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UMH.48 (NCBI JN807465) the Fungus causing Rhinosporidiosis is sensitive to anti fungal polyene drug Amphotericin B

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UMH.48, JN807465, a fungus causing Rhinosporidiosis was isolated in pure culture from biopsies from patients with nasal Rhinosporidiosis. It was identified as a lower aquatic fungus by 18S rRNA gene sequencing which compared 100% similar to the sequences from fungal extract of the tissue, thus establishing the etiologic role of UMH.48 in Rhinosporidiosis. UMH.48 18S rRNA sequence showed significant similarity with Synchytridium minutum and very low varying percentages of similarity with Mycobacterium sps., Corynebactrium sps., and Actinomycetales. The organism was tested for susceptibility and sensitivity to antibiotics and antifungal drugs such as Norfloxacin, Dapsone, Rifampicin and Amphotericin B. UMH.48 was highly sensitive to Amphotericin B and Rifampicin. It was resistant to Dapsone at the concentrations tested.

Key Words: UMH.48, Rhinosporidium seeberi, antibiotics, Amphotericin B

Rhinosporidiosis is a chronic muco-cutaneous granulomatous infection characterised by large tumours or wart like lesions cauliflower like in appearance highly vascularised, hyperplasic and friable. The most common site is the nose, and conjunctiva (Chao and Loh, 2004; Grover, 1970; Karunaratne, 1964). Systemic dissemination is believed to be uncommon or rare. The only treatment is wide excision surgery. Recurrence is very high and there are hospital records showing patients who have undergone repeated surgery up to 49 times! (Protilla Aguilar et al., 1977)

The fungus had defied isolation since its first report by Guillermo Seeber in 1893 in patients with large nasal polyps, in Buenos Aires. Later Ashworth concluded it to be a fungus based on detailed analysis of the development stages of the causative agent in stained tissue sections. Ashworth named the organism *Rhinosporidium seeberi* in honour of Guillermo Seeber in 1903 (Grover, 1970).

All attempts to grow the etiologic agent had failed since then. There have been several reports on the cause of the disease and taxonomy of the etiologic agent of Rhinosporidiosis which have been much debated and have still remained highly controversial with widely divergent views, inconclusive and unscientific experimental data.

Brief review on aetiology of Rhinosporidiosis and taxonomy of the microbe:

Ahluwalia reported that the sporangia seen in Rhinosporidiosis tissues in sections were "wrongly believed to be so and they were only accumulation of metabolic waste end products of ingested

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dietary cellulosic tubers (tapioca) in the nasal mucosa and they advocated redesignation of the sporangia as "spherules". The conclusion was based on SEM analysis of tissues and a weak illogical presumption that the 5 patients studied hailed from the State of Kerala, India where tapioca is consumed as a staple diet by many! (Ahluwalia et al, 1997). Subsequently this view was withdrawn and it was claimed by the same group of authors that the causative agent of Rhinosporidiosis was a *Cyanobacterium, Microcystis sp.*, based on laser-scanning con focal microscopy, light and electron microscopy. Ahluwalia thus tried to confirm that Rhinosporidiosis was the first human disease shown to be caused by a *Cyanobacterium* (Ahluwalia et al, 1999).

Further, sequence alignments and phylogenetic analyses of 16S rRNA gene from the "Round bodies" from infected tissues were performed. They showed 99% similarity with 16S rDNA of plant chloroplasts. With this study she confirmed that a pigmented prokaryote like *Cyanobacteria* was the etiologic agent of the disease (Ahluwalia et al, 2010).

A group of researchers assigned the *R.seeberi* to a pathogenic clade DRIPS an acronym derived from *Dermocystidium, Rosette agent, Ichtyophonus* and *Psorospermium* (Ragan et al., 1998). *The addition of R.seeberi* to DRIPS forced the researchers to modify the nomenclature and replace DRIPS with a novel protracted taxonomical group "Mesomycetozoa" that stood between the fungi and animals (Herr et al, 1999).

