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Investigation of Antibiotic Resistance of Bacteria Isolated from Insuyu Cave, Burdur-Turkey

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ABSTRACT

Studies for the discovery of microorganisms with different metabolic properties in extreme environments are increasing every year. Within the scope of this study, samples were taken from, an extreme environment, İnsuyu cave. Antibiotic resistance in the obtained isolates was investigated by cultural and molecular biology methods. As a result of Minimum Inhibition Concentration (MIC) test, 64 cefpodoxim, 6 rifampicin and gentamycin, 18 vancomycin, 15 ampicillin, 44 clindamycin, 48 penicillin resistant strains were detected. *Pseudomonas spp.* (2G-2), *Pseudomonas jesseni* (2J), *Sphingopyxis fribergensis* (4D), *Microbacterium yannicii* (4M), *Flavobacterium chungangense* (6B), *Rhodococcus spp.* (7A), *Flavobacterium resistens* (9D), *Pseudomonas spp.* (K-4F), *Pseudomonas spp.* (K-4G), *Buttiauxella agrestis* (K-15A) were found to have multidrug resistant by cultural methods. These bacteria and susceptible strains, *Flavobacterium chungangense* (K-1E), *Rhodococcus erythropolis* (K-11G) and *Pseudomonas spp* (K-15G), as negative controls were selected for the identification of antibiotic resistance genes by PCR based methods. Eight different gene regions, aminoglycoside 2''-O-nucleotyltransferase (aadB), beta lactamase (bla_{CTXM3}, bla_{SHV}, bla_{TEM}), aminoglycoside resistance protein (strA), rifampin ADP- ribosyltransferase (arr2- int2a), vancomycin (vanC) have been detected in *Pseudomonas jesseni* (2J) and *Pseudomonas spp.* (K-4G).

Keywords: Antibiotic resistance, extreme environment, multidrug resistance

İnsuyu Mağarası'ndan İzole Edilen Bakterilerin Antibiyotik Dirençliliğinin Araştırılması

ÖZ

Ekstrem çevrelerde farklı metabolik özellikteki mikroorganizmaların keşfi için yapılan çalışmalar her geçen yıl artmaktadır. Bu çalışma kapsamında ekstrem çevre olarak İnsuyu mağarasından örneklemeler yapılmıştır. Elde edilen izolatlarda antibiyotik dirençliliği kültürel ve moleküler biyoloji yöntemleri ile araştırılmıştır. Çoklu direnç gösteren 10 izolat ve duyarlı 3 izolat seçilerek 16S rRNA dizi analizi ile tür tayini yapılmıştır. Çoklu direnç gösteren *Pseudomonas spp.* (2G-2), *Pseudomonas jesseni* (2J), *Sphingopyxis fribergensis* (4D), *Microbacterium yannicii* (4M), *Flavobacterium chungangense* (6B), *Rhodococcus spp.* (7A), *Flavobacterium resistens* (9D), *Pseudomonas spp.* (K-4F), *Pseudomonas spp.* (K-4G), *Buttiauxella agrestis* (K-15A) ve duyarlılık gösteren *Flavobacterium chungangense* (K-1E), *Rhodococcus erythropolis* (K-11G) ve *Pseudomonas spp* (K-15G) suşlarında antibiyotik direnç genleri taranmıştır. *Pseudomonas jesseni* (2J) ve *Pseudomonas spp.* (K-4G)' de aminoglikozit 2''-O-nükleotiltransferaz (aadB), beta laktamaz (bla_{CTXM3}, bla_{SHV}, bla_{TEM}), aminoglikozit direnç proteini (strA), rifampin ADP- ribosiltransferaz (arr2- int2a), vankomisin (vanC) olmak üzere 8 farklı gen bölgesi saptanmıştır.

