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ARAŞTIRMA MAKALESİ /**RESEARCH ARTICLE**

MICROBIAL POPULATION OF HOT SPRING WATERS IN ESKİŞEHİR/TURKEY

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ABSTRACT

In order to investigate and find out the bacterial community of hot spring waters in Eskişehir, Turkey, 7 hot spring water samples were collected from 7 different hot springs. All samples were inoculated using four different media (nutrient agar, water yeast extract agar, trypticase soy agar, starch casein agar). After incubation at 50 ºC for 14 days, all bacterial colonies were counted and purified. Gram reaction, catalase and oxidase properties of all isolates were determined and investigated by BIOLOG, VITEK and automated ribotyping system (RiboPrinter). The resistance of these bacteria was examined against ampiciline, gentamisine, trimethoprime-sulphamethoxazole and tetracycline. As a result, heat resistant pathogenic microorganisms in addition to human normal flora were determined in hot spring waters (43-50 ºC) in investigated area. Ten different species belong to 6 genera were identified as *Alysiella filiformis, Bordetella bronchiseptica, B. pertussis, Molexalla caprae, M. caviae, M. cuniculi, M. phenylpyruvica, Roseomonas fauriae, Delftia acidovorans* and *Pseudomonas taetrolens.*

Keywords: Hot spring, Eskişehir, BIOLOG, VITEK, Ribotype.

ESKİŞEHİR/TÜRKİYE'DEKİ ILICA SULARININ MİKROBİYAL POPULASYONU

ÖZ

Eskişehir/Türkiye'deki ılıca sularındaki bakteriyel topluluğun ortaya konulması ve incelenmesi için 7 ılıca su örneği 7 farklı ılıcadan toplanmıştır. Tüm örnekler dört farklı besi ortamına (nutrient agar, su-maya ekstraktı agar, triptik soy agar, nişasta-kazein agar) inoküle edilmiştir. 50 °C'de 14 günlük inkübasyondan sonra tüm bakteri kolonilerinin sayımı yapılıp saflaştırılmıştır. Tüm izolatların gram reaksiyonları, katalaz ve oksidaz özellikleri belirlenmiş ve BIOLOG, VITEK ve otomatik ribotiplendirme sisteminde (RiboPrinter) incelenmişlerdir. Bu bakterilerin ampisilin, gentamisin, trimetoprim-sülfametoksazol ve tetrasiklin antibiyotiklerine karşı dirençliliği incelenmiştir. Sonuç olarak normal insan florasına ilaveten ısıya dirençli patojenik mikroorganizmalar incelenen alandaki ılıca sularında (43-50 °C) belirlenmiştir. Altı genusa ait 10 farklı tür *Alysiella filiformis, Bordetella bronchiseptica, B. pertussis, Molexalla caprae, M. caviae, M. cuniculi, M. phenylpyruvica, Roseomonas fauriae, Delftia acidovorans* ve *Pseudomonas taetrolens* olarak tanımlanmıştır.

Anahtar Kelimeler: Ilıca, Eskişehir, BIOLOG, VITEK, Ribotip.

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1. INTRODUCTION

Turkey is one of the 7 countries in the world in terms of thermal source richness with almost 1300 thermal springs throughout Anatolia (Anonymous, 2009). Thermal springs of Eskişehir are among the famous ones, most of the thermal springs are near Hamamyolu Street in the center of Eskişehir. The waters (38-45 ºC) contain bicarbonate, sodium, and calcium and suitable for both drinking and bathing cures of rheumatism, neuralgia, post-operational problems, digestive problems, kidney stones and gynecologic complaints, and for the metabolism (Anonymous, 2009). In addition, these spring waters are also consumed as drinking water for health.

Generally, hot springs differ in their physicochemical characteristics, such as water temperature (WT), pH, oxidation–reduction potential (ORP, Eh), electrical conductivity (EC), dissolved oxygen, and in their composition of main anions and cations. Previously, interest in these hydrothermal systems has been restricted to chemical and geological investigations. Both microbiologists and geologists have showed great interest in diversity, structure, and function of the resident microbial communities and most research on the microbiology of the hot springs has concentrated on cultivating and isolating extreme strains (Belkova et al., 2007). Geothermal hot spring streams provide favorable conditions for the development of microbial mat, which may contain physiologically and phylogenetically different groups of prokaryotes, such as chemotropic sulfur bacteria, cyanobacteria, and anoxigenic phototrophic bacteria, depending on the temperature, pH, sulfide (30-60 μM) concentration, and some other environmental conditions (Hiraishi et al., 1999, Wahlund et al., 1991).

Bacterial abundance and biomass in marine, freshwater, and soil ecosystems have been quantified parallel to the enumeration of active bacterial cells. However, bacterial enumerations in the hot-spring water are scarce and restricted to total bacterial abundance (TBA). Furthermore, most of the reported data concern low-density bacterial populations. Nevertheless, the effect of microorganisms on geochemical processes occurring in hot springs is evident. Accordingly, measuring the TBA, especially the fraction of active bacteria in the microbial community of the hot springs, is particularly important.

