International Journal of Advanced Biotechnology and Research ISSN 0976-2612,
Vol 2, Issue 1, 2011, pp 224-229 http://www.bipublication.com

CHARACTERIZATION, ANTIBIOTIC SENSITIVITY OF A THERMOSTABLE AMYLASE PRODUCING *HAEMOPHILUS HAEMOLYTICUS* ISOLATED FROM UNKESHWAR HOT SPRING AND PREDICTION OF ORIGIN USING ANTIBIOTIC TARGET SITE

[1]Bhagwan N. Rekadwad and [1]Anupama P. Pathak

[1]DST-FIST Sponsored School of Life Sciences,
Swami Ramanand Teerth Marathwada University, Nanded 431606, India.

ABSTRACT:

Unkeshwar hot spring is located in Nanded district of Maharashtra State, India. It is SE Deccan continental basaltic rock is terrestrial, perennial, having neutral pH,contains phosphate and sulphur. Water samples have been collected by composite sampling method. Haemphilus haemolyticus were isolated from hot spring at Unkeshwar. It is thermopile, haemolytic, non-pigmented, Gram negative rod, non motile, and positive for endospore staining. The growth temperature ranges from 45 to 80 ^{0}C with an optimum 67^{0}C. It also shows positive amylase, gelatinase, catalase, and urease activity. It is very sensitive to ciprofloxacin (5mcg/disc) and tetracycline (30mcg/disc). It is predicted that H. haemolyticus has the fungal origin.

Keywords: Vertical evolution, *H. haemolyticus*, hot spring, clustalW2, penicillin binding proteins.

[I] INTRODUCTION

Unkeshwar hot springs is the natural outlet of ground water discharge having temperature of water fall above the mean of annual air temperature of that region [1, 2]. It possesses sufficient nutrients that allow bacteria to survive which is called thermophiles [3, 4]. The thermophiles amongst first living things on earth, during evolution and thus been called the Universal Ancestor [5]. In this paper we have studied hot spring situated at Unkeshwar in Kinwat Tahsil is positioned on south east corner of Maharashtra State, India, 1 km south of **Penganga** River near Nanded-Nagpur highway and is connected by air at Nagpur, Maharashtra airport near Godavari rift zone [6, 7]. With its climatic conditions, attitude similarities and by its geographical position occupies major portion of South East Deccan Continental basalt of India. It is unique, terrestrial on Deccan basalt amongst presently active systems. It has small surface area in about 2.13 m in diameter. No detail geological description and published data are available. Geographically, Unkeshwar lying between [19^0 34' to 19^0 40'] N lat. & [78^0 22' to 78^0 34'] E long. from mean sea level. We have initiated microbiological analysis of Unkeshwar hot spring, which aims to study hot spring biodiversity.

In present study *In Vitro* antibiotic sensitivity of thermophilic *Haemophilus haemolyticus* has increased to antibiotics as amphoterecin B, bacitracin, polymyxin B, tetracycline and ciprofloxacin. Amphotercin B and polymyxin B both are antifungal drugs, having common ergosterol target site showed the bactericidal effect. From the result, the new domain of classification will be initiated by showing phylogenetic relationship with yeasts and fungi using bioinformatics approach.

[II] MATERIALS AND METHODS The entire chemicals used are of **Himedia** Laboratories Pvt. Ltd. Mumbai, India. Sampling is done by composite sampling method in monsoon 2009 in early hours of the day 09.00am to 11.00 hours of day and stored in ice carried out to laboratory, refrigerated at four to eight degree Celsius until use. Wide mouth [two centimeters] polypropylene [one liter] bottles were used for sampling. The media used for isolation is tryptone yeast extract medium composed of tryptone bacto 20g/L, yeast extract bacto 5g/L, sodium chloride 5g/L and agar-agar bacteriological 20g/L. The microorganisms were isolated using spread plate techniques at 67 ± 2 ^{0}C. Pure culture of isolated species is preserved in refrigerator. The organism is identified using morphological and biochemical techniques by following the criteria of Bergey's manual of systematic bacteriology [Table 1]. Tests of susceptibility towards different antibiotics were established by Kirby-Bauer disc diffusion method on Muller-Hinton agar and plates were incubated for 24 hours. After 24-48 hours incubation the zone of inhibition measured in millimeters from periphery of disc [Table 2] [8].

