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Serological and Molecular characterization of Bacterial isolates using 16S Ribosomal DNA Restriction Analysis from soil sediments of Kotumsar Cave Ecosystem, Chhattisgarh, India

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ABSTRACT

Bacterial communities exist everywhere in the universe so in the caves. The ever increasing human activities inside any cave, in the form of ecotourism exert a major impact on its native microbial communities, which often stops its growth and pollutes the whole ecosystem. The situation is often found to be responsible for producing some human pathogenic bacteria inside it, which might pose a threat of infection to the other tourist. Kotumsar cave is a well known tourist pulling limestone cave of central India. In the present study the soil bacterial communities earlier isolated and characterized from different microhabitats of Kotumsar cave have been further confirmed by molecular identifications by applying 16S rDNA analysis and serotyping. All bacterial strains were also assayed for antibiotic resistance. Among the tested strains, support the PIB-win results and also shows the maximum resistance (about 69.23%) to Vancomycin and Polymyxin B.

Introduction

Subterranean cave's biospheres are usually characterized by high humidity (almost saturated), complete darkness and low energy sources. Kotumsar Cave is one of the most biologically explored limestone caves of India. Time to time several troglobiotic as well as troglophilic species have been reported from this cave. It is situated in the Kanger Valley National Park (18°52'09" N; 81°56'05" E), which became a major eco-tourist spot of Chhattisgarh, after it has been developed as a new state of India (figure 1). The main entrance of this cave is formed by a vertical fissure in the wall of a hill which further leads inwards via a narrow, twisted tubular path, measuring about 15 m in length. The cave is honeycombed in its structure, consisting of several irregular chambers. The main tunnel of the cave is nearly 500 m long and has several lateral and downward passages. The roofs and walls of the different chambers are lined with colorful dripstone formations resulting from the precipitation of calcite-dissolved carbonate lime. The chambers of the cave are floored with either rocks or pebbles of various dimensions or by surface-derived soil/clay deposits. The cave is subject to frequent flooding during the monsoon season which generally begins in the middle of June and continues till the mid of October.

In the present study, earlier characterized bacterial strains (Rajput et al. 2012a) have been confirmed by conventional method that is serotyping and also by molecular level applying amplified ribosomal DNA restriction analysis (ARDRA) technique. Antimicrobial sensitivity relates to microbes (usually bacteria) sensitivity to a specific antibiotic.

Materials and methods

Initially the bacterial species were isolated from the cave soil, sampled from the various depth dependant microhabitats of the cave. On the basis of external environmental impact to any subterranean cave, the total subterranean biotope could be divided into four different zones.

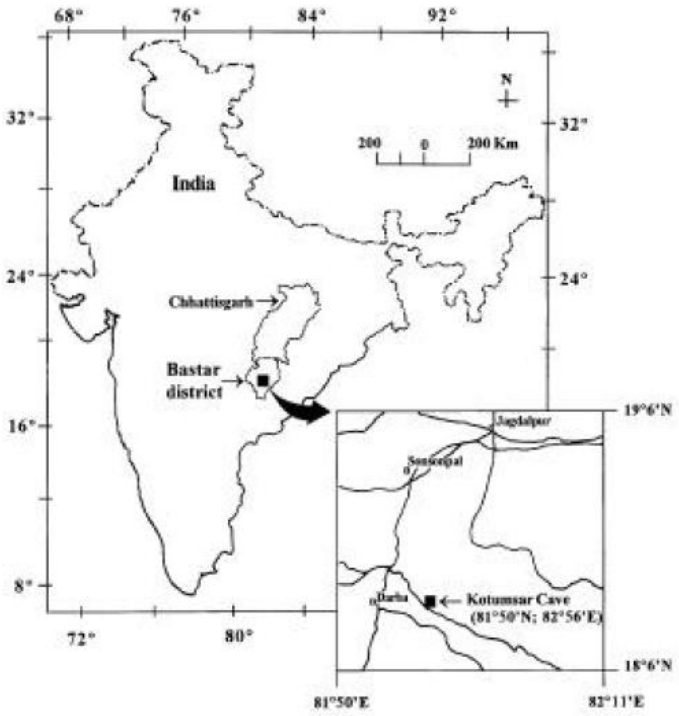

Figure 1: Location map of the Kotumsar cave located in the Kanger Valley National Park, Jagdalpur, Baster, India (Pati and Agrawal, 2002*)*

(1) The entrance zone is the area adjacent to the cave entrance, which always gets tuned by their immediate epigean environmental conditions.

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(2) The twilight zone starting from the entrance towards inner side where light intensity, humidity and temperature vary.
(3) The transition zone of almost complete darkness with variable humidity and temperature.
(4) The deep zone of complete darkness with almost 100% humidity and constant temperature.

Isolated bacterial strains were characterized by employing the PIB Win software (Bryant 2003) on the basis of morphological, biochemical and physiological identification, were re-cultured on nutrient agar medium at 28 ± 1°C and maintained by subculturing every fortnightly at 4°C for employing ARDRA technique and other studies.