Anahtar Kelimeler: Antibiyotik direnci, ekstrem çevreler, çoklu ilaç direnci

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INTRODUCTION

Antimicrobial agents are chemical substances that kill or prevent the growth of microorganisms. Resistance occurs as a result of two factors: the antibiotic itself and resistance genes. Antibiotic resistance occurs by many different mechanisms such as target change, drug intake and excretion and highly efficient enzyme inactivation. Resistance may occur rapidly due to certain mutations in target genes and there is evidence that antibiotics support such mutations. However, resistance to most antibiotics takes place through enzymes. These factors are the result of evolution through natural selection. Therefore, it is believed that antibiotic resistance has a long evolutionary history (Yücel and Yamaç, 2010; Rajput et al., 2012; Stankovic et al., 2012; Cheeptham et al., 2013). Numerous recent studies indicate that resistance genes are often caused by increased use of antibiotics (Levy, 2002; Davies, 2010; Bhullar, 2011). However, some studies suggest that some resistant genes exist prior to the use of antibiotics for treatment of people (White, 2013). Researchers have performed functional studies to identify resistance gene sources of bacteria isolated from different environments (Bhullar, 2011; White, 2013). Natural environments have been identified as reservoirs for antibiotic resistance genes. The caves are closed, protected and important working environments for understanding the discovery of new antibiotics and antibiotic resistance. Therefore, the aim of this study was to investigate the antibiotic resistance of bacteria isolated from Insuyu Cave (Burdur/ Turkey) by culture-based and PCR-based methods.

MATERIALS AND METHODS

Isolation

Insuyu cave was discovered in 1952 and officially opened to the touristic activities in 1965 as the first show cave in Turkey. It is located on Mediterranean Region of Turkey and the coordinates are 37°39′34″N 30°22′27″E.

In the study, very small fractions, soil, water and sediment samples were taken into sterile falcon and ependorfs tubes under aseptic conditions, transferred to the Molecular Biology and Genetics Department Laboratories and kept at -20^{0}C until forward studies. 0.1x Nutrient agar, M9 and R2A media were used for isolation studies and incubated at 25^{0}C for 48-72 hours in dark and light conditions. Specifically characterized colonies were identified and purified. The cell wall characteristics of the isolates were examined by Gram staining method. Bacterial cultures were maintained as a stock in a 0.1xNutrient broth medium containing 25% glycerol. Samples and locations are shown in Table 1.

Table 1. Sampling locations in Insuyu Cave

Sample Number	Location	Material
1	Great Lake	Soil
2	Great Lake	Soil
3	Great Lake- stalactite	Water
4	Great Lake - Cave wall	Soil
5	Great Lake - Cave wall	Soil
6	Cave ceiling	Water
7	Walking track- Cave wall	Soil
8	Visitor Path- Cave ceiling	Water
9	Visitor Path- Cave wall	Soil
10	Visitor Path- Cave ceiling	Soil
11	Visitor Path	Soil
12	Visitor Path	Soil
13	Cave wall	Water
14	Dilek Lake	Water
15	Cave wall	Water

Determination of Minimum Inhibitory Concentration (MIC)

Antibiotic resistance to ampicillin, clindamycin, rifampicin, vancomycin, cefpodoxime and penicillin were investigated. The antibiotics used for the MIC test are shown in Table 2. In order to determine the susceptibility to ampicillin, clindamycin, rifampicin, vancomycin, cefpodoxim antibiotics for MIC experiments, 0.1x Nutrient Agar medium containing 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.625 g/ml antibiotics were used. To determine the susceptibility to penicillin antibiotics, 0.1x Nutrient Agar medium containing 40, 20, 10, 5, 2. 5, 1.25, 0.125 U/ml concentrations were used. The isolates were activated in 0,1XNutrient Broth. The suspension was prepared in accordance with 0.5 MacFarland turbidity in media containing 0.9% NaCl. MIC test results were compared with the standard determined by the Clinical and Laboratory Standards Institute (CLSI).