Herr et al (1999) taking queue from the above also claimed that the causative agent was not a fungus but a protist belonging to a novel group of fish parasites. All patients with Rhinosporidiosis had a history of bathing in stagnant pools and polluted natural water bodies where cattle too were bathed. Possibly this observation and isolation of the protist both from the pool and fish could have been the basis to associate the infection with a fish protozoan, upheld and confirmed by others (Fredericks et al., 2000). Mendoza et al opined that the taxonomic relationship *of Lacazia loboi* and *Rhinosporidium seeberi*, had always been controversial. But with molecular approaches they found that *L.loboi* was more likely to be a dimorphic fungus phylogenetically closer to *P. brasiliensis* and the other dimorphic fungal pathogens (*A. dermatitidis, A.capsulatum and E. parva*). They concluded that *R. seeberi* was a protist in the new class *Mesomycetozoea*, taxonomically situated between animals and fungi.

To the above publication Ahluwalia responded stating that "the conclusion of Herr et al. (1999) that *R. seeberi* was related to the DRIP clade, based on 18SrDNA and mitochondria from human cells, was unwarranted. She argued that evidence drawn from superficial criteria such as spherical parasites, endospores, the in ability to culture, and the aquatic habitat has very little meaning. Inadequate knowledge about the various manifestations of the microbe, after acquisition of the pathogenic state, might also have led to the erroneous conclusions" (Ahluwalia et al, 2001).

The subject of causation of Rhinosporidiosis has been one of the hottest raging spots rife with open views, debates, arguments, statements and counter statements galore among major groups of scientists engaged in research on Rhinosporidiosis. To quote the major players themselves "We thank Dr.Ahluwalia for the opportunity to address her position on the prokaryotic nature of *Rhinosporidium seeberi* (Ahluwalia, 1999). We differ with the arguments presented in her letter regarding our phylogenetic analysis of *R.seeberi* and her assertion that this hydrophilic pathogen is a *cyanobacterium* and not a *Mesomycetozoan*. Our position is based on our studies (Herr

et al, 1999) and a report by Fredericks et al (2000) which confirmed our phylogenetic analysis (Mendoza et al, 2001)".

Amidst the chaotic milieu described above, *Rhinosporidium seeberi* described as "a yet un isolated unclassified organism till date" belonging to lower aquatic group of fungi the chytridiales, was successfully grown in a selective enriched culture media for the first time ever by Thankamani in 1992 and reported in 2005 (Thankamani V, 2005a, 2005b).

The source of the isolate was biopsies and nasal swabs from patients with clinically diagnosed and histopathologically confirmed nasal Rhinosporidiosis from Medical College Trivandrum, Kerala, India. Later the isolate was characterised in detail with respect to cultural characteristics, microscopic morphology, staining properties, cytochemistry by special stains, and induction of developmental stages in vitro in selective enriched media. Structures such as spore-like bodies, huge sporangia (250-300 μm dia.) filled with characteristic spores found in Rhinosporidiosis tissue sections, transformation of the single celled spores into a "sorus" delimited by multilayered walls made of transient beaded mycelia forms, various stages and forms of the isolate closely resembled the life cycle of Synchytridium endobioticum a lower aquatic fungus belonging to Chytridiales causing black wart disease in potatoes (Thankamani V, 2005b). The isolate believed to be *Rhinosporidium seeberi* was coded as UMH.48 and preserved on agar slopes at 4°C in the year 2000.

UMH.48 (*Rhinosporidium seeberi*) resembles members of Chytridiales, the lower aquatic fungi - surviving in the fully virulent form for decades outside the body

The culture on an agar slope, after 10 years at 4°C was tested for viability, microscopic and cultural characters, life cycle and developmental stages possessed by the organism in comparison with those in the first isolate (Thankamani, 2005b). Media contained natural carbohydrates (Thankamani & Lipin Dev, 2011). Over a period of 3-4 weeks at 20-25°C, exactly similar nucleated spores (3-5 μm in dia.), uni and bipolar flagellation, multilayered beaded mycelia encapsulating the young sporangia, pro-sorus, repeated division resulting in formation of sporangia (250-300 μm dia.) etc., could be demonstrated in UMH.48 colonies on culture media . The lesion in humans resembled the wart disease in potatoes caused by *S.endobioticum*, a member of Chytridiales (Plate 1a and 1b) All stages conformed to original H&E pictures (Plate 1c - e). The SEM pictures of the tissue also showed the typical sporangia and the unique spores present in the Rhinosporidiosis biopsy (Plate 1f-i) which were very much similar to those obtained in culture in the earlier work in 1990 (plates 1j-o) (Thankamani, 2005).

