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An Investigation of Microsporidia Species in Humans Living in Düzce and Bolu Province of Türkiye

Düzce ve Bolu İllerinde Yaşayan İnsanlarda Mikrosporidia Türlerinin Araştırılması

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ABSTRACT

Objective: The literature about microsporidia clearly shows that the prevalence of microsporidia varies at different rates depending on the sampling status and region in the studies. However, the majority of studies have been conducted in immunocompromised individuals. It is vital to determine the prevalence of pathogens in society in order to protect immunocompromised individuals from opportunistic pathogens. Consequently, the present study investigated stool samples from Düzce and Bolu provinces for microsporidia, irrespective of disease group.

Methods: A study of 400 stool samples from Düzce and Bolu was performed. The samples were first stained with trichrome and examined under a microscope at 100x magnification. DNA was then isolated, amplified with polymerase chain reaction primers, and the samples were identified. The relationship between microsporidia presence and age, sex, and diarrheal status was statistically investigated.

Results: Following microscopic examination, microsporidia spores were detected in 25 of the 400 (6.25%). Microsporidia spores were detected in 6.6% (14/212) of the Düzce samples and 5.8% (11/188) of the Bolu samples. Consequently, molecular analysis revealed the presence of microsporidia in 49 of the 400 (12.25%). The positivity rates were found to be 11.3% (24/212) for Düzce and 13.3% (25/188) for Bolu. The spores detected in this study were identified as belonging to the species *Enterocytozoon bieneusi*. A statistical analysis was conducted, revealing no significant association between the presence of the pathogen and diarrhea complaints, gender, or age.

Conclusion: It is seen that the presence of microsporidia in our study is at a level similar to rates reported in immunocompetent individuals around the world. Additionally, a correlation between the presence of microsporidia and complaints has been reported, especially in immunocompromised individuals, but no relationship has been determined with complaints in this study. Furthermore, the present study demonstrated that neither age nor gender exhibited any correlation with the presence of microsporidia.

Keywords: Microsporidia, *Enterocytozoon bieneusi*, prevalence, diarrhea, molecular

ÖZ

Amaç: Literatüre bakıldığında, fırsatçı patojen mikrosporidiaların yaygınlığının çalışmalardaki örneklem durumuna ve bölgelere göre farklı oranlarda raporlandığı görülmektedir. Bununla birlikte çalışmaların büyük bir çoğunluğu bağışıklığı yetersiz kişilerde gerçekleştirilmiştir. Bağışıklığı yetersiz kişilerin fırsatçı patojenlerden korunması için toplumda o patojenin yaygınlığının belirlenmesinin çok önemli olduğunu düşünmekteyiz. Bu sebeple, mevcut çalışmada hastalık grubu gözetmeksizin Düzce ve Bolu illerinden gaita numuneleri mikrosporidia açısından araştırılmıştır.

Yöntemler: Bu çalışmada Düzce ve Bolu illerinde yaşayan insanlardan toplam 400 gaita numunesi temin edilmiştir. Örnekler ilk olarak trikrom boyama ile boyanarak mikroskopta 100x büyütmede incelenmiştir. Yine her örnekten DNA izolasyonu yapılmış ve

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Türkiye PARAZİTOLOJİ Dergisi

ÖZ

sonrasında türlere özgü primeler kullanılarak polimeraz zincir reaksiyonu ile tür tespiti gerçekleştirilmiştir. Mikrosporidia varlığının yaş, cinsiyet ve ishal durumu ile ilişkisi istatistiksel olarak araştırılmıştır.

Bulgular: Mikroskobik incelemeler sonucunda 400 örneğin 25'inde (6,25) mikrosporidia sporları saptanmıştır. İllerin kendi içerisinde değerlendirilmesi sonucunda Düzce örneklerinin %6,6'sında (14/212) Bolu örneklerinin %5,8'inde (11/188) mikrosporidia sporları görülmüştür. Moleküler analizin sonucunda 400 örnekten 49 tanesinde (12,25) mikrosporidia saptanmıştır. Pozitiflik oranlarının Düzce için %11,3 (24/212) ve Bolu için %13,3 (25/188) olduğu görülmüştür. Tespit edilen sporların *Enterocytozoon bieneusi* türüne ait olduğu saptanmıştır. Yapılan istatistiksel analiz sonucunda patojenin varlığı ile ishal şikayeti, cinsiyet ve yaşın arasında anlamlı bir ilişki olmadığı görülmüştür.