Serotyping of bacteria:

Serology is a method to investigate the molecular architecture of the bacterial cell, depend on the ability of the chemical constituents of the same to behave as antigens, i.e. to elicit the production of antibodies in vertebrate animals. Live antigen was used to raise antiserum against *Micrococcus luteus3*. The cell concentration was 7×10^{10} CFU/ml. Rabbit was immunized as per the Prescott, 1981. For agglutination 100µl bacterial suspension and 100µl of polyclonal antibody were placed on slide. Preimmunized serum and saline were used as a control.

Amplified Ribosomal DNA Rastriction Analysis (ARDRA):

For ARDRA, the genomic DNA was extracted by the following method of Sambrook, et. al (1989). Oligonucleotide primer was derived from conserved region present at the edges of the 16S rDNA. The sequences of primers were 16SrDNA F–5' AGAGTTTGATCCTGGCTCAG-3'and 16SrDNA R–5'AGGAGGTGATCCAGCCGCA-3' Thorn and Tsuneda 1996. The template DNA (1 ml, around 10 ng DNA) was added to 49µl aliquots of PCR mixture containing 5 µl of 10X PCR buffer supplied with enzyme (Taq polymerase), 2.5 µl each forward and reverse primer from a 10 pico mol stock (*i.e*. 12.5 pico mol of each primer), 2.5 µl of 10mM (each) deoxynucleoside triphosphates, and 0.5µl of DNA polymerase (3 unit/ml) final volume was made to 50µl by adding sterile glass distilled water. After initial denaturation at 74°C for 3 min, the reaction mixture was run through 35 cycles of denaturation at 94°C for 1min, annealing at 51°C for 1 min, and finally a 10 min extension period at 72°C was carried out. The 16S rRNA type was determined by digestion of the amplicon with *EcoRI* (G/AATTC) and *AluI* (AG/CT) enzymes (Banglore Genei, India) and analyzed by electrophoresis on 1.5% agarose gel with staining by ethidium bromide (0.5µg/ml). ARDRA bands were scored as either present (1) or absent (0). All binary data were entered and genetic distances were calculated through Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), version 2.02 and calculating Euclidean distance and then assembling a dendogram using "Unweighted Paired Group Method using Arithmetic average criterion" (UPGMA).

Antibiotic sensitivity test:

Antibiotic sensitivity was tested according to the method of Kirby-Bauer (Bauer et al. 1966). Antibiogram plays an important role in the preliminary characterization of the organisms. Ten antibiotic discs such as chloramphenicol (30 µg), erythromycin(10 µg), gentamycis (10 µg), kanamycin (30 µg), neomycin (30 µg), novobiocin (30 µg), penicillin-G (10 units), polymyxin-B(100 units), streptomycin (10 µg), vancomycin (30 µg) were obtained from Hi-Media Pvt. Ltd., Mumbai, India.The response of the bacetria to antibiotic discs were determined by spreading broth cultured suspension on Nutrient agar medium and antibiotic discs were placed with the help of disc dispenser. All the plates were incubated at 28°C for 24-48h. On the basis of zone diameter, the isolates were classified as resistant, sensitive and intermediate. Two-way ANOVA were performed for statistical analysis.

Results

Overall one forty six bacterial isolates were characterized from soil of Kotumsar cave out of which only twenty one bacteria were identified with the help of PIB-win software. PIB-win results (figure 2 & 3) were further cross checked by a conventional method through serotyping.

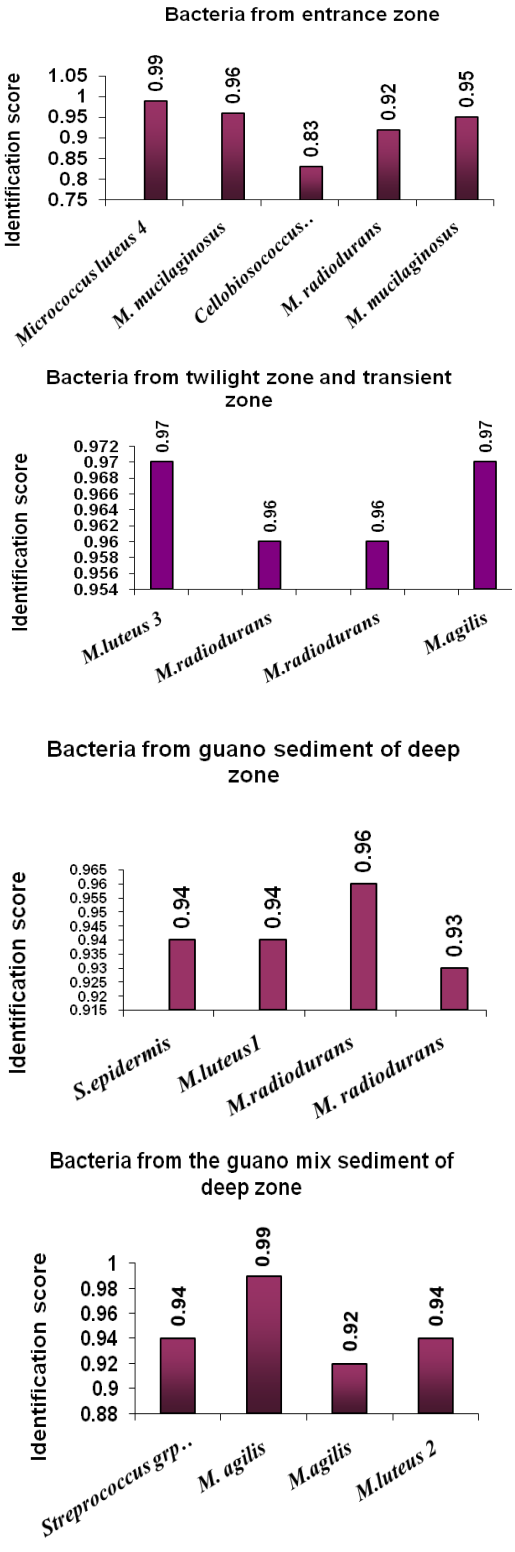
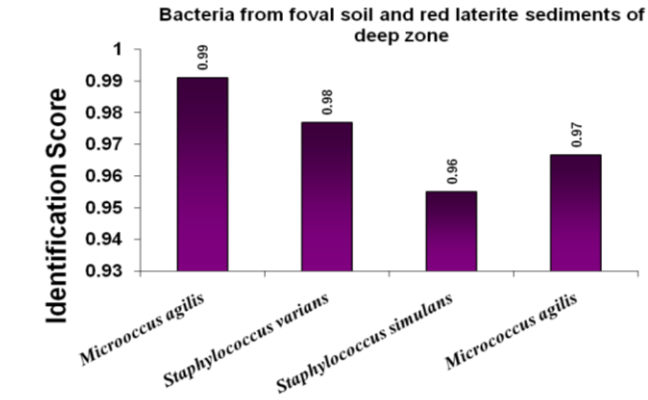