Table 2. Antibiotics used for the MIC test

Antibiotic	Universal symbol	Antibiotic group
Ampicillin	AMP	Beta Lactam
Streptomycin	STR	Aminoglyco-sides
Gentamicin	GEN	
Rifampicin	RIF	Aminoglyco-sides
Vancomycin	VAN	Ansamycin
Penicillin	PCN	Glycopeptide
Cefpodoxime	CP	Beta Lactam Cephalosporin

Preparation of Genomic DNA and Determination of Antibiotic Resistance by PCR Method

10 multiple resistance and 3 sensitive strains were selected for molecular studies. DNA isolation was carried out by Thermo Scientific GeneJET Genomic DNA Purification Kit according to manufacturer instructions. Extracted DNA samples were kept -20 ^{0}C until they were needed for PCR. Selected strains were identificated by 16S rDNA gene sequencing. Sequence analysis was performed by BMlabosis. Sequence analysis results were evaluated using ApE and Finch TV programs and Blasted in NCBI.

Gene region specific primers were used for the detection of resistance genes by PCR are shown in Table 3.

Table 3. List of primers used for detection of antibiotic resistance

Gene Region	Primers (5'→3')	Antibiotic	Amplicon size and temperature	References
Aac(3)-IIa	F-ACTGTGATGGGATACGCGTC R-CTCCGTCAGCGTTTCAGCTA	Gentamycin	237 bp- 58 C^0	Teixeira et al. 2016
AphA1	F-ATGGGCTCGCGATAATGTC R-CTCACCGAGGCAGTTCCAT	Gentamycin	600 bp- 55 C^0	Teixeira et al. 2016
AphA2	F-GAACAAGATGGATTGCACGC R-GCTCTTCAGCAATATCACGG	Gentamycin	680 bp- 56 C^0	Teixeira et al. 2016
Aac(6´)-Ib-cr	F-TTGCGATGCTCTATGAGTGGCTA R-CTCGAATGCCTGGCGTGTTT	β –lactam	482 bp-55 C^0	Teixeira et al. 2016
AadB	F-CGTCATGGAGGAGTTGGACT R-CGCAAGACCTCAACCTTTTC	Gentamycin	350 bp- 57 C^0	Teixeira et al. 2016
Bla$_{TEM}$	F-GAGTATTCAACATTTTCGT R-ACCAATGCTTAATCAGTGA	β –lactam	857 bp- 48 C^0	Bhattacharya et al. 2015
Bla$_{SHV}$	F-TCGCCTGTGTATTATCTCCC R-CGCAGATAAATCACCACAATG	β –lactam	768 bp- 56 C	Bhattacharya et al. 2015
Bla$_{CTX-M-3}$	F-AATCACTGCGTCAGTTCAC R-TTTATCCCCCACAACCCAG	β –lactam	701 bp- 53 C^0	Bhattacharya et al. 2015
Bla$_{OXA1}$	F-GCAGCGCCAGTGCATCAAC R-CCGCATCAAATGCCATAAGTG	β –lactam	198 bp- 58 C^0	Bhattacharya et al. 2015
Bla$_{OXA7}$	F-AGTTCTCTGCCGAAGCC R-TCTCAACCCAACCAACCC	β –lactam	591 bp- 51 C^0	Bhattacharya et al. 2015
Bla-TEM2	F-AAAATTCTTGAAGACG R-TTACCAATGCTTAATCA	β- lactam	1080 bp- 40 C^0	Sharma et all. 2010 Lee et al. 2011
Bla-SHV2	F-GGGTTATTCTTATTTGTCGCT R-TAGCGTTGCCAGTGCTCG	β - lactam	929 bp- 54 C^0	Lee et al. 2011
Bla-CTX-M	F-TTTGCGATGTGCAGTACCAGTAA R-CGATATCGTTGGTGGTGCCATA	β - lactam	544 bp- 62 C^0	Mir et al. 2016
StrA	F-TCAATCCCGACTTCTTACCG R-GCTAACGCCGAAGAGAACTG	β- lactam	240 bp- 57 C^0	NC_005205.1, 487-506 NC_005205.1, 726-745 (This study)