Sonuç: Çalışmamızdaki mikrosporidia varlığının bağışıklığı yeterli kişilerde raporlanan oranlarla benzer düzeyde olduğu görülmektedir. Bu durum, mikrosporidia türlerine maruz kalmanın yaygın olduğunu ve sağlıklı bireylerde herhangi klinik belirti göstermeden enfeksiyonun toplumda sürekli devam ettiğini göstermiştir. Literatürde mikrosporidia varlığı ile gastrointestinal şikayetlerin ilişkili olduğunu bildiren çalışmalar varken tam tersi durumun da varlığı raporlanmıştır. Özellikle bağışıklık sistemi açısından kronik bir rahatsızlığı olmayan kişilerde mikrosporidia türleri kendini sınırlayan ve klinik belirti göstermeyen enfeksiyonlar oluşturması sebebiyle bazı çalışmalarda şikayetlerle mikrosporidia ilişkisinin olmadığının raporlanması normal bir durumdur.

Anahtar Kelimeler: Mikrosporidia, *Enterocytozoon bieneusi*, prevalans, ishal, moleküler

INTRODUCTION

Microsporidia species are spore-forming unicellular eukaryotes and are obligate intracellular pathogens. To date, more than 1500 species belonging to 200 genera have been identified within phylum microsporidia which have broad spectrum of host. Of the 17 human pathogen microsporidia species, *Enterocytozoon bieneusi* is most common agent in humans followed by *Encephalitozoon intestinalis*. They are responsible for more than 90% of human microsporidosis and are often associated with gastrointestinal tract infections (1,2).

It is established that microsporidia species are responsible for opportunistic infections and severe clinical symptoms, particularly in individuals with suppressed or insufficient immune systems, such as organ transplant recipients and human immunodeficiency virus (HIV) positive individuals. Consequently, majority of studies have been conducted on immunocompromised individuals (3-6).

It is greatly important to ascertain the prevalence of the pathogen in society and the environment in order to protect immunocompromised individuals afflicted with diseases such as HIV and cancer. We think that determining the frequency of microsporidia species in the society and the environment through studies conducted irrespective of disease group will help the implementation of appropriate measure for protection of immunodeficient people.

For this reason, stool samples were obtained from individuals residing in Düzce and Bolu provinces, irrespective of their disease status, and subjected to microscopic and molecular examination to ascertain the prevalence of microsporidia species in the aforementioned provinces.

METHODS

In this study 400 fecal samples were obtained from Düzce University (DU) Health Application and Research Facility (212 samples) and the Bolu Abant İzzet Baysal University Training and Research Hospital (188 samples) between April 2021 and July 2021. The sample amounts obtained from the provinces were calculated in proportion to their population. Samples were collected irrespective of any disease group to determine prevalence of microsporidia in society. However, stool samples were obtained from subjects who were not immunocompromised and did not receive either antibiotic or antiparasitic medication. Samples were examined using both microscopic and molecular methods. We commenced the research following the ethics approval from the "DU Non-Invasive Health Research Ethics Committee" (decision no: 2019/41 and date: 16.12.2019). Stool samples were promptly conveyed to the parasitology laboratory, where they were separated into two groups, within every fecal sample, both for microscopic and molecular examinations.

Microscopic Examinations

Parted number of samples for microscopy are put to the formol-ether sedimentation method and subsequently modified trichrome staining (MTS) for determining parasites under microscope at 100X magnification (2).

Molecular Methods

DNA extraction: Genomic DNA was extracted directly from fecal specimens using "GeneAll Stool DNA Minikit" (GeneAll, Seoul, South Korea) following the manufacturer's instruction. All reagents necessary for the extraction process were present within the kit.

Polymerase chain reaction (PCR) amplification: C1 and C2 primers were selected for use in to amplifying a con-served region of the small-subunit rRNA of four microsporidia species namely *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. cuniculi* and *E. hellem* (7). Subsequently, a second PCR reaction was conducted using an alternative primer pair with positive specimens from the first PCR application for the purpose of identifying the species (Table 1). The ATB 2X PCR Master Mix brand mixture was used in the PCR reaction and the final volume was calculated to be 50 µL. The size of the bases was determined with the Thermo Scientific GeneRuler 1 kb Plus DNA Ladder marker (Figure 1).