The purpose of this study is to determine and characterization of bacterial populations in hot spring water from the Eskişehir, Turkey with classical and molecular based methods. For this aim, the majority of isolates are detected and typed by biochemical tests on BIOLOGY and VITEK systems. In addition to these systems, Ribotyping was used to characterize the isolates.

2. MATERIALS AND METHODS

2.1 Samples, Cultures, Media and Growth Conditions

A total of 7 different thermal water samples were collected from thermal springs around the Eskişehir/Turkey. After sampling, temperature and pH values of all samples were measured (Table 1). Samples were transported in dark conditions for analysis within 6 h of sampling. Membrane filtration method was used for the bacterial isolation and 26 isolates were obtained. Different media; nutrient agar (NA), water yeast extract agar (WA), trypticase soy agar (TSA), starch casein agar (CSA) were used to obtain the bacterial population. After incubation at 50 ºC for 14 days, all bacterial colonies were counted and transferred to NA medium for purifying (Table 2). For identification and investigation, sub-cultures were performed on NA for 24 h at 50 ºC.

2.2 Biochemical Tests

Gram staining reaction, oxidase and catalase tests of these isolates were determined with basic microbiological methods (Wistreich, 1997). The tolerances against to different temperatures of these bacterial isolates were investigated by inoculation on NA and incubation at 40 and 60 ºC for 48 h (Table 2). In addition, according to their gram staining reaction, morphology, temperature tolerances, catalase and oxidase properties, some isolates were select and the resistance of these bacteria was examined against ampiciline, tetracycline, gentamisine, trimethoprime-sulphamethoxazole (Table 3).

Two different identification systems, BIOLOG (GP or GN) (OOA 002, 2001; OOA 003, 2001) and VITEK (BAC or GNI) (Laboratory Manager Vitek Manual, 2004), were used to identification of selected isolates according to manufacturing manual. In addition, carbon source metabolic fingerprints of these isolates were determined by these systems. Results of these systems were exported for analysis in NTS files and imported into NTSYSpc 2.1. Clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) method based on the SM coefficient.

Table 1. Sampling points, temperature, pH and total colony counts on different media and isolates number of thermal water samples.

Sample No	Sampling point	Temperature (ºC)	pH	Media/Total Colony Counts	Isolate Number*
1	A	50	7.8	NA: 470 cfu/ml	S6
				WA: 3 cfu/ml	S10
2	B	50	7.5	NA: 340 cfu/ml	S23
				WA: 1 cfu/ml	
3	C	43	7.9	No Growing	
4	D	48	7.3	NA: 173 cfu/ml	S22, S4, S5
				TSA: 1 cfu/ml	
				CSA: 21 cfu/ml	S11
5	E	43	7.4	NA: 3 cfu/ml	S19, S20, S21
				TSA: 2 cfu/ml	S18
6	F	43	7.4	NA: 36 cfu/ml	S16, S15, S14
				CSA: 90 cfu/ml	S17
				TSA: 24 cfu/ml	S13
				WA: 92 cfu/ml	S12
7	G	45	7.5	NA: 571 cfu/ml	S7, S8, S9
				CSA: 127 cfu/ml	S24, S25, S26
				TSA: 24 cfu/ml	S1, S2, S3

***S=Strain**

2.3 Ribotyping and Ribotype Analysis

As a molecular method based on the analysis of the restriction fragment length polymorphisms (RFLPs) of rRNA genes (Cordevant et al., 2003), Ribotyping were used for identification and/or characterization by analyzing ribosomal DNA banding pattern of isolates. For this aim, all isolates were characterized by use of the RiboPrinter system (Qualicon, Wilmington, Del., USA), as described previously (Ito et al., 2003), with respect to the procedures and conditions recommended by the manufacturer. EcoRI and PVUII were used as the restriction enzymes. At the end of the process, densitometry scan and molecular weight of the restriction fragments were obtained for each samples analyzed.

Restriction fragments of ribotypes were exported for analysis in NTS files and imported into NTSYSpc 2.1. Clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) method based on the SM coefficient for band matching. Bands for analysis with the SM coefficient were allocated manually, according to densitometry curves and the accompanying hard copy photograph.

3. RESULTS AND DISCUSSION

Thermal water samples in this study have a temperature value between 43-50 ºC and pH value between 7.3 and 7.9 (Table 1). While these temperatures are suitable for growing of mesophilic and thermophilic microorganisms, pH values of investigated samples are supporting to microbial growing. Optimum pH value for many microorganisms is between 6 and 8 already many natural environments have pH value between 5 and 9 (Madigan and Martinko, 2006). We counted to colony forming unit between 36 and 571 cfu/ml at different media (Table 1). The highest mean numbers of microorganisms were obtained from sample G with 571 cfu/ml at NA and 127 cfu/ml at CSA. We determined 470 cfu/ml at NA from sample A, 340 cfu/ml at NA from sample B, 173 cfu/ml at NA from sample D, 92 cfu/ml at WA and 90 cfu/ml at CSA from sample F. Only sample E had colony forming unit out of 30-300 but this sample were took to isolation process because of this microorganisms could be pathogenic microorganisms. By the sampling process, we couldn’t found colony forming unit at sample C (Table 2).

