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Identification and β -lactam resistance in aquatic isolates of *Enterobacter cloacae* and their status in microbiota of Domica Cave in Slovak Karst (Slovakia)

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Abstract: Domica Cave is located in the Slovak Karst National Park in southern Slovakia. Heterotrophic cultivable psychrophilic and mesophilic microbiota were confirmed to be prevalent in this cave. *Escherichia coli* was the most abundant bacterium, followed by *Enterobacter* species and *Serratia* species. *Enterobacter cloacae* isolates belong to the group of faecal contaminants (coliforms) of concern in water. Their status in cave ecosystem is questionable, but we observed *E. cloacae* in water samples all years round suggesting an autochthonous origin. We were concerned with possible contamination of the cave water with resistant *E. cloacae* from animal farms located 2-3 km from the cave. We tested 36 aquatic isolates of *E. cloacae* from Domica Cave to β -lactam resistance. The majority of tested isolates were resistant to more antibiotics (from 3 to 10), 5 isolates were resistant to more than 10 antibiotics, and 1 isolate was resistant to ampicillin. Resistance to the broad spectrum of β -lactams correlated with resistance mechanisms due to an expression of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes. The majority of isolates possessed a combination of tested resistance genes. We assume a direct impact of long-term human agricultural activities in the area of the Domica Cave to the conserved microbiota of karst system.

Keywords: Domica Cave; karst water system; *Enterobacter cloacae*; β -lactam resistance

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INTRODUCTION

In spite of extended research in microbiota of caves located in different sides of the world over the past 10–20 years, our knowledge has still been limited (Jurado et al., 2010; Saiz-Jimenez, 2012; Vanderwolf et al., 2013). Microorganisms play a significant role in cave ecosystems, mainly due to their contribution in building speleothems, circulation of matter in aphotic ecosystem and their participation in biotic interactions (Barton & Northup, 2007; Jones, 2010; Bauermeister et al., 2012). There is a difference between natural endokarst communities of microorganisms, so called autochthonous microbiota and transient allochthonous microbiota (Farnleiter et al., 2005; Pronk et al., 2008). The ratio between both groups can change in regard to many stimuli like season and weather, rain events (Dussart-Baptista et al., 2003; Goldscheider et al., 2010), cave tourism (Fernandez-Cortes et al., 2011; Saiz-Jimenez, 2012), or due to using chemical components to control *lampenflora* growth

in show caves (Bastian et al., 2009). An important factor affecting protected locations is building villages or animal farms nearby which may be a source of pollution (Martinez, 2009). The karst system is extremely sensitive to infiltration with polluted water contaminated by bacteria or pharmacologically active substances (Brandon et al., 2010; Einsiedl et al., 2010; Morasch, 2013). *Escherichia coli* and total coliform bacteria including members of the genus *Enterobacter* are conventional indicators of microbial contamination (WHO, 2006). Antibiotics used in prevention and treatment of animal diseases, and to increase animal growth have still been one of the major factors of pollution despite the recommendation of the European Union on the prudent use of antibiotics and on a coordination between human and veterinary antibiotic policy (EFSA, 2010; Obeng et al., 2012; Harnisz, 2013). Prevalence of antibiotics in agriculture may result in violation of ecosystem with an impact to micro- and macrobiota located in these conserved biotopes due to a dissemination of bacteria carrying

different resistance determinants (Wright et al. 2010, Eisenberg et al., 2011; Wellington et al., 2013).

Our research studied groundwater karst system of the Domica show cave (Fig. 1) located in the south of Slovakia in which resistant *Enterobacter* species (spp.) have been detected. Previous information about microbial composition of this cave suggested that members of the genus *Enterobacter* belong to the dominant mesophilic microbiota isolated from water (Seman & Gaállová, 2009). Generally, among *Enterobacter* spp., *Enterobacter cloacae* has occupied a leader position as bacterium of an environmental origin as well as a source of human diseases (Baudart et al., 2011; Pavlovic et al., 2012). This species (sp.) has been frequently identified like nosocomial pathogen with a high ability to adopt and spread resistance against a variety of antibiotics (Mezzatesta et al., 2012; Radu et al., 2012). These bacteria have very often possessed resistance to β -lactams, the most frequently used antibiotics (Danziger & Neuhauser, 2011; Page, 2012) due to TEM or SHV ESBLs (extended spectrum β -lactamases) production (Coque et al., 2008; Dhillon & Clark, 2012; Korzniewska & Harnisz, 2013), and CTX-M ESBLs have been identified more frequently as well (Livermore et al., 2007; D'Andrea et al., 2013).