PCR reactions were run using the "BİO-RAD T100" thermal cycler device. Following the denaturation of the DNA at 94 °C for a period of 5 minutes, 35 cycles were conducted in following manner: Denaturation at 94 °C for 1 minute, annealing 56 °C for 1 minute, and elongation at 72 °C for 1 minute. Following the completion of 35 cycles of process, PCR reactions were terminated at 72 °C for a period of 5 minutes. The amplified products were subjected to electrophoresis on an agarose gel and stained with ethidium bromide. The stained gel was examined under ultraviolet light.

Statistical Analysis

Chi-square tests were employed to evaluate the correlation between microsporidia positivity and factors such as age and gender. A Kappa test was also applied to ascertain the sensitivity and specificity of the methods. A p-value of less than 0.05 was considered to indicate a statistically significant result.

Table 1. Primer pairs sequences

Primers	Base squences	Species
C1 (F)	5′-CACCAGGTTGATTCTGCC-3′	*Enterocytozoon bieneusi, Encephalitozoon intestinalis, E. cuniculi* and *E. hellem*
C2 (R)	5′-GTGACGGGCGGTGTGTAC-3′	
EBIEF1 (F)	5′-GAAACTTGTCCACTCCTTACG-3′	*Enterocytozoon bieneusi*
EBIEF1 (R)	5′-CCATGCACCACTCCTGCCATT-3′	
EIMCF (F)	5′-GTCTATGGTGAATGCTGCAGTT-3′	*Encephalitozoon intestinalis*
EIMCR (R)	5′-ATTTCGATCCTCTAGCCTTCGT-3′	

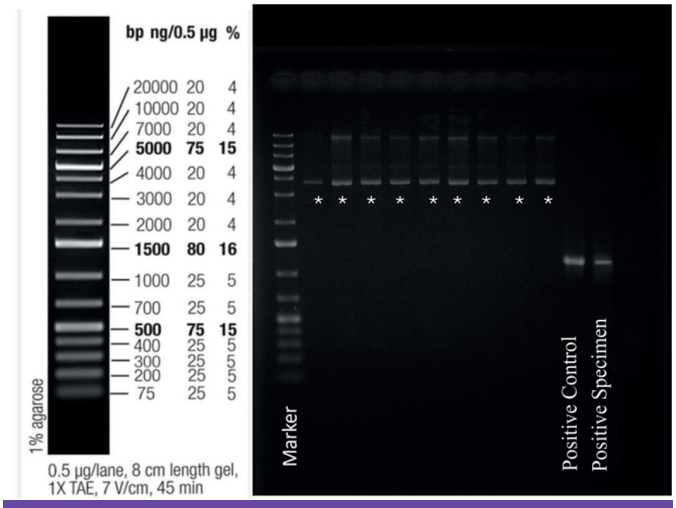

Figure 1. Image of PCR products of 1% agarose gel electrophoresis (*signed strips belong another study)
PCR: Polymerase chain reaction

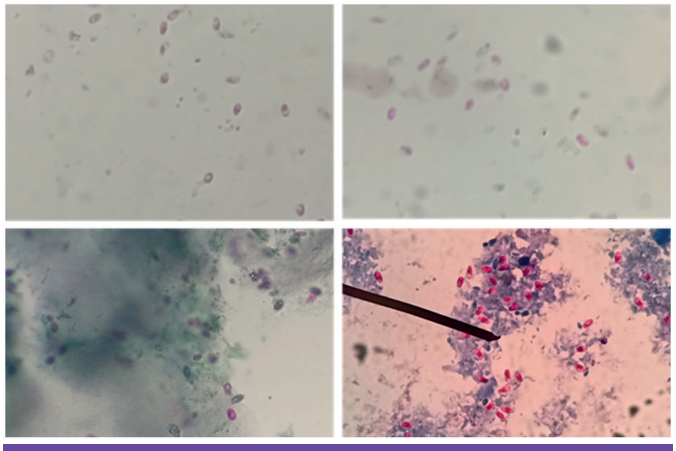

Figure 2. MTS-stained microsporidia spores from different individuals (100x magnification)
MTS: Modified trichrome staining

RESULTS

Microscopic Results

As a result of examination of the MTS-stained slides at 100X magnification, microsporidia spores were observed in 25 out of 400 (6.25%) samples (Figure 2). Upon separation of the cities where the patients come from, the positivity rate in Düzce was determined to be 6.6% (14/212) while in Bolu it was 5.8% (11/188).