Figure 2: Identification score of bacterial isolates from soil of Kotumsar cave



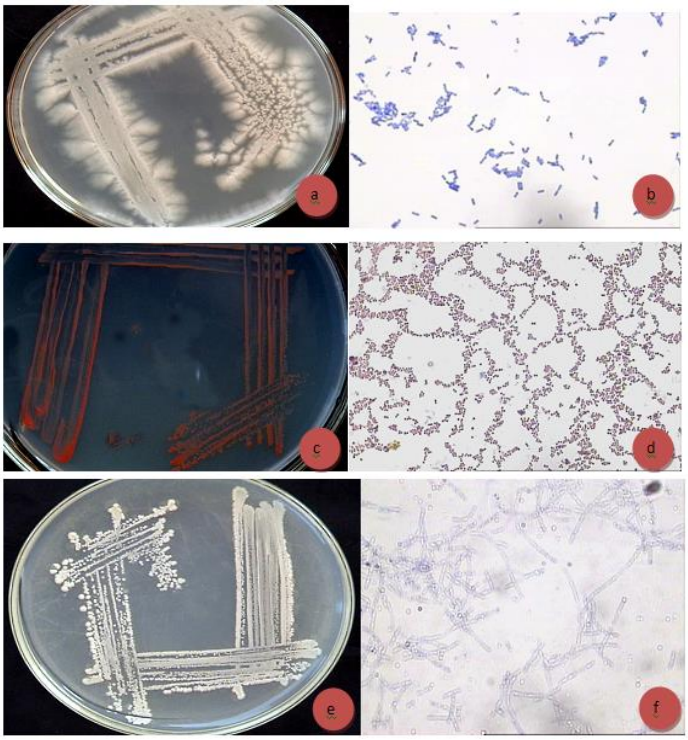


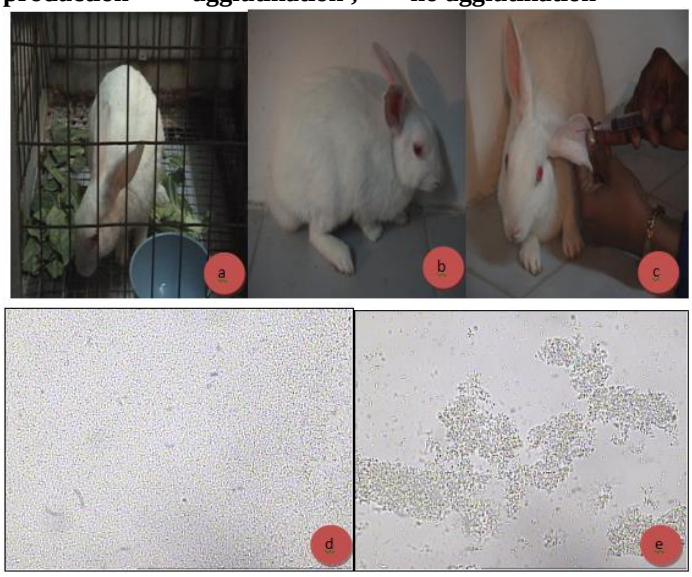
Fig 3(a - f): Subculture and microscopic view of bacteria isolated from soil of Kotumsar cave. Subculture of *Micrococcus mucilaginosus* KCB14 from outside of the entrance gate (a), Microscopic view (1000X) (b), Subculture of *Staphylococcus varians* KCB134 from inner zone (brown soil) (c), Microscopic view (1000X) (d), Subculture of *Streptococcus grp Q1* KCB109 from inner zone (black soil) (e), Microscopic view (1000X) (f).