StrAF-R	F-AGCAGAGCGCGCCTTCGCTG R-CCAAAGCCCACTTCACCGAC	β- lactam	500 bp- 62 C^0	Carattoli, 2002
Arr2- int2a	AACCGAGGATGCGAACCACT	Rifampicin	3602 bp- 54 C^0	Houang et al. 2003
İnt6b	CCG AGC CGC TCG TAT AG	Rifampicin	3602 bp- 54 C^0	Houang et al. 2003
Arr-4 CATB3	CACTGGAGAAGATCAAAGCG	Rifampicin	406 bp	Houang et al. 2003
Arr4-INB	GGGCAGACTTGACCTGAT	Rifampicin	450 bp- 54 C^0	Fonseca et al. 2008
Arr-5-INF	GGCATCCAAG CAGCAAG	Rifampicin	450 bp- 54 C^0	Fonseca et al. 2008
Van A	F-ATGAATAGAATAAAAGTTGCAA-TAC R-CCCCTTTAACGCTAATACGAT	Vancomycin	1029 bp- 57 C^0	Miele et al. 1995
Van B	F-GTGACAAACCGGAGGCGAGGA R-CCGCCATCCTCCTGCAAAAAA	Vancomycin	433 bp- 63 C^0	Clark et al. 1993
Van C	F-GAAAGACAACAGGAAGAC CGC R-ATCGCATCACAAGCACCAATC	Vancomycin	796 bp- 57 C^0	Clark et al. 1993

RESULTS AND DISCUSSION

Isolation and strains

A total of 75 bacterial isolates were isolated from Insuyu Cave. Of the 75 bacterial isolates, 50 were Gram negative and 25 were Gram positive. Studies on cave microflora indicate that various Gram-negative bacteria are more common than Gram-positive bacteria in cave ecosystem (Palmer et al., 2000). In the study conducted in Lechuguilla Cave in 2012, the resistance of 93 bacterial strains to antimicrobial agents was investigated. Of the 93 strains, 33% were Gram positive and 63% were Gram negative. In our study, it is seen that Gram negative bacterias are formed from the samples which were isolated from Insuyu Cave and their resistance against antibiotics were examined.

Antibiotic MIC Test Results

In order to evaluate the results of antibiotic MIC test, the standard of CL Performance Standarts for Antimicrobial Susceptibility Testing "published by the Clinical and Laboratory Standards Institute (CLSI) was used. The resistance of the isolated bacteria against cefpodoxime, vancomycin, ampicillin, gentamicin, rifampicin, clindamycin and penicillin antibiotics were investigated by MIC test. As a result of the Minimum Inhibition Concentration (MIK) test, 64 cefpodoxime, 6 rifampicin and gentamycin, 18 vancomycin, 15 ampicillin, 44 clindamycin, and 48 of the 75 isolates were found to be resistant to the highest concentration of penicillin antibiotics. Of the samples, 39 were resistant to rifampicin, 20 to gentamicin, 40 to clindamycin, 18 to vancomycin, 17 to ampicillin, 55 to penicillin, 68 to cefpodoxime antibiotics. The antibiotic MIC test results are shown in Table 4 and Table 5.

Table 4. MIC test results for light zone samples

İsolate No	**Gram Staining**	**Antibiotics***						
		Cli	**Rif**	**Gen**	**Van**	**Pen**	**Cpdx**	**Amp**
2A	Gram +	32+	4	32+	0,25	10	32+	32
2B	Gram (+)	32+	2	8	0,5	40+	32+	16
2C	Gram (+)	32+	1	8	32+	40+	32+	16
2D	Gram (-)	32+	4	2	4	40+	32+	32+
2F	Gram (-)	32+	4	0,25	1	40+	32+	0,5
2G-2	Gram (-)	32+	32+	32	0,25	40+	16	32+