This work represents an original and exclusive research documenting a total number of cultivable microorganisms and a spectrum of collected enterobacteria in the Domica Cave. Additionally, 3 animal farms are situated nearby this cave, so β -lactam resistance in selected *E. cloacae* originating from the cave water was investigated.

DESCRIPTION OF DOMICA CAVE

The Domica is a part of Domica-Baradla cave system on the Slovakian-Hungarian boundary. The length of Domica Cave in Slovakian side is 5,368 m, whereas the whole system with Baradla is 25,564 m long. The protected area of the Domica Cave is 616,6892 ha. The main cave channel is created by underground stream Styx, with an older, dry cave level in fragments above it. The youngest phreatic level is also composed of fragments and it is situated about 20-47 m below the main active passage (Gaál, 2008). The underground waters of Styx and younger phreatic channels rise to the surface in Jósfa Spring near Jósfa Village in Hungary. The main part of the catchment area of Styx stream overlies in Slovakia and in past, it was polluted several times due to agriculture activities. Establishment of a protected area in the year 2005 improved this unfavorable state. The cave system was created in Triassic limestone but its development was strongly influenced by non-karstic rocks of old river cover (gravel, sand and clay), in its direct vicinity. Some occasional streams flow on this cover sinking into underground spaces through ponors at the limestone/gravel contact. These occasional streams create some lateral channels in the cave system ending with the Styx stream (e.g., Domický Brook in Slovakia and Little Baradla, Acheron stream and Retek branch in Hungary). The air temperature in the Domica Cave is between 9-10°C. The Domica Cave is well-known as

a wintering place for bats, site of rare underground invertebrates, and a famous locality of Neolithic man (Bella, 1997; Jakál et al., 2008).

MATERIAL AND METHODS

Sample collection

The water samples were seasonally collected aseptically into sterile 100 ml dark glass bottles from 2007 to 2011 from various sites of the subterranean streams in Domica Cave (Fig. 1). The first sampling site was the groundwater stream Styx near Roman Spa (D1), the second was the Ponor of the Domický

Fig. 1. The map of the Domica Cave with selected sampling sites. D1 – groundwater stream Styx near Roman Spa; D2 – the Ponor of Domický Brook, D3 – the Diamond Cruise (II. Cruise).

Brook, surface brook after entering into the cave (D2), and the third sampling site was the Diamond Cruise (II. Cruise), mixed water of the Styx and Domický Brook (D3). Samples were transferred in a portable refrigerator (5–8°C) and processed within 24 h after collecting (according to ISO standard for microbiology analysis of water quality, EN ISO 19458, 2007).

Cultivation of microorganisms

One ml of each water sample was inoculated to Mueller-Hinton Agar (HiMedia, India). Colonies of microorganisms were formed at 22°C (psychrophilic microorganisms) for 72 h or at 36°C (mesophilic microorganisms) for 48 h according to Häusler, 1994. After incubation, the colony forming units (CFU) were counted.

Identification of enterobacteria

Bacteria were cultivated on MacConkey Agar (HiMedia, India) at 36°C for 48 h. They were identified by the biochemical identification system ENTEROtest 24 with TNW Lite 6.5 program (Erba-Lachema, Czech Republic). *Serratia marcescens* CCM 303 and *Enterobacter cloacae* CCM 1903 were used as reference standard strains for verification of biochemical identification sets. Additionally, 6 reference strains of the genus *Enterobacter*: *E. cloacae* CCM 1903, *E. aerogenes* CCM 7797, *E. amnigenus* CCM 3430, *E. cowanii* CCM 7015, *E. gergoviae* CCM 3459, and *E. asburiae* CCM 4032 were used in the study. Reference strains were obtained from the Czech Collection of Microorganisms (CCM).