The immune sera from rabbit when subjected to direct agglutination with the Kotumsar's bacterial isolates were non-specific, as shown in table 1 & figure 4, when serum reacted with its homologous bacterial isolate by agglutination. All fourteen bacterial isolates were tested by agglutination and double diffusion, using a soluble, particulate and sonicated antigen. Further, only nine *Micrococcus sps.* exhibited agglutination with antisera of *Micrococcus luteus3,* but rest of the isolates did not show agglutination. However, sonicated antigen and soluble antigen of bacteria did not show any precipitation through double diffusion. Two isolates of *Micrococcus mucilaginosus* KCB26 & KCB14, *Micrococcus luteus 4* KCB11 and *Micrococcus radiodurans* KCB 21 from entrance zone whereas *Micrococcus luteus 3* KCB38, *Micrococcus radiodurans* KCB39 from twilight zone, *Micrococcus radiodurans KCB50* from transient zone likewise *Micrococcus agilis* KCB125 from foval soil, *Micrococcus luteus1* KCB90 and *Micrococcus radiodurans* KCB 93 from guano mixed soil and also *Micrococcus agilis* KCB140 from red laterite soil of deep zone exhibited agglutination with antisera. Therefore, serological relationship exists between these organisms. Rest of the three bacterial isolates *Staphylococcus simulans* KCB129, *Staphylococcus varians* KCB134 from deep zone (foval soil), *Streptococcus grp Q1* KCB109 from deep zone (guano mix soil) did not show agglutination reaction.

Table 1: Serotyping of bacteria from soil of Kotumsar cave by agglutination reaction

S.N.	Zone	Culture code	Agglutination reaction	Name of the bacteria
1.	Entrance zone	KCB26	+	*Micrococcus mucilaginosus*
		KCB21	+	*Micrococcus radiodurans*
		KCB14	+	*Micrococcus mucilaginosus*
		KCB11	+++	*Micrococcus luteus4*
2.	Twilight zone	KCB38	++++	*Micrococcus luteus3*
		KCB39	+	*Micrococcus radidurans*
3.	Transient zone	KCB50	+	*Micrococcus radidurans*
4.	Deep zone (foval soil)	KCB134	-	*Staphylococcus varians*
		KCB125	+	*Micrococcus agilis*
		KCB129	-	*Staphylococcus simulans*
5.	Deep zone (guano mix soil)	KCB109	-	*Staphylococcus grp Q1*
		KCB90	++	*Micrococcus luteus 1*
6.	Deep zone (guano soil)	KCB93	+	*Micrococcus radidurans*
7.	Deep zone (red laterite soil)	KCB140	+	*Micrococcus agilis*

❖ ***Micrococcus luteus3*** **KCB38 was used for antibody production + = agglutination ; - = no agglutination**



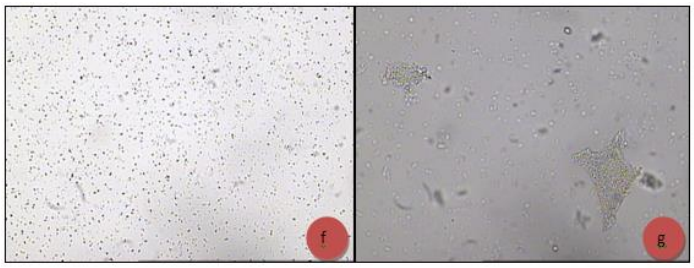


Figure 4 (a - g): Serotyping of bacterial isolates from soil of Kotumsar cave. Rabbit (a& b), intravenous injection of live particulate antigen of *Micrococcus luteus 3* KCB38 (c); Agglutination reaction of particulate live antigen with antiserum of *Micrococcus luteus 3* KCB*38*, control (d), test agglutination reaction (e); Agglutination reaction of *Micrococcus agilis* KCB 125, control (f), agglutination (g).

ARDRA technique was applied for further confirmation of the selected bacteria. Amplified fragments of 16SrDNA were digested with *EcoRI*. Restriction fragment were analyzed by the using 2.5% agarose gel. In entrance zone four bacterial isolates were studied for ARDRA pattern *i.e. Micrococcus mucilaginosus* KCB14, *Micrococcus luteus 4* KCB11, *Micrococcus mucilaginosus* KCB26, *Micrococcus radiodurans* KCB21. 16SrDNA of *Micrococcus mucilaginosus* KCB14 was cut into 6 fragments i.e. 5000bp, 4000bp, 3900bp, 3000bp, 2600bp and 2500bp whereas in *Micrococcus mucilaginosus* KCB 26 4000bp fragment is absent. *Micrococcus luteus 4* KCB11 DNA was fragmented into 7 part i.e. 4500, 3800, 3500, 2400, 2300, 2200, 2000 bp, but *Micrococcus luteus 3* KCB38 from transient zone observed 6 fragments 3800, 3600, 3500, 3000, 2600 and 2500bp. *Micrococcus radiodurans* KCB21 isolated from entrance zone and guano soil of deep zone shows same number of bands these were 4500, 3800, 3500, 2500, 2400, 2300, 2000bp. *Micrococcus agilis* from foval soil and alluvial soil of deep zone shows the same number of band pattern. 16SrDNA of *Staphylococcus simulans* KCB 129 was fragmented into 6 parts i.e. 4200, 3800, 3600, 3500, 3100, 3000 bp while *Staphylococcus varians* KCB134 shows 4200, 4000, 3900, 3100 and 3000bp. And *Streptococcus grp* Q1 KCB109 shows 4200, 3500, 2600,2500, 2400, 2200 (table 2, Figure 6a&b).