Molecular Results

As a result of electrophoresis for PCR products, strips of parasite DNA were observed in 49 out of 400 (12.25%) samples in total. When the cities were evaluated separately, the prevalence in Düzce was found to be 24/212 (11.3%) while in Bolu was 25/188 (13.3%). Following the PCR reactions conducted with species-specific primers (*E. bieneusi* and *E. intestinalis*), the presence of strips was observed exclusively in those, primers selected for *Enterocytozoon bieneusi*.

Statistical Results

In this study, the relationship between the presence of microsporidia and age, gender and diarrhea status was evaluated based on PCR results because of its reliability. Although certain differences were observed among the factors compared in terms of presence of microsporidia, these differences were not found to be statistically significant.

While 26 of 204 samples (12.7%) obtained from women were found to be microsporidia positive, 23 of 196 samples (11.7%) from men were found to be positive in total. In the separate evaluation of the cities, positivity was observed in 15 of 118 women samples (12.7%) and 9 of 94 men samples (9.6%) in Düzce. Furthermore, 11 of the 86 women samples (12.8%) and 14 of the 102 men samples (13.72%) were positive in Bolu. The overall level of positivity is notably elevated among the sampled female population. Nevertheless, while the positivity rate is high in females within the Düzce province, it exhibits a higher frequency among males residing in the Bolu province.

In one sample, microsporidia positive was found among 16 diarrheic individuals (6.3%) while 48 of 384 (12.5%) non-diarrheal patients were positive. Microsporidia were identified in one of 7 diarrheal samples (14.3%) and 24 of 181 non-diarrheal samples (13.3%) in Bolu province. The presence of microsporidia was not detected in any of the nine diarrheal samples from Düzce province, yet 24 of 203 (11.8%) non-diarrheal samples were positive. It is noteworthy that the prevalence of microsporidia in individuals exhibiting diarrhea is lower in comparison to non-diarrheal. Nevertheless, the limited number of diarrheal samples collected precludes the ability to make precise comparisons.

When the results were evaluated in terms of age groups, it was seen that 3 of 32 (9.4%) in the “1-14” age group, 5 of 50 (10%) in the “15-24” age group, 27 of 214 (12.6%) in the “25-64” age group and 14 of 104 (13.5%) in the “65 and over” age group. An examination of these results demonstrates a clear increase in the frequency of microsporidia as the age of the subjects increases but the chi-square analysis revealed that there was no statistically significant relationship between gender, diarrhea and age groups with the presence of microsporidia in this study (Table 2).

Table 2. Distribution of microsporidia according to gender, age and diarrhea

Groups		Total	Positive	Percentage	p-value
Gender	Women	204	26	12.7%	0.439
	Men	196	23	11.7%	
Age	1-14	32	3	9.4%	0.705
	15-24	50	5	10%	
	25-64	214	27	12.6%	
	65 and over	104	14	13.5%	
Diarrhea	Yes	16	1	6.25%	0.885
	No	384	48	12.5%	

Table 3. Crosstab of molecular and microscopic results

Groups		Molecular				
		Positive		Negative		Total
		n	%	n	%	
Microscopic	**Positive**	25	51	0	0	6.3
	Negative	24	49	351	100	93.7
Total		49	100	351	100	100

Considering the potential limitations of a single-method approach, this study employed a dual-method design, assessing the compatibility of these methods. As a result of analysis, the sensitivity and specificity of MTS was calculated to be 51% and 100%, respectively (Table 3). The Kappa test yielded a compatibility score of 0.646 for the two methods employed in this study. This result indicates that the compatibility between the two methods is moderate.