DNA extraction

All strains were grown on Mueller-Hinton Agar (HiMedia, India) at 37°C for 24 h. One full loop of colonies was suspended in 300 µl of sterile deionised water and boiled for 10 min at 95°C. After centrifugation at 12,000 x g for 8 min at 4°C, the supernatants were recovered and 2 µl was directly used as the template for PCR.

tDNA and universal PCR, restriction with BstBI and DdeI enzymes

For tDNA, specific set primers T3B (5'- AGG TCG CGG GTT CGA ATC C-3') and T5A (5'- AGT CCG GTG CTC TAA CCA ACT GAG-3') (Invitrogen, USA, Clementino et al., 2001) were selected. Universal primers for 16S rRNA U1 (5'- CCA GCA GCC GCG GTA ATA CG-3') and U2 (5'- ATC GGY TAC CTT GTT ACG ACT TC-3') (Invitrogen, USA, Lu et al., 2000) were used for PCR. The PCR reaction (20 µl) contained 4 µl of 5x HOT FIREPol Blend Master Mix (with 12.5 mM MgCl₂) (Solis BioDyne, Estonia), 0.4 µl of relevant primers (all per 2 pmol), 13.2 µl of sterile deionised water, and 2 µl of DNA template. Conditions of tDNA PCR were as follow: denaturation at 95°C for 12 min, then denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 2 min (35 cycles), and additional extension at 72°C for 10 min. The program for universal PCR consisted of denaturation at 95°C for 12 min followed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C

for 2 min (35 cycles), and additional extension at 72°C for 10 min. The PCR products were used for restriction with BstBI (2500 U BMI Fermentas, Germany) and DdeI (500 U BMI Fermentas, Germany) enzymes. Restriction mix contained 0.2 µl of BstBI enzyme or DdeI enzyme, 2 µl of 10x Buffer Tango with BSA (MBI Fermentas, Germany), 12.8 µl of sterile deionised water, and 5 µl of PCR product. Restriction was performed in thermoblock MD-01 (Major Science, USA) at 37°C for 2 h. Then 2 µl of 6x DNA Loading Dye (MBI Fermentas, Germany) were added to stop the reaction. Ultra Power™ Nucleic Acid Stain (5 µl) (BioTeke, China) was applied into gel during its preparation. A sample of 10 µl of the PCR product (or restriction product) was loaded onto 1% agarose gel. Electrophoresis was performed at 100 V for 120 min in 1x TBE (Tris-borate-EDTA buffer, pH 8.3). For comparison, 100 bp Plus DNA Ladder Gene Ruler™ (MBI Fermentas, Germany) was used. DNA was visualized with UV-transilluminator MUV21-312-220 (Major Science, USA) at wavelength 254 nm.

Disk diffusion assay and Combination disk diffusion assay

Assays were performed according to the recommendation of CLSI, M2-A10, 2009a, and CLSI, M100-S19, 2009b. Briefly; one loop of 24 h old culture was suspended in broth (Mueller-Hinton Broth, HiMedia) and incubated to log phase growth. The suspension was diluted in sterile water (1:1000) and then 100 µl was inoculated on MHA plate. The disks containing antimicrobial agents were applied on plates within 15 min of inoculating. For disk diffusion assay, following antibiotics (Bio-Rad, France) were tested: ampicillin (10 µg), carbenicillin (100 µg), amoxycillin-clavulanic acid (20/10 µg), ampicillin-sulbactam (10/10 µg), cefazolin (30 µg), cefalotin (30 µg), cefaclor (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), cefprozil (30 µg), cefonicid (30 µg), ceftazidime (30 µg), ceftizoxime (30 µg), and cefepime (30 µg). Plates were incubated at 37°C for 18 h and the inhibition zones were measured. For combination disk diffusion assay, disks with cefotaxime (30 µg), cefotaxime-clavulanic acid (30/10 µg), ceftazidime (30 µg), and ceftazidime-clavulanic acid (30/10 µg) were tested (Bio-Rad, France).

PCR for detection of bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes

For each type PCR, appropriate pairs of primers (Metabion, Germany) were selected: GRA1 (5'- ATG CGT TAT ATT CGC CTG TG-3') and GRA2 (5'- GTT AGT GTT GCC AGT GCT CG-3') for detection of the bla_{SHV} gene (Babini & Livermore, 2000); TEM-A (GTA TGG ATC CTC AAC ATT TCC GTG TCG) and TEM-B (ACC AAA GCT TAA TCA GTG AGG CA) for detection of the bla_{TEM} gene (Stapleton et al., 1995); MA1 (ATG TGC AGY ACC AGT AA) and MA2 (ACC GCR ATA TGR TTG GT) for detection of the bla_{CTX-M} gene (Saladin et al., 2002). Each PCR reaction (20 µl) consisted of 4 µl of 5x HOT FIREPol Blend Master Mix (with 12.5 mM MgCl₂) (Solis BioDyne, Estonia), per 2 pmol of appropriate primers, 13.2 µl of sterile deionised water, and 2 µl of DNA. The programs for every PCR were designed independently according to publications

with mentioned primer sets. PCR products were visualized as described.