Molecular identification of UMH.48 by 18S rRNA gene sequencing and comparison with fungus extracted from the Rhinosporidiosis biopsy

The 18S rRNA gene sequencing of UMH.48 (1990) as well as the fungal extracts of biopsy from new (2009) cases of nasal Rhinosporidiosis were done (Chromous services, Chennai, India). Both the sequences were deposited in Genbank with accession numbers JN807465 (UMH.48) and JN807466 (UMH.48.T) respectively.

Sequences from UMH.48 and the fungus DNA extracted from fresh biopsies of nasal Rhinosporidiosis showed 100% identity. Based on BLAST analysis (NCBI) on *Mesomycetozoa* and *Synchytrium* along with the unique morphology and life cycle UMH.48 the organism was categorized as a Fungus. Chytridiales is a group of lower aquatic fungi containing members like *Synchytridium endobioticum* causing black warts in potatoes (Plate 1a).

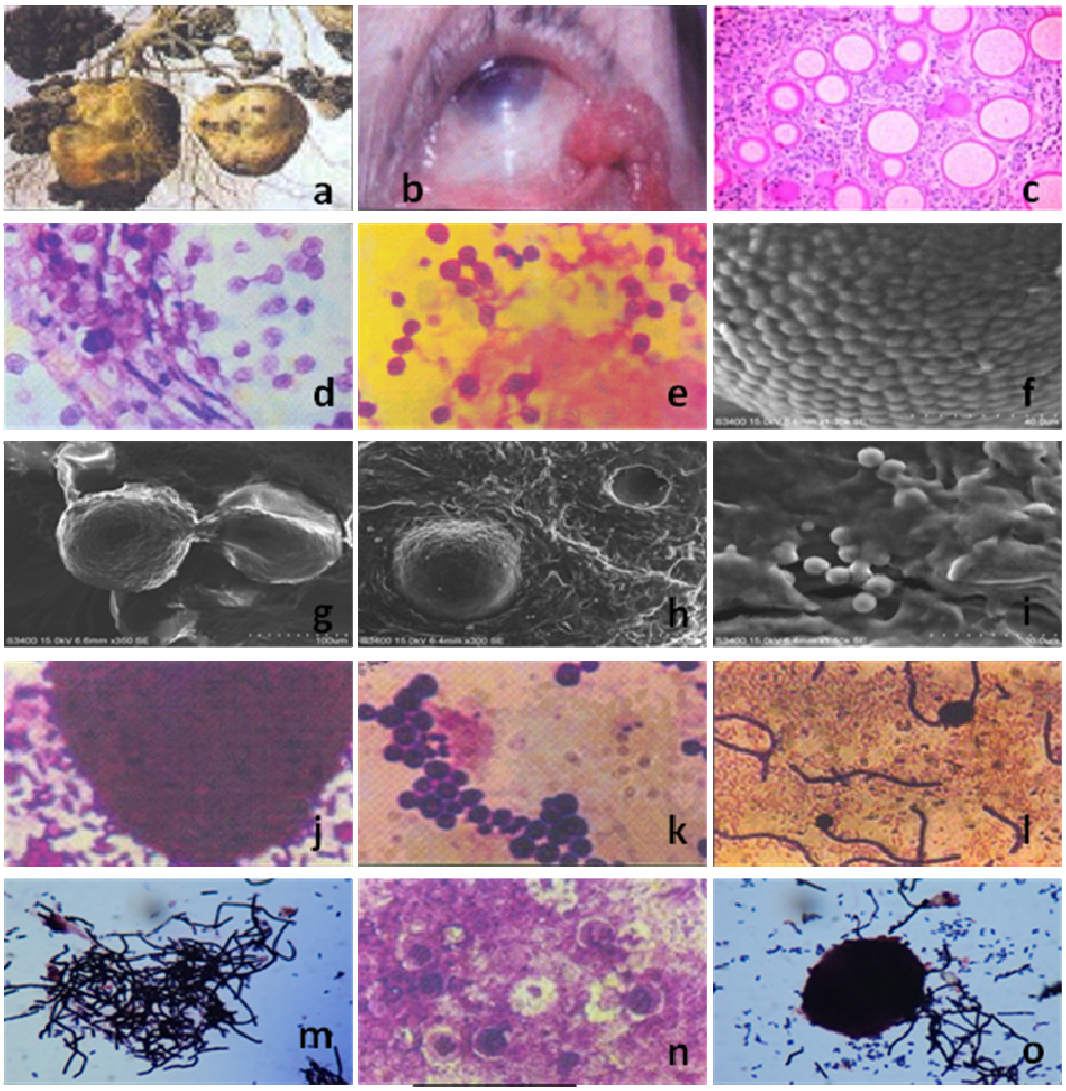

Plate 1 – (a) Black wart in potatoe caused by *S.endobioticum*; (b) – Rhinosporidiosis in the eye; (c-e) – H&E stained formalin fixed sections of Rhinosporidiosis biopsy showing spherical young sporagia , multilayered thick wall of mature sporangium, mature spores in characteristic groups; (f-i) – scanning electron microscopy of fresh rhinosporidiosis biopsy showing large fully mature sporangium filled with spores, sporangia in formation, sites of young developing sporangium appearing as cavities, mature spores liberated from sporangium in typical arrangement; (j-o) – UMH.48 (R.seeberi) in culture resembling structures seen in tissues c.e,f and g.

The absence of a perfect sexual phase or any typical asexual fungal spores in UMH.48, its rare, unique microscopic morphology, life cycle and remarkable resemblance with the morphology and stages in developmental cycle of *Synchytridium endobioticum*, a member of lower aquatic fungi led us to surmise (also confirmed by a personal communication with NCBI, Taxonomy expert) that the isolate UMH.48 was a Fungus (unknown) [Fungal sp. UMH.48 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene,

partial sequence, NCBI] (Thankamani and Lipin Dev., 2011). It is worth noting that UMH.48 had also shown similarity, though to a lesser score, with *Synchytrium minutum* of Order Chytridiales. This finding superseded the initial identification of UMH.48 as *Colletotrichum truncatum and Glomerulla sps* (Ascomycetes) based on 99% 18S rRNA gene sequence identity (fig 1). The outcome of the 18S r DNA sequence study proved that Rhinosporidiosis is caused by a lower aquatic fungus, not belonging to DRIPS Clade or Ascomycetes, but resembling the morphology and phases of life cycle described by Seeber in 1893 . It has been classified and identified as a fungus (unknown) (Thankamani & Lipin Dev, 2011).