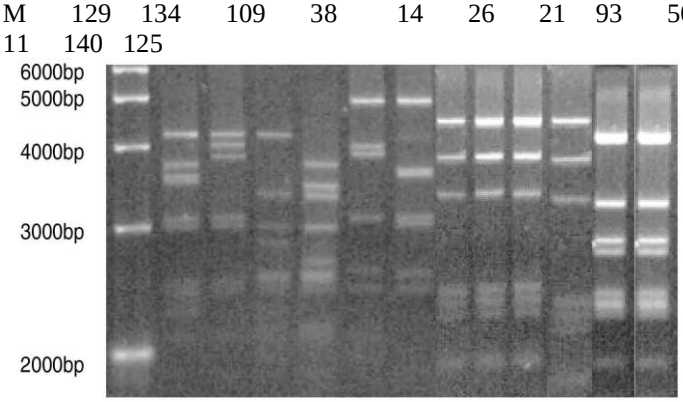


Figure 6a: 16S rDNA amplification (ARDRA) was using *EcoRI* from bacterial samples with universal primer

Amplified product of these bacterial 16SrDNA were also treated with *aluI* restriction enzyme. *Micrococcus mucilaginosus* KCB14 showed 3 fragments *i.e.* 4900bp, 3100bp and 2900bp whereas *M. mucilaginosus* KCB26 recorded one more band that was 4800bp from entrance zone. *Micrococcus luteus 4* KCB11 from entrance zone showed 6000bp, 3900bp, 3100bp, 3000bp and 2500bp while *Micrococcus luteus 3* from twilight zone KCB38 recorded 7000bp, 4300bp, 2500bp, 2400bp and 2200bp. Now again *Micrococcus radiodurans* from entrance zone, transient zone and guano soil of deep zone were show same number and size of band pattern i.e. 7000, 2900, 2600, 2500 and 2000bp. *M. agilis* from foval soil and alluvial soil of deep zone recorded same pattern of bands which were 4200bp, 3300bp, 3200bp, 2500bp, 2400bp, 2300bp and 1800bp. *Staphylococcus simulans* KCB 129 exhibited 8 fragments *i.e.* 6000bp, 4800bp, 4200bp, 4100bp, 3200bp, 3100bp, 2500bp and 2000bp whereas *Satphylococcus varians* KCB134 showed only 6 fragments *i.e.* 4500bp, 4000bp, 3900bp, 3000bp, 2600bp and 2500bp.One isolate of guano mix soil that was *Sreptococcus grpQ1* showed completely different band pattern *i.e.* 6000bp, 5500bp, 5000bp, 3200bp, 3100bp, 2500 and 2000bp (table 3, Figure 7a&b).

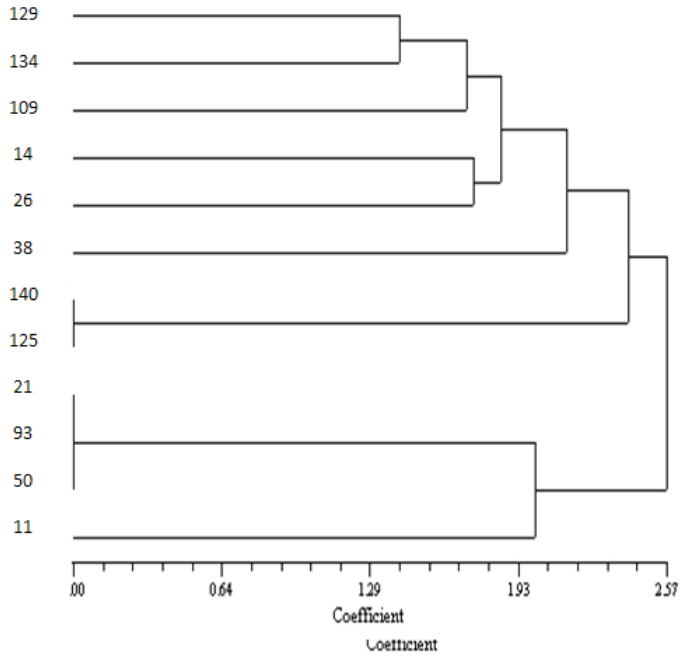


Figure 6b: Dendogram showing relatedness among bacterial isolates based on restriction digestion of genomic DNA using *Eco*RI. Where; M: DNA Marker; 129: *Staphylococcus simulans* KCB129; 109: *Streptococcus grp Q1* KCB109; 11: *Micrococcus luteus 4* KCB11; 134: *Staphylococcus varians* KCB 134, 14: *Micrococcus mucilaginosus* KCB 14; 26: *Micrococcus mucilaginosus* KCB26; 93: *Micrococcus radiodurans* KCB93; 21: *Micrococcus radiodurans* KCB21; 50: *Mcrococcus radiodurans* KCB50; 38: *Micrococcus luteus 3* KCB38; 140: *Micrococcus agilis* KCB140; 125: *Micrococcus agilis* KCB125.