2I	Gram(-)	32	32+	0,5	0,5	40+	32	4
2J	Gram(-)	32+	32	0,5	32+	40+	32+	0,5
2L	Gram(+)	32+	4	32+	16	40+	32+	16
2M-2	Gram(+)	8	8	32+	32+	40+	32+	32
2G-1	Gram(-)	32+	32+	4	16	40+	32+	32
4B	Gram(v)	32+	1	2	0,5	40+	32+	0,25
4D	Gram(-)	32	8	16	32+	40+	32+	4
4E	Gram(+)	32	4	2	32+	40+	32+	32+
4F	Gram(+)	32+	16	16	0,25	40+	32+	1
4O	Gram(+)	32+	0,25	32+	8	40+	32+	32
4H	Gram(+)	32+	4	16	32+	40+	32+	32+
4M	Gram(+)	32+	32+	8	4	40+	32+	32+
6B-1	Gram(-)	0,25	32	32	32+	40+	32+	32+
6D	Gram(-)	32+	8	32	32+	40+	32+	16
6I-1	Gram(-)	32+	16	0,25	8	40+	32+	16
7A	Gram (+)	32+	32+	1	32	40+	32+	4
7B	Gram (+)	32+	8	32	1	40+	32+	32
7C	Gram(-)	32+	0,5	4	0,5	40+	32+	32+
7D-1	Gram (-)	32	0,25	2	0,25	2,5	32+	0,25
7J	Gram (+)	32+	0,5	4	4	40+	32+	4
9A	Gram(-)	32+	16	0,25	16	40+	32+	1
9D	Gram(-)	32	32	32+	32+	0,25	32+	8
10B	Gram(-)	32	16	4	32	40+	32+	32
11L	Gram (+)	4	0,25	4	8	40+	32+	0,25
11M	Gram(+)	32+	0,25	8	0,25	0,625	4	0,25
12C	Gram(+)	32+	8	4				
12F	Gram (+)	32	8	4	0,5	40+	32+	8
12G	Gram (-)	16	0,25	0,25	0,5	40+	32+	8
12M	Gram (+)	32	16	4	0,25	40+	32+	0,25
13A	Gram (-)	32+	8	4	0,5	0,625	32+	16
13C-1	Gram (-)		16	4	8	40+	32+	16
13G	Gram (-)	32	4	4	8	0,625	32+	32+
13H	Gram (-)	32+	32	8	0,5	40	32+	0,5
13I	Gram (-)	32+	4	4	0,5	20	32+	16
13J	Gram (-)	32	16	8	0,25	40	32+	0,25
15F	Gram (+)	32	8	1	0,25	0,25	32+	0,25
15D	Gram(-)	32+	4	2	0,25	40+	32+	32

Table 5. MIC test results for dark zone samples

İsolate No	Gram Staining	Antibiotics*						
		Cli	Rif	Gen	Van	Pen	Cpdx	Amp
K-1A	Gram (+)	0,25	32+	8	32+	1,25	32+	32+
K-1C	Gram (+)	32+	4	16	0,25	40+	32+	32+
K-1E	Gram (-)	0,35	0,25	4	32+	0,625	32+	0,25
K-2A	Gram (-)	32+	0,25	4	0,5	40+	32+	32+
K-2B	Gram (-)	32+	0,25	32	0,5	40+	32+	16
K-2E	Gram (+)	32	4	32	16	40+	32+	32+
K-2G	Gram (-)	32+	1	8	32+	40+	32+	4
K-2H	Gram (-)	32+	0,25	8	0,5	10	0,25	32
K-2I	Gram (-)	32+	8	4	0,5	40+	32+	8
K-2J	Gram(-)	32+	8	4	2	5	32+	0,25
K-2N	Gram (-)	32+	4	4	0,5	0,625	0,25	4
K-4B	Gram(+)	8	16	2	0,25	0,625	32+	0,25
K-4C	Gram(-)	32+	4	2	0,25	40+	32+	32+
K-4E	Gram (-)	0,25	32	4	4	0,625	32+	0,25
K-4G	Gram(-)	32	32	8	16	40+	32+	16
K-4H	Gram(-)	32+	4	4	32+	40+	32+	0,25
K-4F	Gram(-)	32+	16	8	32+	40+	32+	32+
K-7B	Gram (-)	0,25	8	4	32+	40+	32+	0,25
K-7K	Gram (+)	32+	8	2	32+	40+	32+	4
K-9M	Gram(-)	32+	0,25	8	32+	1,25	32+	2
K-11A	Gram(-)	32+	4	4	4	20	32+	8
K-11D	Gram(-)	32+	0,25	8	8	40+	0,25	32+
K-11G	Gram(+)	0,25	0,25	0,25	0,25	0,25	32+	0,25
K-12E	Gram(-)	32+	8	32+	8	40+	0,25	32+
K-13B	Gram(-)	0,25	8	0,25	0,25	0,25	32+	0,25
K-13J	Gram(-)	32	0,25	16	0,25	1,25		0,25
K-13I	Gram(-)	32+	8	1	0,25	40	32+	16
K-13F/1	Gram(-)	0,25	32	32+	32+	40+	32+	0,25
K-15A	Gram(-)	32+	32+	32+	0,25	40+	32+	32
K-15C	Gram(-)	32+	0,5	4	0,25	40+	32+	32
K-15F	Gram(-)	8	4	16	0,25	0,625	32+	0,25
K-15G	Gram(-)	32+	0,25	8	0,25	10	0,25	4