Table 1: Phylogenetic analysis and comparison of UMH.48 with various biological groups

Description	Accession number	Max score	Total score	Query coverage	E value	Max identity
UMH.48 Vs Biopsy		1107	1107	99%	0.0	99%
Colletotrichum sp	JN807465.1	1122	1122	100%	0.0	100%
Colletotrichum sp	JN807466.1	1107	1107	99%	0.0	99%
Colletotrichum sp	JN717227.1	1103	1103	98%	0.0	100%
Colletotrichum sp	AF451899.1	1103	1103	100%	0.0	99%
Colletotrichum sp	AF451906.1	1103	1103	100%	0.0	99%
Colletotrichum sp	AY266372.1	1101	1101	99%	0.0	99%
Colletotrichum sp	AJ301945.1	1098	1098	100%	0.0	99%
Colletotrichum sp	AJ301944.1	1098	1098	100%	0.0	99%
Synchytrium sp	EF053263.1	118	423	46%	4e-30	100%
Synchytrium sp	EF053262.1	118	394	42%	4e-30	100%
Synchytrium sp	EF053261.1	118	342	39%	4e-30	100%
Mesomycetozoa sp	AF399715.2	165	305	43%	7e-42	91%
Mesomycetozoa sp	AY372365.1	165	310	44%	7e-42	93%
Mesomycetozoa sp	AY610945.1	161	301	44%	9e-41	91%
Mesomycetozoa sp	AY372367.1	161	301	44%	9e-41	91%
Mesomycetozoa sp	AY378083.1	161	301	44%	9e-41	91%
Mesomycetozoa sp	AY378082.1	161	301	44%	9e-41	91%
Mesomycetozoa sp	AY378081.1	161	301	44%	9e-41	91%
Mesomycetozoa sp	AY486143.1	161	301	42%	9e-41	91%
Uncultured Mycobacterium sp.	HQ212610.1	46.4	46.4	7%	0.001	84%
M.leprae	M20905.1	46.4	46.4	7%	0.001	84%
Uncultured M. sp.	HQ213412.1	37.4	37.4	7%	0.74	79%
Uncultured M.sp.	HQ212455.1	37.4	37.4	7%	0.74	79%
M.sp.	CP002329.1	35.6	35.6	4%	2.6	89%
M.marinum M,	CP000854.1	35.6	35.6	3%	2.6	92%
M. elephantis	GU142939.1	33.7	33.7	3%	9.0	95%
M. abscessus	CU458896.1	33.7	33.7	5%	9.0	85%
M. hassiacum	NR_026011.1	33.7	33.7	7%	9.0	77%
M.smegmatis	U07955.1	33.7	33.7	3%	9.0	95%
Corynebacterium glutamicum	EU520168.1	273	312	35%	3e-71	100%
Uncultured *Janibacter sp.*	HE798176.1	46.4	46.4	7%	0.007	84%
Uncultured Actinomycetales bacterium	HQ213721.1	46.4	46.4	7%	0.007	84%
Uncultured Actinomycetales bacterium	HQ213603.1	46.4	46.4	7%	0.007	84%
Uncultured Actinomycetales bacterium	HQ213316.1	46.4	46.4	7%	0.007	84%
Uncultured Actinomycetales bacterium	HQ213156.1	46.4	46.4	7%	0.007	84%
Uncultured Solirubrobacterales bacterium	HQ213130.1	46.4	46.4	7%	0.007	84%
Uncultured Actinomycetales bacterium	HQ212783.1	46.4	46.4	7%	0.007	84%

