 69.1%) were obtained from the samples submitted by the ICUs. This finding is consistent with many studies in this field and the literature[17-19]. The more frequent occurrence of infections caused by *Acinetobacter* spp. in the ICUs can be explained by the follow-up of critical patients in these units and the more frequent application of invasive procedures such as mechanical ventilation, tracheostomy, intubation, central catheterization, and urinary catheter[20].

In recent years, *A. baumannii* isolates from clinical specimens have started to exhibit MDR against many antibiotics such as β-lactams, fluoroquinolones, tetracyclines, aminoglycosides, and carbapenems, which is alarming[21]. They are generally resistant to all agents other than a few antibiotics such as polymyxin and tigecycline. However, *A. baumannii* isolates that are resistant to colistin and tigecycline have been increasingly reported in recent years. As a result, many clinical isolates appear pan-resistant[22]. The highest colistin rate of resistance was reported from India (53%), followed by Iran (48%), Spain (40.7%), and Korea (30%) [4]. The use of different methods in studies conducted in our country is directly reflected in the results reported in a wide range of rates. The striking point is the high rates of resistance to colistin found by broth microdilution, which is accepted as the standard method. In our study, susceptibility to colistin was investigated by the broth microdilution method as the standard method recommended by European Committee on Antimicrobial Susceptibility Testing. Resistance to colistin was detected in 17 (15.5%) of the 110 *Acinetobacter* spp. isolates in our study. When the studies using the broth microdilution methods were examined, multidrug-resistant *Acinetobacter* spp. isolates were further tested for susceptibility against colistin by microdilution and the rate of colistin resistance was 27.5%[23]. In another study, the rate of resistance to colistin in 97 *Acinetobacter* spp. isolates was 8.2% by broth microdilution[24].

Besides the increases observed in the resistance rates detected in *Acinetobacter* spp. isolates, another concern is the heterogeneous colistin resistance selection and the subsequent development of resistance. In recent years, isolates with colistin heteroresistance have been increasingly detected. However, these studies have limitations because of wide variations in sample sizes, detection methods, and rates of colistin heteroresistance[9-14]. Colistin heteroresistance cannot be detected by standard susceptibility tests as disk diffusion method, and it causes problems in determining the MIC. The concerning issue occurs in antibiotic-resistant cases, where all bacterial populations and even subpopulations with the lowest MIC values are resistant. In that case, gene transfer occurs from highly resistant bacteria to bacteria showing a low level of resistance[7-11].

Colistin heteroresistance in *A. baumannii* was first described by Li et al.[9] in 2006. To investigate colistin heteroresistance, the researchers used the population analysis profile (PAP) method on 16 *A. baumannii* isolates. Although all isolates were susceptible to colistin with MIC values from 0.25 mg/L to 2 mg/L, most of the isolates (15/16) yielded growth in the presence of 3-10 mg/L colistin. The rate of heteroresistance to colistin was 93.8%, and it was argued that heteroresistance could be due to the inappropriate use of colistin, affecting treatment success unfavorably. Srinivas et al.[10] screened heteroresistance and showed that the heteroresistance rate was 83% [92% (11/12) for blood cultures and 75% (9/12) for respiratory tract] *A. baumannii* isolates. The MIC values of those isolates at baseline ranged from ≤0.25 mg/L to 0.5 mg/L by the broth microdilution method indicating that they were susceptible. However, MIC values were not confirmed for colonies, which yielded growth in colistin-containing MHA media and consequently were accepted as heteroresistant in that study. Thet et al.[25] investigated colistin resistance by the broth microdilution method and heteroresistance rate by the PAP method in 75 carbapenemase-resistant *A. baumannii* isolates. The colistin MIC range for the 75 isolates was 0.5-2 µg/ml, with MIC_{50} and MIC_{90} of 1 and 2 µg/ml, respectively. Moreover, 44% of the isolates were considered colistin-heteroresistant with subpopulations growing at 3-8 µg/ml of colistin. The high rate of colistin heteroresistance in *A. baumannii* isolates may lead to the failure of treatment with colistin due to the selection of colistin-resistant subpopulations. In the study by Yau et al.[11]., a total of 30 isolates from 10 clinical centers in various countries were examined. Except for one isolate (MIC 128 mg/L), the MIC values of all isolates ranged from 0.5 mg/L to 2 mg/L by the broth microdilution method indicating that they were susceptible. Performing the PAP analysis, subpopulation growth was shown in the presence of colistin >2 mg/L in seven susceptible isolates, and the heteroresistance rate was 23%. Rodriguez et al.[13] observed colistin resistance selection in a heteroresistant *A. baumannii* isolate from a patient receiving colistin therapy because meningitis developed after neurosurgery. Gazel and Tatman Otkun[26] could not detect heteroresistance on MHA media containing 4 mg/L colistin inoculated with 31 carbapenem-resistant *A. baumannii-calcoaceticus* complex isolates from blood cultures of patients from the ICU. When isolates were exposed to colistin at sub-inhibitory concentrations to reveal latent heteroresistance/resistance, heteroresistance/resistance to colistin developed in all isolates. That study has demonstrated that strains that do not appear to be heterogeneously resistant can easily develop heteroresistance/resistance after exposure to colistin. In the same study, the effects of different antibiotic combinations on the heteroresistance ratio were investigated on two isolates. Colistin-rifampicin and colistin-tigecycline combinations prevented the development of heteroresistance/

