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Rhinosporidium seeberi proven as a fungus for the first time after a century since its discovery

Thankamani V* and Lipin Dev M.S.

Department of Biotechnology, University of Kerala, Kariavattom, Trivandrum 695 581, India
Corresponding author Email: dr.thankamani@gmail.com

The 18S rRNA gene sequencing of a pure microorganism isolated in pure culture from human rhinosporidiosis cases coded UMH.48 and preserved at 4^{o}C, and, the fungal extracts of biopsy from new cases of nasal rhinosporidiosis were done. Both the sequences were compared for the presence any identical regions by BLAST tool. Astonishingly both the sequences showed 100% identity with each other. The sequences were further compared with the sequences present in NCBI database, followed by sequences of specific organisms like *Mesomycetozoa sp* and S*ynchytrium sp*. Based on the morphological features, life cycle and BLAST analysis the organism UMH.48 was categorized as a Fungus. The sequences of UMH.48 and sequences from the fungus extracts from new tissue biopsies were deposited in Genbank with accession numbers JN807465 and JN807466 respectively. This paper reports the identity of 18S rRNA sequences between the pure, preserved, isolate with those obtained from biopsies of nasal rhinosporidiosis obtained from totally new cases. Our isolate has been tentatively identified as a lower aquatic fungus with 100% alignment with *Colletotrichum truncatum* and *Glomerulla sps* and lesser score similarity with *Synchytrium minutum*. Yet the absence of a perfect sexual phase or any asexual fungal spores, very rare microscopic morphology, life cycle and remarkable resemblance with members of lower aquatic fungi led us to surmise (also through personal communication with NCBI, Taxonomy expert) that the isolate is a Fungus (unknown) and not an *Ascomycete*.

Rhinosporidiosis is a chronic mucocutaneous granulomatous infection characterized by large wart like tumors highly vascularised, pedunculate, and friable, bleeding to touch and suggested by Ashworth to be caused by a lower aquatic fungus Rhinosporidium seeberi. Predominant site of infection is the nose. The aetiologic agent has challenged medical microbiologists ever since its discovery by Guillermo Seeber in 1893 in histo-pathological sections of biopsy from a patient with nasal polyp in Buenos Aires. Majority of the cases have been recorded in India and Sri Lanka (Chao and Loh, 2004). India is followed by South America, Brazil, Argentina, Mexico and a few reports are from Columbia, Venezuela, United States, Uganda, Madagascar, Ghana, Iran, Russia, Europe and South East Asia (Grover, 1970; Karunarathne, 1964; Allen and Dave, 1936; Protilla Aguilar et al, 1977; Brygoo et al, 1959). Rhinosporidiosis of other sites eye, face, skin, genitals and osteolytic lesions are rare (Christian and Kovi, 1966; Karpova, 1964; Owor and Wamukola, 1978; Sukumaran and Zachariah, 1975; Suseela and Subramaniyam, 1976; Chatterjee and Khatua, 1977; Samaddar and sen, 1990; Aravindan et al, 1989; Patnaik and Vasal,

1994; Mahantha et al, 1992; Luca Morelli et al, 2006; Kishan Prasad et al, 2011). It had evaded culture in the laboratory for more than a century. Attempts all over the world to isolate the incriminated agent and growth in artificial media, cell lines and experimental animals had failed (Luca Morelli et al, 2006; Kishan Prasad et al, 2011, Thankamani and Lipin, 2011).

The first ever successful attempt to grow the organism believed to be Rhinosporidium seeberi was carried out in 1992 by Thankamani (2005). It was isolated from biopsies from clinically diagnosed and histopathologically confirmed nasal rhinosporidiosis at Medical College Thiruvananthapuram, Kerala, India and coded as UMH.48. Later a detailed description was given about UMH.48, its cultural characteristics, microscopic morphology, staining properties, cytochemistry by special stains, growth characteristics, and various stages of development with illustrations to correlate and prove absolute resemblance of the isolate to structures seen in Rhino-sporidiosis tissues (Thankamani, 2005). The morphology of small spore-like bodies, sporangia, germination, formation of a sorus, transient beaded mycelial structures forming multilayered walls around sporangia, repeated multiplication of the spores within, formation of large swollen bodies filled with spores, stages of germination of spore like structures etc were clearly shown (Thankamani, 2005). The 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, 2005; Thankamani and Lipin, 2011).