RESULTS AND DISCUSSION

Microorganisms are regular inhabitants of different niches including caves. The composition of cave microbiota is affected by many environmental factors independent of human activities, but also those ones directly associated with human impact like an import of microorganisms through mass tourism in show caves (Saiz-Jimenez, 2012). Another possible risk effect represents nearby animal farms because of groundwater contamination (Sapkota et al., 2007; Martinez, 2009). Our research studied a proportion of heterotrophic cultivable bacteria isolated from waters of the Domica Cave; it was mainly focused at diversity among enterobacteria, and resistance to β -lactams in *Enterobacter cloacae*, one of the dominant mesophilic bacterial species. In the period of 2007-2011, 17 sample collections were realized from 3 sampling sites (D1, D2, D3, Fig.1) during the years 2008 - 2009 the water samples were collected seasonally. In the years 2007 - 2010 and in the year 2011, the samples were taken only in autumn and winter because of rainfall deficiency. The data confirmed two dominant groups of heterotrophic cultivable microbiota belonging to psychrophilic and mesophilic microorganisms that are able to survive at low temperature (9-10°C) in cave during a year. Numbers of psychrophilic microorganisms ranged from 10^2 to 10^3 CFU/ml and numbers of mesophilic microorganisms ranged from 10^1 to 10^2 CFU/ml. Both groups of microorganisms are widely distributed in the karst caves (Khyzniak et al., 2003; Semikolennykh et al., 2004). The majority of microorganisms were recovered in summer and spring, periods which are generally characterized by frequent rainfall or snow melt. From the hydrochemical point of view, the groundwater stream Styx near Roman Spa (D1) belongs to Ca-HCO₃ type, with dominant HCO₃⁻ anions and Ca²⁺ cations. The average daily temperature of the water is around 9.3°C. The primary chemical composition of the Ponor of the Domický Brook (D2) largely depends on quality of rainwaters. Water of the Ponor of the Domický Brook predominantly belongs to Ca-HCO₃ type, or less Ca-Mg-HCO₃, Ca-HCO₃-SO₄ or Ca-Mg-HCO₃-SO₄ type, with dominant Ca²⁺ and Mg²⁺ cations and HCO₃⁻ anions, less SO₄²⁻, Cl⁻ and NO₃⁻ anions. The average daily temperature of the water in this location is around 9.8°C. For the water from Diamond Cruise (II. Cruise, D3), there are dominant HCO₃⁻, Ca²⁺ and Mg²⁺ ions, which determine the Ca-HCO₃ type (Gazda, 1974; Haviarová et al., 2010). The average daily temperature of the water is around 10°C. The water pH of all sampling sites is slightly alkaline. Haviarova et al. (2010) summarized more chemical parameters and microbiological indicators of water quality in the Domica Cave water system. The problem concerning water contamination has also been described from the hydrochemical and microbiological point of view in other related caves in Slovak karst: Krásnohorská Cave, Diviáča Chasm and Attilova Chasm (Motyka et

al., 2005), Milada Cave (Haviarová et al., 2011), Silica-Gombacek cave system (Haviarová et al., 2012), and in Demänovský Cave System, Low Tatra Mountains (Motyka et al., 2005).

Enterobacteriaceae is a group of mesophilic bacteria associated with environmental pollution. Many studies from cave environments are attended to this group, mainly in regard to an association with faecal contamination of soil, karstic waters or cave walls (Personé et al., 2004; Lavoie & Northup, 2006; Pronk et al., 2008). In this study, 285 isolates of enterobacteria were identified among mesophilic microorganisms (Fig. 2) with *Escherichia coli* (112 isolates) as the most abundant bacterium. This microorganism was collected mainly in summer of 2008 and 2009 that probably resulted from the several rainfalls during those years. *E. coli* belongs to the coliform bacteria which are monitored in waters as faecal indicators of water pollution (WHO, 2006). The second most dominant *Enterobacteriaceae* isolated belonged in the *Enterobacter* genus (53 isolates). *E. cloacae* (36), *E. amnigenus* (5), *E. dissolvens* (4), enteric group 69 (4), *E. gergoviae* (3), and *E. cancerogenus* (1) were identified.