Sequence Analysis Results

According to the results of the MIC Test, 10 strains with the highest concentration and multiple resistances and 3 sensitive strains were used for negative control. For these samples, sequence analysis was performed for the 16S rRNA gene region. The NCBI registration number of the sequence analysis are shown in Table 6.

Table 6. 16S rDNA based identification of strains and NCBI registration numbers

Strain Code	Species	NCBI registration numbers
2G-2	*Pseudomonas sp.*	MH548897
2J*	*Pseudomonas spp.*	
4D	*Sphingopyxis fribergensis*	MH549184
4M	*Microbacterium yannicii*	MH794238
6B	*Flavobacterium chungangense*	MH553177
7A	*Rhodococcus erythropolis*	MH685563
9D	*Flavobacterium resistens*	MH549189
K-1E	*Flavobacterium chungangense*	MH549208
K-4F	*Pseudomonos sp.*	MH549212
K-4G	*Pseudomonas sp.*	MH549227
K11-G	*Rhodococcus erythropolis*	MH685563
K-15A	*Buttiauxella agrestis*	MH558274
K-15G	*Pseudomonas sp.*	MH549406

*: culture includes two species, sequencing results not clear

Detection of Antibiotic Resistance Genes by PCR

Antibiotic resistant gene PCR results are shown in Table 7. In our study, at least one of these gene regions was detected in multiple resistant strains; *Pseudomonas sp.* (2G-2), *Pseudomonas sp.* (K-4F), *Pseudomonas jesseni*, *Pseudomonas sp.* (K-4G) and *Flavobacterium chungangense* (6B).

In our study, the selected gene regions are chromosomal origin. The presence of the plasmid integration was investigated for rifampicin resistance. One of the selected gene regions was determined in *Flavobacterium resistense* (9D). This strain was not evaluated as rifampicin resistant according to MIC Test results. The *Rhodococcus erythropolis* (K-11G) strain identified as a negative control revealed a plasmid-derived rifampicin gene region.

Although as a result of MIC test 3 strains were determined as rifampicin resistant; the arr genes which are effective in rifampicin resistance could not be determined by PCR. In these strains, resistance is thought to be achieved by the mutation in rpoB gene, which is also known to be effective in rifampicin resistance formation.

The blaSHV, blaOXA-1 genes have been identified in Flavobacterium chungangense (K-1E), which is determined as resistant to vancomycin and cefodoxime by culture-based methods used as negative control. The blaSHV genes were determined in *Pseudomonas sp*. (K-15G), which was determined as clindamycin and penicillin resistant. These genes are genes that encode beta-lactamase. These genes encode beta-lactamase and are effective in resistance to beta-lactam group antibiotics.