2.1 Phylogenetic analysis

Cell wall protein and ergosterol sequences freely available were downloaded from http://www.ebi.ac.uk/Tools/msa/clustalw2/ [16] and www.uniprot.org/ [17] websites. These proteins and their accession numbers are penicillin binding protein of *Haemophilus haemolyticus* [AK3CC7 and AK3CC8], ergosterol biosynthetic protein Erg 28 putative of *Aspergillus nidulans* [C8UZZ5], ergosterol biosynthetic protein 28 putative of *Candida dubliniensis* strain CD36 [B9WAF9] and squalene synthase of *Schizosaccharomyces prombe* [Fission yeast] [P36596]. After BLAST, phylogenetic trees were constructed, from the neighbour joining plot the relationship between two organisms is shown [Fig: 2.].

Fig: 1. *In Vitro* antibiotic sensitivity of *H. hemolyticus*

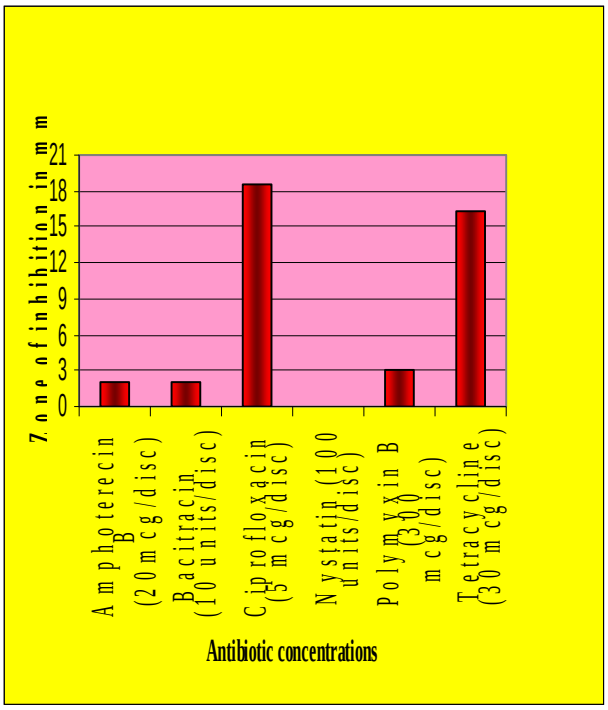


Fig: 2(a).

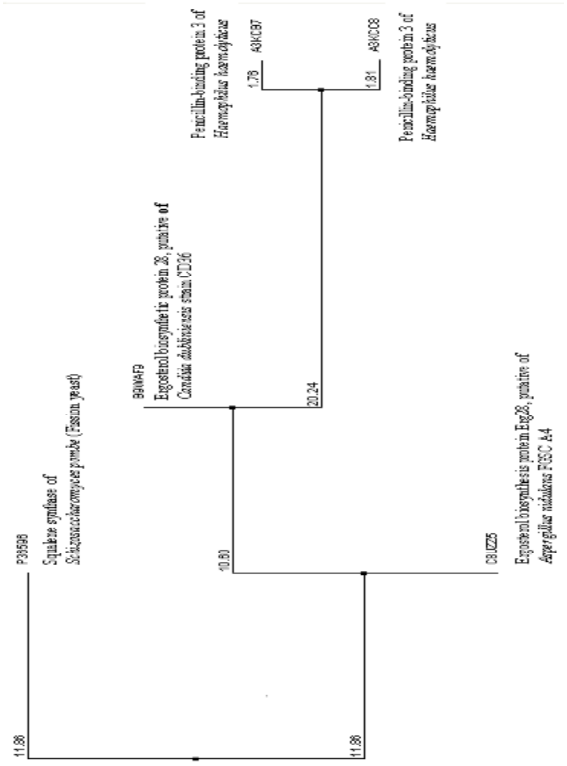


Fig: 2(b).