According to Leclerc et al. (1997), *Enterobacter* spp. have been included in allochthonous microbiota like *E. coli* in both drinking and ground waters. However, in this research, *Enterobacter* spp. were not found to be seasonable present in the water samples collected from all sampling sites. This observation suggests an autochthonous origin of collected *Enterobacter* spp., as these bacteria were recovered regardless of sampling season and occurred in various areas of cave.

Concerning this information, it seemed to be interesting to search for possible origin and resistance profile of *E. cloacae* from Domica Cave. In the immediate area of Domica Cave, there are 3 animal farms located within 2-3 km (Fig. 3), thus probable contamination from these farms might be assumed. In the 1970s, more than 500 cows were kept in animal farms Dlhá Ves and Kečovo villages. During that time, veterinary drugs containing the combinations of amoxicilline with clavulanic acid were served on a mass scale to animals. At present, the livestock in Dlhá Ves has been stopped. Ferdi-Ranch (horse and cow farm between Dlhá Ves and Domica) still has cradled 75 pieces of black cattle and Kečovo village has several cows and sheep which are pastured on the watershed of the Domica Cave. Until now, commercially available preparations containing amoxicillin with clavulanic acid has been still applied, but in a limited amount and only for therapeutic reasons (personal communication with local veterinarian).

Thirty six *E. cloacae* showing the same profile as the reference strain *E. cloacae* CCM 1903 in ENTEROtest 24 were selected for the resistance study; 9 isolates were collected from the site D1, 12 from the site D2 and 15 from the site D3. Prior to the study of β -lactam resistance, identification of *E. cloacae* was completed by molecular biology: the universal PCR with restriction by BstBI and DdeI enzymes and the tDNA PCR. Universal PCR amplified fragment of the gene encoding 16S rRNA was used for restriction analysis

Fig. 2. Spectrum of isolated enterobacteria from the Domica Cave.

in order to discriminate the genus *Enterobacter* from the other bacterial genera (Lu et al., 2000). All our tested isolates showed the same profile like the majority of the reference *Enterobacter* strains (Fig. 4). Only *E. cowanii* CCM 7015 showed a different profile. The tDNA PCR method (Clementino et al., 2001) allows distinguishing some spp. of *Enterobacter* like *E. cowanii*, *E. aerogenes*, *E. gergoviae*, *E. asburiae*, *E. amnigenus*, and *E. cloacae* (Fig. 5). Both methods confirmed that selected *E. cloacae* were correctly identified. All these isolates were submitted for the study of β -lactam resistance.

At present, an increasing prevalence of the problematic clinical isolates of *E. cloacae* with the broad spectrum β -lactam resistance has been documented (Jaskulski et al., 2013). Among *bla* genes, *bla*_{TEM} has been identified as the most frequently occurring type

of gene encoding β -lactamases (Jaskulski et al., 2013).

All *E. cloacae* were tested for susceptibility/resistance to penicillin and β -lactam antibiotics and the results are summarized in Tab. 1. There was only 1 isolate resistant to ampicillin. Other isolates were resistant to more antibiotics (mostly from 3 to 10) and 5 isolates were resistant to more than 10 antibiotics. We did not observe differences in susceptibility of tested isolates in regard to isolation site. Additionally, 18 isolates were detected as the ESBL producers by combination disks assay when ceftazidime and combination of ceftazidime-clavulanic acid was used, and 1 isolate when cefotaxime and combination of cefotaxime-clavulanic acid was applied. These results suggested the presence of *bla* genes. Therefore, another aim of our work was

Fig. 3. Map of catchment area of the Domica Cave (the cave is marked with black color, red line is the surface of protected cave area. Red rectangles characterize agricultural areas: 1. Agricultural cooperation in Dlhá Ves village, 2. "Ferdí ranch" (horse farm), 3. Agricultural cooperation in Kečovo village.

Fig. 4. Electrophoresis of the universal PCR (upper line) and the restriction with BstBI and DdeI enzymes (bottom line) M- DNA ladder 100 bp Plus, 1. *E. cloacae*, 2. *E. aerogenes*, 3. *E. gergoviae*, 4. *E. asburiae*, 5. *E. amnigenus*, 6. *E. cowanii*, 7 - 9. aquatic isolates of *E. cloacae*.