Taxonomy and biology of UMH.48

It was important to assign a definite taxonomic position and comprehend the biological properties of UMH.48, isolated by us repeatedly in pure culture from clinical samples of human Rhinosporidiosis cases and proved to be the etiologic agent based on the above experimental data (Thankamani & Lipin Dev 2011). This was vital for searching for a treatment with suitable antibiotics or drugs to replace the repeated surgery and its associated physical and psychological trauma of Rhinosporidiosis patients. With this view further BLAST analysis were done to find out the 18S rRNA gene sequence similarity with a few microbial classes and genera. The Phylogenetic analysis of UMH.48 with *Colletotrichum sp*,*Synchytrium sp*, *Mesomycetozoa sp* (Thankamani and Lipin, 2011), *Mycobacterium sp, Coryne bacterium sp* and Actinomycetales are shown (Table 1).

The results also assigning the causative role to Cyanobacteria, taxonomy under fish protozoan pathogen or the theory of accumulation of metabolic waste end products of ingested cellulosic tubers (tapioca) in the nasal mucosa and their re designation as "Spherules" etc (Ahluwalia, 1999; Mendoza, 2001) could be set aside. This also reiterated the significance of the findings of Guillermo Seeber and Ashworth as early as 1893-1900s in deducing the lower aquatic fungal aetiology of Rhinosporidiosis based on very meticulous studies and recordings of H &E stained tissues from the nasal lesions.

Our strong experimental data cited above have proved that UMH.48 is the causative agent of human Rhinosporidiosis and overlook all the controversies and widely divergent views on the aetiology of Rhinosporidiosis, and, taxonomy of the infectious agent. The identity of UMH.48 has been established to be a lower aquatic fungus with 18S rRNA gene sequence. Since the only treatment mode is wide excision surgery and recurrence rate being very high, a search for an alternative drug was attempted by testing the susceptibility of UMH.48 to antibiotics, anti fungal drug Amphotericin B and Dapsone.

MATERIALS & METHODS:

Microorganism: UMH.48 inoculated on nutrient agar slopes containing natural carbohydrates pH 7-7.4 was sub cultured on plates and a single colony was inoculated into 5 ml of liquid nutrient carbohydrate rich medium. After incubation for 4 hours at 25^{o}C it was used as the inoculum.

Antibiotics and drugs: Norfloxacin, Rifampicin, Dapsone and Amphotericin B IP [(i) Amphocare - (Medispec), (ii) Fungisome- Liposomal (Life care), (iii) Fungitericin (Life care)] were used. The lyophilised powders were dissolved in suitable volumes of sterile distilled water to prepare known concentrations for the tests.