Table 7. Detection of antibiotic resistance genes in multi resistance and negative control strains

Gene Region	Strains												
	2G-2	**K-4F**	**2J**	**K-4G**	**4D**	**4M**	**K 15A**	**6B**	**7A**	**9D**	**K-1E**	**K-11G**	**K-15G**
aac (3)-IIa	-	X	-	-	-	-		-	-	-	-	-	-
aac (6)-Ib	-	-	-	-	-	-	X	-	-	-	-	-	X
aph A1	-	-	-	-	-	-	X	X	-	-	-	-	-
aph A2	X	-	-	-	-	-	-	-	-	-	-	-	-
aadB	X	X	X	X	-	-	-	-	-	-	-	-	-
bla_{TEM}	-	-	-	-	-	-	-	-	-	-	-	-	-
bla_{SHV}	-	-	X	-	-	X	X	-	X	-	X	-	-
$bla_{CTX\ M-3}$	-	-	X	X	X	-	-	X	-	-	-	-	-
bla_{OXA1}	-	-	X	X	-	-	X	X	-	X	X	-	-
bla_{OXA7}	-	-	-	-	-	-	-	-	-	-	-	-	-
$bla\text{-}_{TEM2}$	-	-	X	-	-	-	-	-	-	-	-	-	-
$bla\text{-}_{SHV2}$	-	-	X	X	-	-	-	-	-	-	-	-	X
$bla\text{-}_{CTX\text{-}M}$	-	-	-	-	-	-	-	-	-	-	-	-	-
strA’	-	-	-	X	-	X	-	X	-	-	-	-	-
strA	-	X	X	X	-	-	-	-	-	-	-	-	-
arr2-int2aint6b	-	-	X	X	-	-	X	-	-	-	-	-	X
arr(4)-CATB3arr(4)-INB	-	-	-	-	-	-	-	-	-	X	-	X	-
arr(4)-IN-Barr(5)-INF	-	-	-	-	-	-	-	-	-	-	-	-	-
vanA	-	-	-	-	-	-	-	-	-	X	X	-	-
vanB	-	-	-	-	-	-	-	-	-	X	X	X	-
vanC	X	-	-	X	-	-	-	-	-	-	-	-	X

CONCLUSION

A study on the resistance of cave bacteria to antimicrobial agents has been conducted in Lechuguilla Cave in 2012 by culture based methods. Of the 93 strains used in the study, 33% were Gram positive and 63% were Gram negative. On average, about 65% of Gram-negative strains resistance to 3-4 antibiotic classes. 25% ampicillin, 10% cefpodoxime, 15% clindamycin and 15% gentamicin resistance were observed in Gram positive strains. In Gram negative strains, 50% ampicillin, 20% cefpodoxime, 60% clindamycin and 20% gentamicin resistance were observed (Bhullar et al., 2012). In our study, the resistance to antibiotics was determined as 52% for rifampicin, 20% for gentamicin, 53% for clindamycin, 24% for vancomycin, 22% for ampicillin, 73% for penicillin and 90% for cefpodoxime. Both MIC tests and PCR results indicate that the strains are resistant to beta-lactam group antibiotics. The highest rate was determined in cefpodoxime and penicillin resistance, acting on cell wall synthesis. The majority of the samples formed by Gram negative bacteria are effective on this result.

Plasmid isolation and transfer was not performed in our study. However, plasmid-derived intron regions and genes have been identified. In a study by Chandrasekaran and Lalithakumari (1998), isolated Pseudomonas fluorescens CAS 102 strains isolated from soil were isolated. The tests of plasmid pSCL 61 isolated from this strain as a result of the transfer to *E. coli*

revealed that the resistance mechanism was also prevented by inhibition of the ingestion of the antibiotic into the cell (Chandrasekaran and Lalithakumari, 1998).

The ADP-ribosyltransferase (Arr2) and aminoglycosidyltransferase (Aac (6) genes responsible for rifampicin resistance in K-15A were both identified. Houang et al. (2003) in their study as a result of the sequence analysis, rifampin ADP ribosyltransferase (Arr 2), aminoglycosidyltransferase (Aac (6)) and the gene regions of the class II integron gene regions were found in the same gene cassette (Houang et al., 2013).

The results of the study confirm that extreme environments such as caves are important habitats in the investigation of the environmental origin of antibiotic resistance. The results of this study show that bacteria can gain resistance against antibiotics in the absence of direct antibiotic exposure. It is also consistent with the view that human activities are effective in the resistance of microorganisms to antimicrobial agents.

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