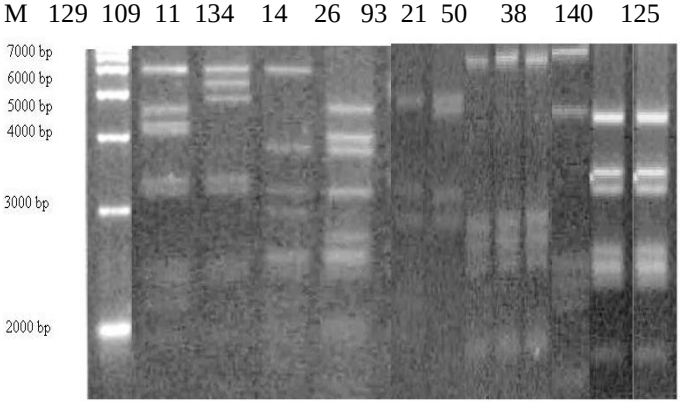


Figure 7a: 16S rDNA amplification (ARDRA) using *AluI* from bacterial samples with universal primer

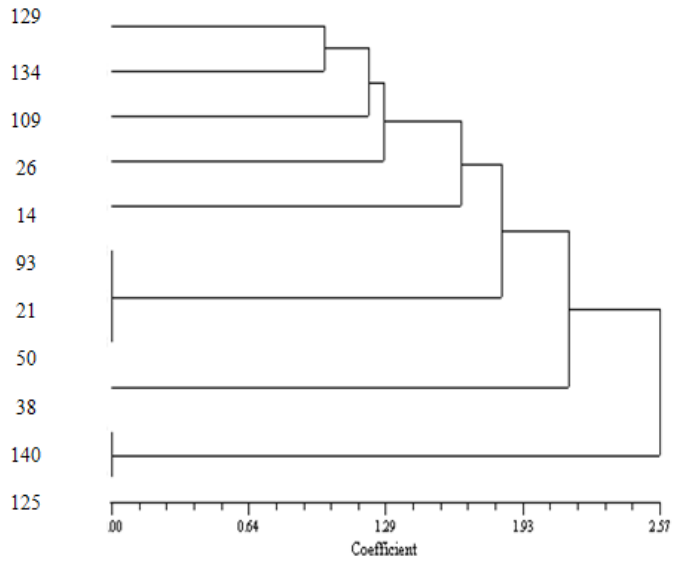
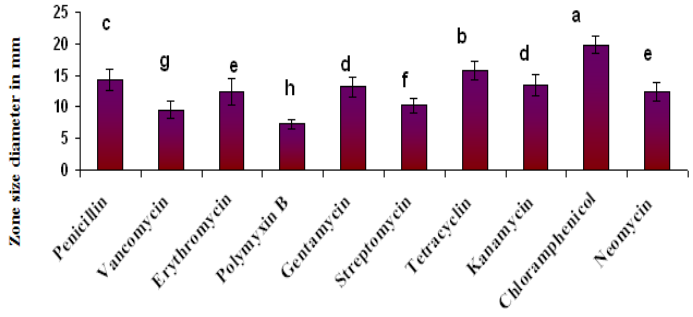


Figure 7b: Dendogram showing relatedness among bacterial isolates based on restriction digestion of genomic DNA using *AluI*.

Where; M: DNA Marker; 129: *Staphylococcus simulans* KCB129; 109: *Streptococcus grp Q1* KCB109; 11: *Micrococcus luteus 4* KCB11; 134: *Staphylococcus varians* KCB 134, 14: *Micrococcus mucilaginosus* KCB 14; 26: *Micrococcus mucilaginosus* KCB26; 93: *Micrococcus radiodurans* KCB93; 21: *Micrococcus radiodurans* KCB21; 50: *Mcrococcus radiodurans* KCB50; 38: *Micrococcus luteus 3* KCB38; 140: *Micrococcus agilis* KCB140; 125: *Micrococcus agilis* KCB125.

Different bacterial isolates were assessed for antibiotic sensitivity tests (table 4). It was observed that 69.23% isolates were resistant against vancomycin and polymyxin B, 61.53% against tetracycline, 53.84% against neomycin and 38.46% against chloramphenicol and kanamycin. (figure 8). Result of two-way ANOVA reveals that both factors, namely antibiotics and bacterial species and their interaction produced statistically significant effect on the zone of inhibition.

Figure 8: Sensitivity of individual antibiotic on total bacteria from Kotumsar cave



ANOVA Summary: Two - way ANOVA

Factor: Antibiotic; df = 9; 389, f = 499.45, p ‹ 0.001***

Factor: Bacterial isolates; df = 12; 389, f = 938.46, p ‹ 0.001***

Factor: Interaction between antibiotic × bacterial isolates; df = 108; 389, f = 227.09, p ‹ 0.001***