Culture media: Sterile agar plates, slopes and nutrient broth were made up of peptone, meat extract, Sodium chloride, soluble cellulose, starch and casein.

Methods

Standard procedures for testing antibiotic sensitivity of microorganisms were employed (Molly Antony et al, 2012 a, b). For Kirby Bauer well-diffusion technique wells of 8 mm dia. were cut out, 10 µl of a 4 hour culture 10^7 c.f.u. per ml, was spread uniformly all over the surface using a sterile L- glass rod, allowed to dry for a few minutes followed by addition of Norfloxacin, Rifampicin, Dapsone (10 µg each) and Amphotericin B (i), (ii), (iii) at 1, 10 and 100 µg of each. The second method adopted was by incorporation of Amphotericin B in agar slopes (1, 10 and 100 µg /ml of medium) followed by inoculation of 10 µl of culture/tube. In tube dilution method, for estimation of MIC (minimum inhibitory concentration) 1, 10

and 100 µg each of Amphotericin B (i), (ii) and (iii) were added to 5 ml of culture medium. Each tube was inoculated with 100 µl of 4 hour culture of UMH.48. All the tubes were incubated for 2 hours at 25°C. 10 µl of each was then spread over agar plates and incubated for 24-72 hours. Zones of inhibition around the wells were measured in well diffusion plates. Presence or absence of growth on agar slopes and number of colonies on plates were recorded. The plate showing more than 95-98% inhibition was taken as the MIC.

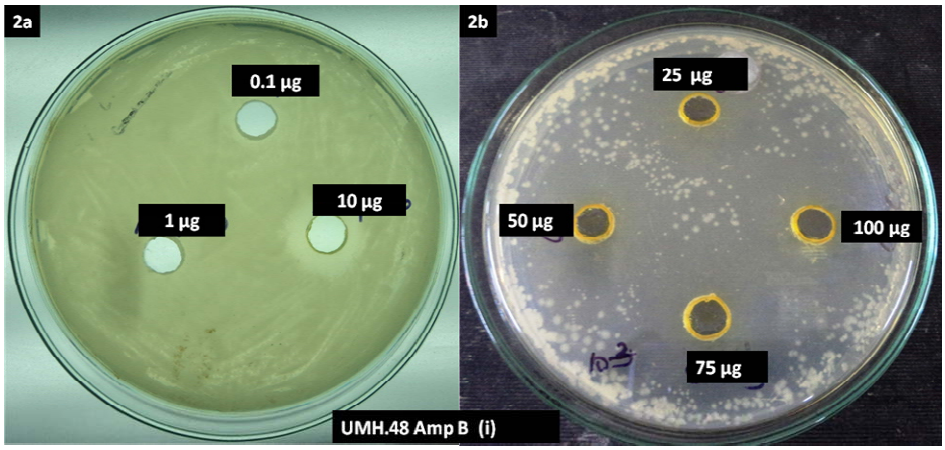

Plate 2a and 2b - Zones of inhibition of UMH.48 at various concentrations (1 to 100 µg/ well) of Amphotericin B (i)

Results and discussion:

The results of cidal activity of Norfloxacin, Rifampicin, Dapsone and Amphotericin B against UMH.48 are shown in plates 2-6. Amphotericin B (i) showed >8, 9, 18, 30, 40, 45 and <=50 mm dia. zones of inhibition at the concentrations 0.1 to 100 µg/well (Plate 2a and 2b). Plate 3 showed 25-30 mm zone of inhibition in Amphotericin B (i) and (iii) at 10 µg while Amp.B (ii) showed 10 mm (Plate 3). In well diffusion technique UMH.48 was found to be resistant to Dapsone and Norfloxacin but highly sensitive to Rifampicin with 22-25 mm dia. zone of inhibition (Plate 4). Dapsone at both 10 and 50 µg did not inhibit UMH.48 and Amp.B (ii) at 100 µg showed 25 -28 mm zone of inhibition (Plate 5). When Amphotericin B was incorporated into agar slopes, there was complete inhibition in 1-100 µg/ml (Plate 6). With respect to MIC, an inoculum with a cell density of 10^7/ml, there was more than 95-98 % killing of UMH.48 at a concentration of 100 µg/5 ml. Amphotericin B (Plate 7).