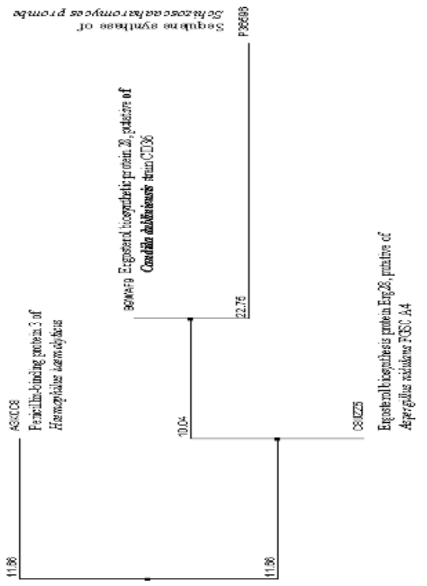
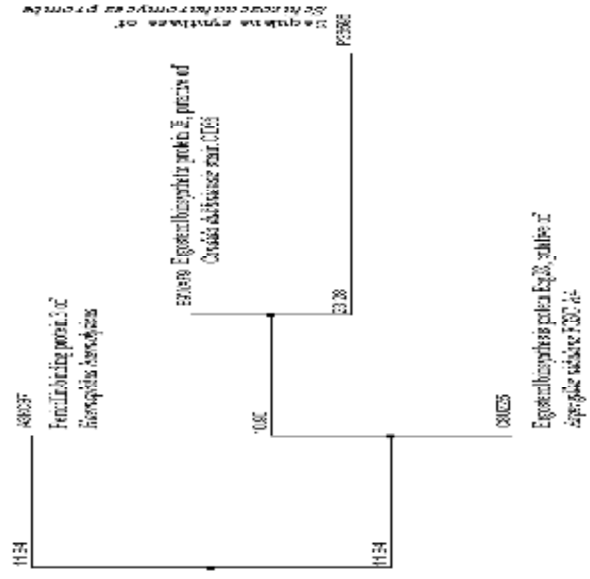


Fig:2(c).

Fig: 2. (a, b and c). Clustalw2 results: Neighbor joining (NJ) plot



[III] RESULTS

The *Haemophilus haemolyticus* isolated from Unkeshwar hot spring, were strictly aerobic haemolytic, non-motile, Gram's negative, long rod with optimum temperature 67^{o}C produces acid in medium (from glucose, cellobiose, and xylose), and exhibits strong amylase, gelatinase, urease, oxidase and catalase activity [8]. The results of an investigation of the phenotypic properties of these organisms [Table 1] allowed distinguishing them from other species that have similar phenotypic characters.

3.1 Antibiotic susceptibility

The *Haemophilus haemolyticus* susceptible to antibiotic and order is as amphoterecin B, bacitracin, polymyxin B, tetracycline and ciprofloxacin and no inhibition done by nystatin to concentrations of antibiotics [Table 2].

3.2 Mechanism of antibiotic susceptibility

Sensitivity test showed particular antibiotic used in the research acts with differing strengths and to different degrees on isolated strain. The ability to be sensitive or resistant depends on composition and mode of action of antibiotic. The strongest antibiotic analyzed in this paper was ciprofloxacin, a synthetic chemotherapeutic fluoroquinolone drug. It is a broad spectrum antibiotic that active against both Gram's positive and negative bacteria [9]. It displays high activity not only bacterial topo-isomerases and toxic to enzymes but also necessary to separate bacterial DNA there by inhibiting cell division [10, 11]. The mechanism is most important and potent in its action, which confirms results obtained in this paper. The second most potent antibiotic is also a broad spectrum polyketide antibiotic, the brand names such as sumycin, terramycin, tetracynin, which together are known as tetracycline antibiotics. Tetracycline works by binding the 30S ribosomal subunit and through an interaction with 16S rRNA [12]. Polymyxin B is a family of polypeptides with attached fatty acid. They are bactericidal for most gram negative bacterial infections. It acts by binding to cell membrane and alters the bacterial outer membrane permeability by binding to a negatively charged sure in the lipopolysaccharide layer, this result in destabilized outer membrane. Fatty acid portion dissolves in hydrophobic region of cytoplasmic membrane and disrupts membrane integrity. It will cause leakage of cellular

molecules and inhibition of bacterial cellular respiration. Bacitracin is a mixture of related cyclic polypeptide produced by organism of the licheniformis group of *B. subtilis*. It acts by interfering dephosphorylation of the C_{55} isoprenyl pyrophosphate [13].