DISCUSSION

The majority of research conducted on these microorganisms has been carried out on immunocompromised people, as microsporidia species are known to cause significant issues in those with these, mentioned individuals. It is of great importance to determine the prevalence of the pathogen in society and the environment in order to ensure the protection of immunocompromised individuals. It is our contention that an investigation of the prevalence of microsporidia in society, conducted without regard to disease group, will facilitate the implementation of appropriate measures for the protection of individuals, especially those with compromised immune systems from these pathogens. Accordingly, samples provided and included in this research were irrespective of any special circumstances.

As a result of microscopic and molecular examination, microsporidia positivity rates were 6.25% and 12.25%, respectively in current study. A review of other studies conducted in Türkiye reveals that the positivity rates of microscopic examinations vary between 5 and 10%, while those of molecular methods range from 10 to 69%. Microsporodiosis rates are higher in immunocompromised individuals, and the course of disease has also been reported to be more serious. Moreover, the researchers provided an explanation for the elevated positivity rates observed in molecular techniques, citing the enhanced reliability of such techniques and the characteristics of the patient cohort in question. A review of the relevant studies reveals that microsporidia positivity in immune sufficient people is close to our study (4,5,8-11). In contrast to the findings of previous studies conducted in our country, which identified *E. intestinalis* as the primary infectious agent, our study observed *E. bieneusi* as the causative agent (4,5,10). This indicates that the predominant microsporidia species may vary according to factors such as geographical location. An analysis of the prevalence of microsporidiosis in the country demonstrates the presence of the pathogen in the population at a certain rate. Indeed, in their meta-analysis of studies conducted within our country, Çetinkaya and Caner (12) documented the mean frequency of microsporidia as 13.4% in human subjects and 15.2% in other vertebrate hosts. This review provides an adequate framework for the frequency of microsporidia in our country and clearly demonstrates the importance of increasing studies on this subject.

The prevalence of human microsporidiosis in European countries is reported to vary between 1-25% (6,13,14). It is noteworthy that the positivity rate is higher in HIV+ patients and organ transplant recipients. The reported positivity rates are suggestive of the prevalence and ubiquity of microsporidia species, which are able to persist in the community without any clinical symptoms in healthy individuals. A survey of European literature reveals the presence of studies that identify *E. bieneusi* as the predominant species, a finding consistent with the results of the present study. However, other studies report a higher prevalence of *E. intestinalis* (6,13). The prevalence of microsporidiosis in Asian countries is approximately 10% (15-17). It can be posited that the reasons for the low prevalence in some studies are the investigation of a single pathogen species and utilization of PCR analysis exclusively in samples that have been microscopically identified as positive (18). The use of single species-specific primer in molecular-based methods precludes the detection of other microsporidia species. Nevertheless, if PCR were performed solely on microscopically positive samples, the resulting prevalence figures would be lower than the actual rates. In the present study, we used species-specific primers and performed PCR on each sample, even if microscopically negative, to try to eliminate these problems. Following a comprehensive meta-analysis of 84 studies investigating the presence of microsporidia in China, Qiu et al. (19) reported that the positivity rate in living creatures (mammals such as humans, cattle, dogs etc.) was 20%, and the rate in water samples was 64.5%. The authors concluded that microsporidia are present at a considerable level in Asian countries (19). Most of the microsporidia prevalence studies conducted on the Africa continent have been conducted in Central African countries and the prevalence appears to vary between 3-67.5%. Furthermore, observed increase in prevalence during rainy seasons is also one of the issues that should be taken into consideration, because the importance of socio-economic status in terms of microsporidiosis

is more effective in African countries (20-23). A consideration of the available literature reveals a decline in microsporidia rates particularly in individuals with HIV positivity. There is a consensus among researchers that the use of highly effective antiretroviral therapy is one of the most significant factors contributing to this condition (6,21). From a global perspective, as Wang et al. (20) have noted, microsporidia have the potential to induce health complications not only in individuals with compromised immune systems (e.g., the elderly, children, and HIV patients) but also in those residing in various regions worldwide. Indeed, an analysis of the results of the present study, as well as those previously conducted in the country, reveals that the frequency of microsporidia in immunocompetent individuals is significant.