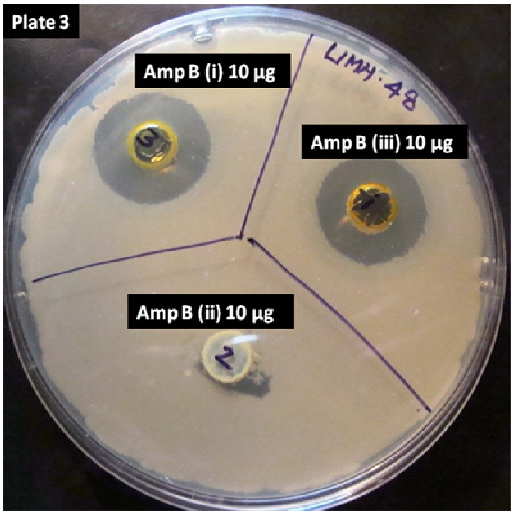

Plate 3 - Zones of inhibition of UMH.48 with Amphotericin B (i) and (iii) (25-30 mm) and (ii) – 10 mm at a concentration of 10 µg/well

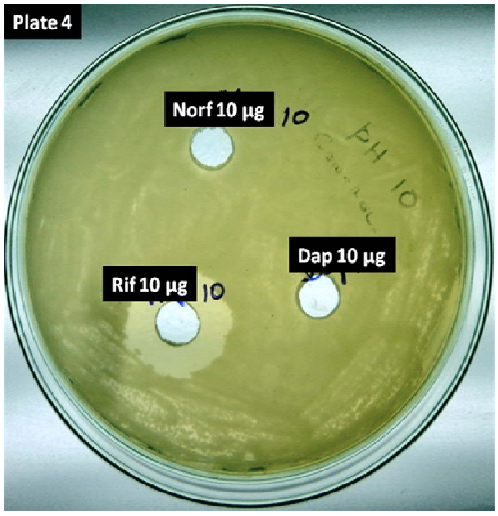

Plate 4 - Zones of inhibition of UMH.48 with Rifampicin, Dapsone and Norfloxacin at a concentration of 10µg/well each

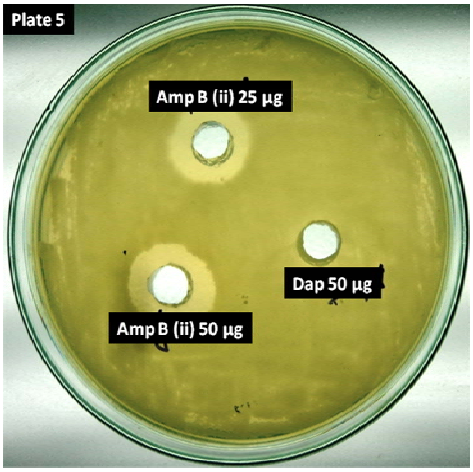

Plate 5- Zone of inhibition by Amphotericin B (ii) at 25 and 50 µg/well and no inhibition by Dapsone even at higher concentrations (50 µg)

This is possibly the pioneering report on culturing of the causative agent, identification as a lower fungus and its susceptibility to antimicrobial drugs in vitro.

Amphotericin B is a polyene antifungal drug used for confirmed serious systemic fungal infections and administered intravenously. It has ampholyte properties and is more active in vivo. The liposomal fraction improves the tolerability of the drug due to a different pharmacokinetics.

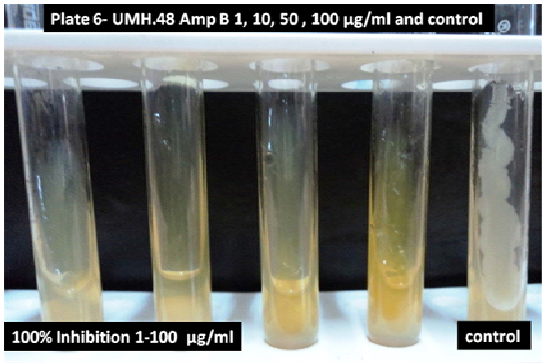

Plate 6 - Complete inhibition of UMH.48 in 1-100 µg/ml Amphotericin B(i) incorporated in agar slopes

UMH.48 has been proved to be a fungus in our work spanning over 20 years. The experimental data clearly demonstrated its sensitivity to Amphotericin B. The mechanism of action of Amp.B causing fungal cell death is understood to be by binding with the ergosterol in the fungal cell membranes, producing a transmembrane channel resulting in vital ion leakage.