Amphoterecin B is a polyne antifungal drug often used intravenously for systematic fungal infections, having sterols as primary target site. Amphoterecin B molecules can form pores in the fungal membrane. This impairment in membrane barrier function can have lethal effects [14, 15]. Results obtained make it that amphotercin B is inhibiting the *H. haemolyticus*, so we predict there are same similar in primary target molecules present in the cell wall having similar unique target site is rembles structure to sterols. These are nothing but penicillin binding proteins 3 and confirmed from alignment, BLAST, NJ plot. Hence amphotercin B molecules can form the pores in bacterial membrane and alter these proteins. This impairment in bacterial membrane leads to death of microorganism.

3.3 Phylogenetic analyses

Clustalw2 results (Fig:2.) meets topology in trees is significantly indicates very close relationship between *H. haemolyticus* and yeasts.

Sr. No.	Characteristics	Results	Sr. No.	Characteristics	Results
I	**Morphological characters**		**III**	**Production of**	
1	Arrangement	Single	**1**	Amylase	+
2	Density	Opaque	2	Catalase	+
3	Elevation	Raised	3	Gelatinase	+
4	Form	Circular	4	Hydrogen sulphide	-
5	Gram's nature	Negative	5	Protease	-
6	Growth in broth medium	Sediment	6	Urease	+
7	Growth on agar	+		**IMViC:**	
8	Margin	Entire	1	Citrate utilization	-
9	Motility	Non motile	2	Indole production	+
10	Optimum temperature	70 ^{O}C	3	Methyl red test	+
11	pH	7.0	4	Voges-Proskaeur test	-
12	Pigmentation	-	**IV**	**Amino acid utilization**	
13	Shape	Rod	1	DL-Phenylalanine deaminase	-
14	Size	Long	2	L-Arginine	-
II	**Utilization of**		3	L-Cysteine	-
1	Arabinose	-	4	L-Glycine	-
2	Cellobiose	+	**V**	Hemolysis	+
3	D-Biotin	-	**VI**	Growth on streptomyces agar	+
4	D-Sorbitol	-	**VII**	Gluconate oxidation test	-
5	Fructose	-	**VIII**	Standard Type Strain	NCTC 10659
6	Galactose	-			
7	Glucose	+			
8	Glycerol	-			
9	Inositol	-			
10	Lactose	-			
11	Maltose	-			
12	Mannitol	-			
13	Resorcinol	-			
14	Ribose	-			
15	Sucrose	-			
16	Xylose	+			

[18]

Table: 1. Morphological and biochemical characterization of *Haemophilus haemolyticus*

Sr. no.	Antibiotics concentrations	Zone of inhibition (in mm)
1	Amphoterecin B (20mcg/disc)	2.0
2	Bacitracin (10 units/disc)	2.0
3	Ciprofloxacin (5 mcg/disc)	18.5
4	Nystatin (100 units/disc)	0.0
5	Polymyxin B (300 mcg/disc)	3.0
6	Tetracycline (30 mcg/disc)	16.25

Table: 1. Morphological and biochemical characterization of *Haemophilus haemolyticus*

[IV] DISCUSSION AND CONCLUSION

We have shown that three deeply concluded result reflects the new insight for classification. From identification of culture it was confirmed that thermophilic hot spring origin H. *haemolyticus*. It is sensitive to antibiotics in order as amphoterecin B, bacitracin, polymyxin B, tetracycline and ciprofloxacin. During the vertical evolution, the organisms were separated. But these organisms are not able to change all the genes though physically/morphologically they assume different. Some macromolecules expressed by unchanged common ancestral gene were as it is and exhibit the expresses previous characteristics. The primary target site for each antibiotic is unique. The positive results are obtained from theoretical prediction and are practically proven. Depending on these results, the *H. Haemolyticus* may be help to classify again the clearer molecular similarities, between *H. haemolyticus* and yeasts, the most closely related species is *Candida dubliniensis* strain CD36, followed by *Aspergillus nidulans* FGSCA4, *Schizosaccharomyces prombe*. It is predicted that *H. haemolyticus* separated during vertical evolution.