Table 1. Testing susceptibility to antibiotics in 36 aquatic isolates of *Enterobacter cloacae*.

	antibiotic resistance													
	penicillin with inhibitors		cephalosporin											
	penicillin		of β -lactamases		1. gen.		2. gen.				3. gen.		4. gen.	
	AM	CB	AMC	AMS	CZ	CF	CEC	CXM	FOX	CPR	CID	CAZ	CIZ	FEP
No. of resistant isolates/	34/94	26/72	21/58	9/25	26/72	31/86	14/39	10/28	23/64	18/50	29/81	18/50	9/25	9/25
% of resistant isolates														

AM – ampicillin (10 μ g); **CB** – carbenicillin (100 μ g); **AMC** – amoxicillin-clavulanic acid (20/10 μ g); **AMS** – ampicillin-sulbactam (10/10 μ g); **CZ** – cefazolin (30 μ g); **CF** – cefalotin (30 μ g); **CEC** – cefaklor (30 μ g); **CXM** – cefuroxim (30 μ g); **FOX** – ceftiofur (30 μ g); **CPR** – cefprozil (30 μ g); **CID** – cefonicid (30 μ g); **CAZ** – ceftazidime (30 μ g); **CIZ** – ceftizoxime (30 μ g); **FEP** – cefepime (30 μ g)

to detect *bla* genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}) encoding TEM-, SHV- and CTX-M type β -lactamases. Genes for *bla*_{TEM} were detected in 8 isolates, genes for *bla*_{SHV} were determined in 7 isolates, and genes for *bla*_{CTX-M} encoding CTX-M type β -lactamases were confirmed in 9 isolates. Three isolates possessed a combination of *bla*_{SHV} and *bla*_{CTX-M} genes and 2 isolates expressed all three types of *bla* genes tested. There were not detected any *bla* genes in 15 isolates. The occurrence of *bla* genes in selected aquatic isolates is presented in the Fig.6. Some previous reports pointed to the phenomenon of the incidence of antibiotic resistance determinants in bacteria originating from caves or extreme biotopes (Brown & Balkwill, 2009, Thaller et al., 2010). Moreover, Bhullar et al. (2012) in their study concerning macrolide resistance suggested that antibiotic resistance is natural and ancient. In contrast, Kemper (2008) concluded that using antibiotics in animals results in occurrence of resistant bacteria in soil and waters. In this relation, there is a question whether resistance to β -lactams of the 3rd and 4th cephalosporin generation could be naturally present in cave microbiota. We implicate observed resistance in *E. cloacae* isolated from the Domica Cave with the long-term activities of neighboring animal farms and

continuous antibiotic pressure, despite the fact, that current animal pharmacy use is more restricted in agreement with the recommendation of the European Union. Moreover, as *E. cloacae* is a characteristic member of the transient allochthonous microbiota and our enterobacteria were isolated regardless of season, it might be expected that they were imported to the cave by underground water during the rains.

CONCLUSIONS

The results of our investigation revealed the prevalence of heterotrophic cultivable microbiota from psychrophilic and mesophilic microorganisms in the Domica Cave. The dominant bacterium was *E. coli* followed by the genus *Enterobacter* from enterobacteria. Showed β -lactam resistance, especially, to the 3rd and 4th cephalosporin generations and resistance mechanisms in isolates of *E. cloacae* obtained from waters of the Domica Cave indicated an external source of resistance gene contamination. The

Fig. 5. Electrophoresis of the tDNA PCR M - DNA ladder 100 bp Plus, 1. *E. cowanii*, 2. *E. aerogenes*, 3. *E. gergoviae*, 4. *E. asburiae*, 5. *E. amnigenus*, 6. *E. cloacae*, 7 - 8. aquatic *E. cloacae* isolates

Fig. 6. Electrophoresis of the PCR detection of *bla* genes in aquatic *E. cloacae* M - DNA ladder 100 bp Plus, 1-2 gene *bla*_{TEM}, 3-4 gene *bla*_{SHV}, 5-6 gene *bla*_{CTX-M}

group of resistance determinants, found also in this work, is usually located on mobile elements and the antibiotic pressure contributes significantly to their horizontal transfer among bacteria. Observation of the resistant *E. cloacae* isolates in protected area of the Domica Cave suggests close relationship between quality of surface (including ground and waters) nearby the cave and its aquatic ecosystem. Taken together, determined resistance in aquatic *E. cloacae* isolated from Domica Cave can be an example of the anthropogenic pressure that might affect natural microbiota of conserved ecosystems.

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