Materials and method

Organism

The original isolate UMH.48 preserved on agar slopes for 2 years was labelled as sample one. The second sample was biopsy from fresh new histopathologically confirmed nasal rhinosporidiosis cases.

Isolation of DNA

The extraction of Genomic DNA from UMH.48 and the tissue samples were done using the Fungal Genomic DNA Isolation Kit (RKT13). The isolated DNA was from both the samples by method was used for the PCR (Neethu et al, 2012).

PCR Amplification for 18S rRNA gene

A cocktail of PCR reaction mixture was prepared with 1 µl (100 ng) of DNA, 400 ng each of forward (ITS5: GGA AGT AAA AGT CGT AAC AAG G) and reverse (ITS4: TCC TCC GCT TAT TGA TAT GC) primers, 4 µl of dNTPs(2.5 mM each), 10 µl of 10X Taq DNA Polymerase Assay Buffer, 1 µl of Taq DNA Polymerase Enzyme (3U/ µl) and the reaction mixture was made up to 100µl with milli Q Water. The initial denaturation was done at 94^0C/ 5 min, denauration at 94^0C/30 sec, annealing at 55^0C/30 sec, extension at 72^0C/3min and final extension was done at 72 ^{0}C/15 min. Mgcl 1.5mM was taken for the final concentration. The reaction was set for 35 cycles. The PCR amplified product was used for gene sequencing (Thankamani and Lipin, 2011; Neethu et al, 2012).

18S rRNA gene sequencing

10 µl Sequencing reaction was set with big dye Terminator (Ready Reaction Mix) 4µl, Template (100ng/ul) 1µl, Primer (10pmol/λ) 2µl and Milli Q Water 3µl. The PCR product with 18SRNA gene was sequence d using ABI 3500 Genetic Analyzer containing POP_7 polymer and 50 cm capillary array (Thankamani and Lipin, 2011; Neethu et al, 2012)

Phylogenetic analysis

A distance matrix was generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions was used, alignment inserts were ignored and the minimum comparable position was 200.

The tree was created using Weighbor with alphabet size 4 and length size 1000. The comparison was made between UMH.48 and the fungus present in the tissue homogenate of Rhinosporidiosis confirmed nasal biopsy. Comparisons were made between UMH.48 and the whole sequences in NCBI database and further a species specific comparison with *Synchytrium sp* and *Mesomycetozoa sp* were done. (Thankamani and Lipin, 2011; Neethu et al, 2012).

Results and discussions

The most remarkable and exciting finding was that the 18S rRNA gene sequencing of the fungal DNA extracted from tissue homogenate of fresh biopsy was 100% identical with UMH.48 (Table 1). This fact viz. 99% identity between our isolate UMH.48 and the fungal DNA from Rhinosporidiosis biopsy with respect to 18S rRNA gene sequence categorically reaffirms that UMH.48 is the aetiology of Human Rhinosporidiosis. The phylogenetic analysis marks an end to the century old debate on the taxonomy of the organism causing Rhinosporidiosis. UMH.48 appeared to be a dimorphic fungus with its morphology and lifecycle resembling *Synchytrium* with spores viable even after 10 years of refrigeration and it appears to follow all the features of *Rhinosporidium seeberi* as described by Seeber and Ashworth. The 18S rRNA gene sequence of UMH.48 and the fungal sequence from tissue biopsy showed 99% identity with *Colletotrichum truncatum* (Table 2). Although in recent years, the well – known plant pathogens of the *Colletotrichum* genus were increasingly reported to cause ophthalmic infections humans, a typical mucosal membrane infection. But only a few are known to be pathogenic for humans, among the 66 species in the *Colletotrichum* genus (Shivaprakash et al, 2011). But the morphology and life cycle of UMH.48 was found to be in total contradiction to that *of Colletotrichum truncatum*, which is a well studied ascomycete that produces ascons and sexual bodies, that was absent in UMH.48 (Thankamani, 2005; Thankamani and Lipin, 2011). Astonishingly, UMH.48 also showed identity with that of *Synchytrium purarie* (table 3) and the mesomycetozoa sp (Table 4) although with less query coverage. The present study states that the causative agent of Rhinosporidiosis is an unknown fungus and needs to be categorized under a new taxonomic classification.

Table 1: Sequence comparison between UMH.48 (JN807465) and causative fungus (JN807466) present in Rhinosporidiosis confirmed nasal tissue biopsies.