resistance even at low doses. Based on these results, resistance and heteroresistance to colistin could easily develop after inappropriate use; therefore, colistin should be used at appropriate doses and in combination with recommended antibiotics to avoid the development of colistin heteroresistance/resistance. Current studies have reported the prevalence of colistin heteroresistance in *Acinetobacter* spp. Isolates, which ranged from 23% to 100%[9-14]. In our study, we inoculated MHA media containing 4 mg/L colistin with 100 µL of aliquots of the 0.5 McFarland adjusted bacterial suspensions of *Acinetobacter* spp. that were identified as susceptible. Growing colony counts were recorded after 24 and 48 h of incubation. While growth was observed in 18 isolates after 24 h of incubation, eight more isolates yielded growth in addition to the former 18 isolates after 48 h. Of the colonies growing on colistin containing MHA media, the ones identified as heteroresistant *Acinetobacter* spp. yielded MIC values of ≥8 mg/L confirming the results. As a result, of the 93 colistin-susceptible *Acinetobacter* spp., resistant subpopulations were detected in 26 (27.96%) isolates. The occurrence of colistin heteroresistance in A*cinetobacter* spp. isolates and concerns about receiving suboptimal results through treatment with colistin, which is considered one of the drugs of last resort, raise the need for studies to further investigate the presence of heteroresistance.

The strengths of our study include the large sample size compared with those of many other studies, detection of heteroresistance reliably through the identification of *Acinetobacter* spp. in the MHA media, and the confirmation of the results in the presence of MIC values of ≥4 mg/L. We used a methodology that was adopted as an alternative option and was reported by previous studies in the literature[10,12,26,27].

The emergence of mcr-1 heralds the breach of the last group of antibiotics, polymyxins, by plasmid-mediated resistance. Although we did not detect *mcr-1* gene positivity in our study, studies have stated that the number is increasing in *Acinetobacter* spp. and Enterobacterales[15,28,29].

Unfortunately, because of the poorly established pharmacokinetics and pharmacodynamics of colistin and suboptimal use, colistin resistance has occurred in clinical isolates. Another important concern is the detection of colistin heteroresistance in *A. baumannii* clinical isolates that are determined to be susceptible to colistin.

Study Limitations

The limitations of the study are the lack of PAP analysis (gold standard method to detect heteroresistance), clonality analysis, and colistin-dependent growth analysis.

Conclusion

Colistin heteroresistance cannot be detected by standard susceptibility tests, and it causes problems in determining the MIC. The concerning issue occurs in antibiotic-resistant cases, where all bacterial populations and subpopulations with the lowest MIC values are resistant. Although not as effective as the PAP method, which is accepted as the gold standard method, but requires ample time and extra effort, the method used in this study can test many isolates and yield results faster as a favorable method for use in clinical practice. Thus, the potential unresponsiveness to treatment will be predicted before starting colistin therapy, and different drugs or combinations of drugs can be suggested to clinicians, contributing to clinical practices. Further experimental and clinical studies are needed to determine the significance of in vitro colistin heteroresistance in clinical therapies, correlating findings across clinical and microbiological results.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Turkey, Dışkapı Yıldırım Beyazıt Training and Research Hospital Clinical Research Ethics Committee (decision no: 60/20, date no: 25.02.2019).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.A.K., E.Ç., Z.D., M.Ç., G.E., Concept: E.A.K., Z.D., M.Ç., Design: E.A.K., E.Ç., Data Collection or Processing: E.A.K., E.Ç., D.Ö., G.E., A.T., Analysis or Interpretation: E.A.K., E.Ç., D.Ö., M.Ç., G.E., A.T., Literature Search: E.A.K., D.Ö., Z.D., M.Ç., G.E., Writing: E.A.K., D.Ö., M.Ç., G.E., A.T.

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