Table 2. Properties of cell morphology, gram staining reaction, catalase and oxidase, temperature tolerances of isolates.

Isolate Number*	**Morphology**	**Gram Staining Reaction**	**Catalase**	**Oxidase**	**Temperature Tolerances**	
					40 ºC	**60 ºC**
S1	Bacil	Gr(+)	-	+	Positive	Poor
S2	Coccobacil	Gr(-)	-	+	Positive	Poor
S3	Bacil	Gr(-)	-	+	Positive	Negative
S4	Coccus	Gr(-)	-	+	Negative	Negative
S5	Coccus	Gr(-)	+	+	Positive	Negative
S6	Coccobacil	Gr(-)	+	+	Negative	Negative
S7	Coccobacil	Gr(-)	+	+	Positive	Positive
S8	Coccobacil	Gr(-)	-	+	Positive	Negative
S9	Coccobacil	Gr(-)	-	+	Positive	Negative
S10	Coccobacil	Gr(-)	+	+	Positive	Negative
S11	Bacil	Gr(+)	+	+	Positive	Positive
S12	Coccobacil	Gr(-)	+	+	Positive	Negative
S13	Coccobacil	Gr(-)	+	+	Positive	Poor
S14	Coccobacil	Gr(-)	-	+	Positive	Negative
S15	Coccobacil	Gr(-)	+	+	Positive	Negative
S16	Coccobacil	Gr(-)	-	+	Positive	Positive
S17	Coccus	Gr(-)	+	+	Positive	Positive
S18	Coccobacil	Gr(-)	+	+	Positive	Negative
S19	Coccobacil	Gr(-)	-	+	Negative	Negative
S20	Coccobacil	Gr(-)	-	+	Negative	Negative
S21	Coccobacil	Gr(-)	-	+	Negative	Negative
S22	Coccus	Gr(-)	+	+	Positive	Negative
S23	Coccobacil	Gr(-)	-	+	Negative	Negative
S24	Coccus	Gr(-)	+	+	Positive	Positive
S25	Coccus	Gr(-)	+	+	Positive	Positive
S26	Coccus	Gr(-)	+	+	Positive	Positive

Table 3. Resistance of some isolates against to ampiciline, tetracycline, gentamisine, trimethoprime-sulphamethoxazole.

Isolate Number*	**Inhibition Zones of Tested Antibiotics (mm)**			
	Ampiciline	**Tetracycline**	**Gentamisine**	**Trimethoprime-sulphamethoxazole**
S1	51.0	29.0	15.5	59.0
S5	0.0	30.0	30.0	52.0
S7	30.0	43.0	20.0	52.0
S8	51.0	25.0	16.0	55.5
S10	27.0	39.5	29.0	43.0
S11	0.0	35.5	30.5	51.0
S12	0.0	34.0	33.5	53.0
S13	0.0	35.0	32.0	53.5
S14	0.0	16.0	30.5	55.0
S15	0.0	16.5	30.5	54.0
S16	0.0	17.5	29.0	51.5
S17	0.0	27.5	30.5	51.0
S18	0.0	16.0	30.5	45.0
S22	0.0	37.5	34.5	54.5
S24	51.0	54.0	43.0	65.0

End of the isolation process, we obtained 26 isolates from samples from 6 samples. While twenty four isolates (92%) were determined as Gram negative, two isolates (8%) were determined as Gram positive cell properties. The sixteen isolates (61.5%) have cocobacil cell shape, seven isolates (26.9%) have coccus cell shape and three isolates (11.6%) have bacil cell shape (Table 2). While all of the isolates showed positive oxidase reaction, 13 isolates showed positive catalase reaction. In temperature tolerances studies, twenty isolates grew very well at 40 ºC and six isolates grew very well at 60 ºC (Table 2). While isolate S6 belong to sampling point A did not grow at 40 °C and 60 °C, S10 grew at 40 °C. Isolate S23 belong to sampling point B did not grow both at 40 and 60 °C; it only grew at 50 °C that is isolation temperature. As S23, S4 belong to sampling point D only grew at isolation temperature. While S5 and S22 grew only at 40 °C, S11 grew both at 40 and 60 °C. Only isolate S18 belong to sampling point E grew at 40 °C, the other isolates did not grow both at 40 and 60 °C. While S16 and S17 isolates belong to sampling point F grew both at 40 and 60 °C, the other isolates grew only at 40 °C. While there are isolates belong to sampling point G which grew at 40 and 60 °C such as S7, S24, S25 and S26, the other isolates of grew only 40 °C (Table 2). According to these results, bacterial community with different properties such as thermo tolerant, thermopile and extreme ther-