Discussion

Our earlier reports have already revealed that *Stomatococcus mucilaginosus, Micrococcus luteus, Staphylococcus simulans, Staphylococcus epidermis* were isolated from soil sediment samples of Kotumsar cave are pathogenic. Semenov, 1990 was also isolated some human pathogens i.e. *Staphylococcus, Enterobacteria, Penicillium, Aspergillus, Gymenoascaceae* fungi from the Kap-Kutan and Tash-Yurak caves (Kugitangtow ridge, Turkmenistan, Russia). Jurado et al., 2010 also reported some pathogenic bacteria in cave ecosystem. The conventional pathogenic microorganisms found in the caves could be dangerous for human. PIB-win identified bacteria were further confirmed by conventional method that was serotyping as well as advanced method such as ARDRA. Both techniques support the PIB-win results. In serological test agglutination would be a useful quick screening test for sorting out *Micrococci sp.* from other bacterial isolates from Kotumsar cave. Antibody was produced against the surface antigen of the *Micrococcus luteus3*. Therefore only particulate antigen showed agglutination with antisera. Serological techniques measure similarities only at the surface of proteins and it is at the protein surface that the greatest number of antigenic sites per protein molecule. Nevertheless, serological techniques of this kind provide a rapid and convenient method for assessing structural similarities between homologous proteins, are useful in the classification of bacteria, and can also cast some light on possible phylogenetic relationships. Lind et al. 1996 reported serotyping as a useful tool in most epidemiological situations but sometimes lack sufficient discriminatory power. DNA fingerprinting can add valuable epidemiological information to that supplied by serotyping and can in some situations provide sufficient epidemiological information. Makrai *et al.,* 2005 reported the plasmid types and serotypes of 164 *Rhodococcus equi* strains obtained from submaxillary lymph nodes of swine from different piggeries in 28 villages and towns located throughout the Hungary. Turkyilmaz, 2005 studied the isolation of *Ornithobacterium rhinotracheale* from poultry in Turkey and serotyping of the isolates and field sera by agar gel precipitation (AGP) tests.

In the present study, ARDRA pattern of bacterial isolates was analyzed using universal primer and endonuclease enzymes *EcoRI* and *AluI*. The Dendogram generated on the basis ARDRA pattern using *EcoRI* showed that three isolates of *Micococcus radiodurans*, each from entrance zone, twilight zone and deep zone (guano soil) recorded exact similarity, likewise *Micrococcus agilis* KCB125 from foval soil and *Micrococcus agilis* KCB140 from red-laterite soil of deep zone exhibited 100% similarity. *Micrococcus luteus 4* and *Micrococcus luteus 3* were present in different clades. The result of *AluI* restriction enzyme exhibited similar result. The results presented here indicate that *EcoRI* and *AluI* restriction enzymes were successful in characterizing the isolated strains belonging to the different genera. Sarra *et al.,* (2004) reported the identification of the numerous species belonging to *Staphylococcus* genus by biochemical characterization and rapid methods as ARDRA and PCR was successfully employed. Lagace *et al.,* (2004) characterized the bacterial community through ARDRA. Results showed a wide variety of organisms with 22 different genera encountered. *Psuedomonas* and *Ralstonia*, of the γ- and β- *Proteobacteria,* respectively,were the most frequently encountered genera. Gram-positive bacteria were also observed, and *Staphylococcus, Plantibacter* and *Bacillus* were the most highly represented genera.

However antibiotic sensitivity test have also shown very interesting results. Most of the bacterial isolates were found to be sensitive against chloramphenicol, then tetracycline. Chloramphenicol is a broad spectrum antibiotic which is effective against a wide variety of gram positive and gram negative bacteria including most anaerobic organisms. Many workers have reported antibiotic sensitivity in bacterial isolates (Goodfellow & Orchard, 1974, Murthy et al. 2008 and Rajput et al., 2012b)

Table 2: ARDRA pattern with restriction enzymes *EcoRI*. Sizes were expressed in bp

S.N.	Zone	Culture code	Bacterial isolates	EcoRI digested 16S rDNA fragment (bp)
1.	Entrance zone	KCB14	*Micrococcus mucilaginosus*	5000, 4000, 3900, 3000, 2600, 2500
		KCB11	*Micrococcus luteus 4*	4500,3800, 3500, 2400, 2300, 2200, 2000
		KCB26	*Micrococcus mucilaginosus*	5000, 3800, 3000, 2600, 2500
		KCB21	*Micrococcus radiodurans*	4500, 3800, 3500, 2500, 2400, 2300, 2000
5.	Twilight zone	KCB38	*Micrococcus luteus 3*	3800, 3600, 3500, 3000, 2600, 2500
6.	Transient zone	KCB50	*Micrococcus radiodurans*	4500, 3800, 3500, 2500, 2400, 2300, 2000
7.	Deep zone (foval soil)	KCB129	*Staphylococcus simulans*	4200, 3800, 3600, 3500, 3100, 3000
		KCB134	*Staphylococcus varians*	4200, 4000, 3900, 3100, 3000
		KCB125	*Micrococcus agilis*	4200, 3500, 2800, 2700, 2500, 2400, 2300, 2000
10.	Deep zone (guano mix soil)	KCB109	*Streptococcus grpQ1*	4200, 3500, 2600,2500, 2400, 2200
11.	Deep zone (guano soil)	KCB93	*Micrococcus radiodurans*	4500, 3800, 3500, 2500, 2400, 2300, 2000
12.	Deep zone (alluvial soil)	KCB140	*Micrococcus agilis*	4200, 3500, 2800, 2700, 2500, 2400, 2300, 2000

Table 3: ARDRA pattern with restriction enzymes *AluI*. Sizes were expressed in bp