Literature survey shows only one work carried out on effect of anti septics on Rhinosporidium so far (Arseculeratne et al; 2006). Arsecularetne has reported the use of MTT assay (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide) to identify the viability of rhinosporidial endospores treated with hydrogen peroxide, glutaraldehyde, chloroxylenol, chlorhexidine, cetrimide, thimerosal, 70% ethanol, iodine in 70% ethanol, 10% formalin, povidone-iodine, sodium azide and silver nitrate. They reported that the reagents caused metabolic inactivation of endospores at 3, 24 or 36 hours after exposure and concluded that anti-Rhinosporidial antiseptics such as

povidone-iodine in nasal packs may be used for nasal and naso-pharyngeal surgery, chlorhexidine and cetrimide-chlorhexidine on the skin, while povidone-iodine and silver nitrate could be applied in ocular Rhinosporidiosis (Arseculeratne et al., 2006).

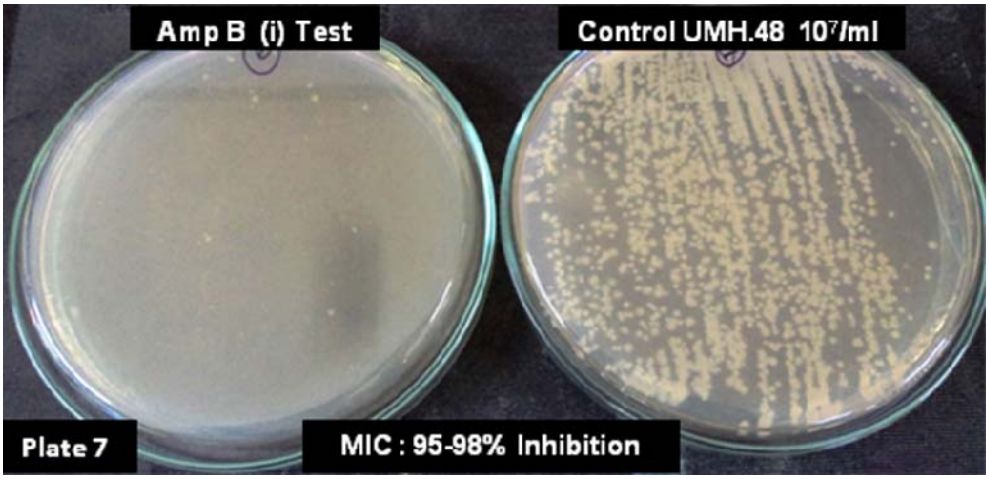

Plate 7- MIC - 95-98 % killing of UMH.48 (10^7 c.f.u./ml) at a concentration of Amphotericin B (i), 100 µg/5 ml medium.

Conclusion

A review of our publications on Rhinosporidiosis culminating in the isolation of *R.seeberi* coded UMH.48 in vitro in laboratory media, demonstration of its growth remarkable and unique developmental stages, identification as a fungus and establishment of its sensitivity to antifungal drugs by standard protocols once again proves beyond any doubt the fact that UMH.48 has an aetiological role in human Rhinosporidiosis and its taxonomy as a member of the lower aquatic fungi. The experimental evidence proving the viability of UMH.48 for over two decades in a fully virulent form also points to its phylogenetic proximity to members of Chytridiales. We have also demonstrated unequivocally the high degree of cidal activity of Amphotericin B against UMH.48 (*Rhino-sporidium seeberi*) isolated repeatedly from several Rhinosporidiosis biopsies. This also helps to conclude that the isolate is a fungus.

This break through discovery is sure to usher in a new era in the therapy of Rhinosporidiosis with antifungal drugs either alone at the initial stages or in combination following wide excision surgery especially in the disseminated Rhinosporidiosis cases. This is sure to alleviate the physical, social and psychological trauma of hundreds of patients subjecting themselves to repeat multiple surgery with no alternative or recluse in view what so ever till date.

Dedication:

This research work is dedicated to my parents, sister and brother in law who breathed me back to life during the most torturous times in my academic life at University of Kerala.

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