FINANCIAL DISCLOSURE

Special thanks to registrar, SRTM University, Nanded, [M.S.] INDIA for providing all necessary facilities and UGC, New Delhi, INDIA for financial assistance in the form of RGNFs.

ACKNOWLEDGEMENT

We are thankful to School of life Sciences, SRTM University, Nanded for to carry out our work.

REFERENCES

[1] Sen S, Mohpatra S, Satpathy S, Gopal Rao TV, [2010]. Characterization of hot water spring source isolated clones of bacteria and their industrial applicability. *Int. J. Chem. Res.* 2:1-7.

[2] Thompson I, (2008).*Clostridium difficile*-associated disease: update and focus on non-antibiotic strategies, *Age and Ageing*. 37:14–18.

[3] Aneja KR, [2003]. Experiments in microbiology, plat pathology and biotechnology, 4th Ed. *New Age International.* pp51-134.

[4] Madigan MT, Martinko JM, Dunlap PV, Clark D, [2008]. Brock Biology of Microorganisms. 12th Ed., Pearson Education Inc Pearson Prentice Hall.

[5] Woese C, [1998]. The Universal Ancestor. *Proc. Natl. Acad. Sci.* 95:6854–6859.

[6] Jay JE, Widdoson M, [2008]. Stratigraphy, structure, and volcanology of the SE Deccan continental flood basalts province: Implementation for eruptive extent and volumes. *Geo. Soci.* 165:177-188.

[7] Sarolkar PB, [2005]. Geochemical character of hot spring of West Coast, Maharashtra State, India. *Proceedings World Geothermal Congress, Antalaya, Turkey.*

[8] Martel AY, Laurent G, Dansereau LA, Bergeron MG, [1989]. Isolation and biochemical characterization of *Haemophilus* species isolated simultaneously from the oropharyngeal and anogenital areas. *J. Clini. Microbiol.* 27: 1486-1489.

[9] Drlicak, Zhao XK, [1997]. DNA gyrase, topoisomerase IV and the 4-quinolones. *Microbiol. Mol. Bio. Rev.* 61: 377-392.
[10] Sissi C, Palumbo MC, [2003]. The quinolone family from antibacterial to anticancer agents *Curr. Med. Chem.* 3(6): 439-50.
[11] Robinson MJ, Martin BA, Gootz TD, Guirk PR, Osher N, [1992]. Effect of novel fluoroquinolones on the catalytic activities of eukaryotic topoisomearse II: Influence of C-8 fluorine group. *Antimicro. Agents. Chemo.* 36(4): 751-756.
[12] Alekshun MN, Levy SB (2007). Molecular mechanism of antibacterial multidrug resistance. Cell. 128:1037-1050.
[13] Stone J, Strominger JL, [1971]. Mechanism of Action of Bacitracin: Complexation with Metal Ion and C_{55}-Isoprenyl Pyrophosphate. *Proc. Natl. Acad. Sci.* U.S.A., 68:3223–3227.
[14] Baginst M, Czub J (2009). Amphoterecin B and its new derivatives-Mode of action. Curr. Drug Metabolism. 10:459-69.
[15] Laborin LR, Varges C, [2009]. Amphoterecin B: Side effects and toxicity. *Revista Iberoamericana de Mycologia*, 223-27.
[16] European Bioinformatics Institute [EBI], European Molecular biology laboratory [EMBL] http://www.ebi.ac.uk/Tools/msa/clustalw2/ Assessed on 2011-01-20
[17] Universal Protein Resource [2002] www.uniprot.org/ Assessed on 2011-01-20
[18] Mogens K, [2004]. Genus III. Haemophilus. In: *Bergey's Manual of Systematic Bacteriology*, 2nd Ed. part B, Springer New York. 2:883-904.