Description	Max score	Total score	Query coverage	E value	Max identity
UMH.48 Vs Biopsy	1107	1107	99%	0.0	99%

Table 2: Sequence comparison of UMH.48 with the NCBI database showed maximum identity with *Colletotrichum sp* and *Glomarulla sp*

Accession number	Max score	Total score	Query coverage	E value	Max identity
JN807465.1	1122	1122	100%	0.0	100%
JN807466.1	1107	1107	99%	0.0	99%
JN717227.1	1103	1103	98%	0.0	100%
AF451899.1	1103	1103	100%	0.0	99%
AF451906.1	1103	1103	100%	0.0	99%
AY266372.1	1101	1101	99%	0.0	99%
AJ301945.1	1098	1098	100%	0.0	99%
AJ301944.1	1098	1098	100%	0.0	99%

Table 3: Sequence comparison of UMH.48 and *Synchytrium sp.*

Accession number	Max score	Total score	Query coverage	E value	Max identity
EF053263.1	118	423	46%	4e-30	100%
EF053262.1	118	394	42%	4e-30	100%
EF053261.1	118	342	39%	4e-30	100%

Table 4: Sequence comparison of UMH.48 and *Mesomycetozoa sp.*

Accession number	Max score	Total score	Query coverage	E value	Max identity
AF399715.2	165	305	43%	7e-42	91%
AY372365.1	165	310	44%	7e-42	93%
AY610945.1	161	301	44%	9e-41	91%
AY372367.1	161	301	44%	9e-41	91%
AY378083.1	161	301	44%	9e-41	91%
AY378082.1	161	301	44%	9e-41	91%
AY378081.1	161	301	44%	9e-41	91%
AY486143.1	161	301	42%	9e-41	91%

The aetiologic agent of rhinosporidiosis has been a much debated subject with myriads of views and controversies due to lack of concrete experimental data. The researchers engaged in this area have put forth their own strong opinions though scientific basis are tragically lacking for the claims (Mendoza et al, 2001).

When Seeber first described rhinosporidiosis in 1900, he believed that its causative agent was a protist close to the *coccidia* (Seeber, 1900). He did not foresee, however, that R. seeberi, often considered to be a fungus, would remain a taxonomic mystery for the next 100 years. A similar situation occurred with the pathogen L. loboi. When Jorge Lobo described this pathogen in 1931, he believed it to be a fungus very similar to the Latin American dimorphic fungal pathogen *Paracoccidioides brasiliensis* (Lobo, 1930). *Rhinosporidium seeberi* is not a fungus!! Surprisingly, the first result of these studies was to find that R. seeberi was not a fungus but a protist belonging to a novel group of fish parasites (Herr et al, 1999). Shortly thereafter its protistan nature was also confirmed by Fredericks *et al*. (Fredericks et al, 2000). Originally, this group of pathogens was identified by Ragan *et al*. as the DRIP clade, an acronym derived from *Dermocystidum*, Rosette agent, *Ichthyophonus* and *Psorospermium* (Ragan et al, 1996). With the addition of *R. seeberi* to the group, however, the DRIP acronym was no longer appropriate. Herr *et al*. replaced it with the term *Mesomycetozoa* (between fungi and animals) (Herr et al, 1999). The other surprise was the finding that *R.Seeberi*'s closest relatives were *Dermocystidium sp*. This finding was reassuring since these microorganisms like R. seeberi produce endosporulating cells in their infected hosts, have not been cultured and their morphological similarities had been noted earlier by other investigators (Ahluwalia et al, 2001; Mendoza et al, 2001, 2002).

Conclusion

The present study is the first report on the taxonomical confirmation that the *Rhinosporidium seeberi* is a fungus with details unknown. The 18S rRNA gene sequences of both UMH.48 and the fungal sequence from Rhinosporidiosis biopsies were deposited in Genbank with accession numbers JN807465 and JN807466 respectively. Meticulous systematic approach to Rhinosporidiosis and its aetiology and recorded observations on the

biological property of the microorganism in the tissue sections by Sir Guillermo Seeber and Ashworth more than a century ago, when sophisticated instruments or techniques were totally lacking, could unambiguously describe the disease in minute details. The clinical presentation, major sites of infection, predilection of the pathogen for special niches, histopathology, treatment and epidemiology were described by them. The outcome of the present 18S r DNA sequence study proves 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).

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