S.N.	Zone	Culture code	Bacterial isolates	*AluI* digested 16SrDNA fragment (bp)
1.	Entrance zone	KCB14	*Micrococcus mucilaginosus*	4900, 3100, 2900
		KCB11	*Micrococcus luteus 4*	6000, 3900, 3100, 3000, 2500
		KCB26	*Micrococcus mucilaginosus*	4900, 4800, 3100, 2900
		KCB21	*Micrococcus radiodurans*	7000, 2900, 2600, 2500, 2000
2.	Twight zone	KCB38	*Micrococcus luteus 3*	7000, 4300, 2500, 2400, 2200
3.	Transient zone	KCB50	*Micrococcus radiodurans*	7000, 2900, 2600, 2500, 2000
4.	Deep zone (foval soil)	KCB129	*Staphylococcus simulans*	6000, 4800, 4200, 4100, 3200,3100, 2500, 2000
		KCB134	*Staphylococcus varians*	4500, 4000, 3900, 3000, 2600, 2500
		KCB125	*Micrococcus agilis*	4200, 3300, 3200, 2500, 2400, 2300, 1800
5.	Deep zone (guano mix soil)	KCB109	*Streptococcus grpQ1*	6000, 5500, 5000, 3200, 3100, 2500, 2000
6.	Deep zone (guano soil)	KCB93	*Micrococcus radiodurans*	7000, 2900, 2600, 2500, 2000
7.	Deep zone (alluvial soil)	KCB140	*Micrococcus agilis*	4200, 3300, 3200, 2500, 2400, 2300, 1800

Table 4: Antibiotic sensitivity profile of bacterial isolates from soil of Kotumsar cave

S.N.	Zone	Culture Code	Name of the bacteria	P (mm)	V (mm)	E (mm)	Pb (mm)	G (mm)	Sr (mm)	T (mm)	K (mm)	C (mm)	N (mm)
1.	Entrance zone	KCB 21 KCB 11 KCB 26 KCB 14	*Micrococcus radiodurans* *M. luteus 4* *M. mucilaginosus* *M. mucilaginosus*	R^{7} R^{15} S^{19} R^{20}	R^{O} R^{10} S^{15} R^{O}	R^{O} S^{25} $S^{25.66}$ R^{12}	R^{8} R^{6} R^{O} R^{O}	R^{10} R^{11} R^{2} R^{11}	S^{17} S^{O} S^{15} R^{10}	R^{16} R^{10} S^{27} R^{11}	S^{20} S^{20} $S^{18.65}$ R^{12}	R^{12} R^{10} S^{21} S^{25}	R^{10} S^{17} R^{9} R^{12}
2.	Twilight zone	KCB 38 KCB 39	*M. luteus 3* *M. radiodurans*	S^{30} R^{16}	R^{2} R^{10}	R^{O} R^{0}	R^{4} R^{5}	R^{12} $S^{22.67}$	R^{o} R^{o}	$R^{5.3}$ S^{24}	R^{O} R^{O}	R^{10} S^{23}	R^{10} R^{11}
3.	Transient zone	KCB 50	*M. radiodurans*	S^{30}	S^{31}	S^{30}	S^{15}	S^{34}	R^{10}	S^{30}	S^{30}	S^{32}	S^{24}
4.	Deep zone (guano soil)	KCB 93	*M. radiodurans*	$R^{10.33}$	R^{10}	R^{O}	$R^{8.67}$	$S^{22.67}$	S^{22}	S^{23}	$S^{18.67}$	S^{22}	S^{18}
5.	Deep zone (guano mix soil)	KCB 109	*Streptococcus grpQ1*	I^{O}	S^{20}	S^{24}	S^{13}	S^{21}	R^{9}	R^{17}	S^{20}	R^{20}	S^{18}
6.	Deep zone (foval soil)	KCB 125 KCB 134 KCB 129	*M. agilis* *Staphylococcus varians* *Staphylococcus simulans*	S^{29} R^{17} S^{30}	R^{O} S^{15} R^{O}	R^{O} S^{26} S^{27}	I^{10} $R^{4.3}$ S^{14}	S^{16} S^{15} S^{15}	S^{15} $S^{16.67}$ $S^{16.33}$	R^{16} S^{20} R^{13}	S^{20} $R^{2.67}$ S^{22}	R^{12} S^{22} $S^{24.33}$	R^{10} R^{10} S^{18}
7.	Deep zone (alluvial soil)	KCB 140	*M. agilis*	S^{30}	R^{10}	R^{O}	R^{5}	R^{O}	R^{9}	R^{1}	R^{O}	S^{32}	S^{30}

Where: P=Penicillin G, V=Vancomycin, E=Erythromycin, P=Polymyxin B, G=Gentamycin, Sr=Streptomycin, T=Tetracyclin, K=Kanamycin, C=Chloramphenicol, N=Neomycin. S= Sensitive; R= Resistant; I= I ntermediate; Zone size diameters (in mm) in superscript; means having similar alphabates within the individual bacterial isolates as superscript, were not statistically significant from each other at $p < 0.05$ (based on Duncan's multiple-range test)

Conclusions

Conclusively, this piece of work supports our earlier findings (Rajput et al. 2012a and Rajput & Biswas 2012) significantly that due to high anthropogenic pressure, presently the Kotumsar cave became a harbor of high microbial communities. Among which, few may be beneficial, to keep the cave biologically alive whereas the others are highly pathogenic to the tourist. Thus it is recommended, that tourist visiting the cave and come in contacts with its walls and floor must wash their hands after caving. Though, a single or couple of visits inside the cave may not be so harmful for the visitors but frequent visit may be a health risk factor for the visitor.

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