In one sample, microsporidia positive was found among 16 diarrheic individuals (6.3%) while 48 of 384 (12.5%) non-diarrheal patients were positive. The results of the statistical analysis conducted in the present study indicate that there is no statistically significant difference in the prevalence of microsporidia between individuals with or without diarrhea. The observed phenomenon can be attributed to the limited number of individuals affected by diarrhea. While there are studies showing that diarrhea, indigestion and fatigue are associated with the presence of microsporidia, some studies have reported that there is no relationship between the presence of the pathogen and these complaints (11,24,25). The defining characteristics of microsporidia as an opportunistic pathogen is the ability of the parasite to cause infection and manifest clinical symptoms in individuals with compromised immune systems. Consequently, results of some studies focusing on specific patient groups demonstrate that incidence of complaints is risen in the presence of microsporidia species.

The present study revealed that prevalence of microsporidia in women was 12.7% (26/204) while in men it was 11.7% (23/196). It was observed that the rate of microsporidia was higher in women in the Düzce province (12.7%) and in men (13.72%) in the Bolu province. However, these differences were not statistically significant. As evidenced by studies made worldwide, there is no significant correlation between gender and presence of pathogen. However, it has been observed that in some regions, the rates are higher than one gender or the other, depending on the environmental and working conditions (18,22,25).

It is established that the prevalence of microsporidia differs with age intervals. Especially in childhood and old age, the rate of infection rises because of the lack of personal hygiene and weak immune status (26,27). Despite the absence of a statistically significant difference between age groups in our study, a higher prevalence of microsporidia positivity was observed in individuals aged 65 and above (13.5%).

In many studies, including our own, there have been discrepancies between the rates of microsporidiosis reported by microscopic and molecular methods. This is due to the fact that microsporidia are very small and are frequently overlooked in microscopic examinations when experienced personnel are not available. Furthermore, although fluorescent dyes such as Uvitex 2B and Calcofluor, which bind to the chitin structure in the cell, are employed, it is imperative to exercise caution to avoid misidentifying fungal spores with microsporidia (2,28). The results of our study demonstrated that the positivity of microsporidia was 6.25% by microscopy and 12.25% by PCR. The Kappa analysis yielded a moderate compatibility score (0.646) between both methods. The specificity of MTS was calculated as 100%. The presented results show that the MTS method effectively reduces the occurrence of false positive outcomes. However, it should be noted that this method exhibits moderate sensitivity, necessitating greater care during microscopic examinations to prevent false-negative results.

It is obvious that the positivity rates reported by microscopy are lower than those in molecular methods. While this is linked to the enhanced sensitivity and reliability of molecular methods, it is crucial to proceed with utmost caution in procedures such as DNA isolation and primer selection. Even if the processes are carried out correctly, incorrect results may be obtained from PCR analysis. Indeed, as reported by Ghoyounchi et al. (18) reported the microsporidia spores observed under a microscope could not be detected by PCR. This may be attributed to the inappropriate or thin staining in the microscopic examination or the existence of inhibitor materials in the feces during DNA isolation.

Consequently, it is important to consider the limitations of the methods when designing the study and, if feasible, employ at least two methods to ensure accurate and reliable results.

Study Limitations

In the field of epidemiological studies, the power analysis is of critical importance for the accurate estimation of the prevalence of a pathogen or disease. In the present study, the sample size was set at 400, due to the high costs of molecular methods, and no power analysis was performed. This is considered a limitation of the present study.

CONCLUSION

We believe that our findings contribute to our knowledge about the prevalence of microsporidia in our country. However, it is our contention that a greater understanding can be gained by determining the prevalence of microsporidia in animals and the environmental samples in addition to human specimens. Thus, we posit that valuable insights can be gleaned regarding the phylogenetics of microsporidia species and their transmission routes through ITS genotyping. It is clear that further research on microsporidia is required in our country, where studies on this subject are scarce.

*Ethics

Ethics Committee Approval: We commenced the research following the ethics approval from the "DU Non-Invasive Health Research Ethics Committee" (decision no: 2019/41 and date: 16.12.2019).

Informed Consent: Informed consent was obtained from all subjects involved in the study.

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Footnotes

*Authorship Contributions

Design: M.Ç., E.A., Ş.Ö., Data Collection or Processing: M.Ç, B.D.G., N.E., Analysis or Interpretation: M.Ç., Ş.Ö., Literature

Search: M.Ç., E.A., Writing: M.Ç., E.A., Ş.Ö.

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