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Culture of the organism that causes rhinosporidiosis

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Abstract

The causative agent of rhinosporidiosis is a microscopic round body in polypoidal masses that is believed to be the sporangium of a fungus *Rhinosporidium seeberi*. But fungal aetiology can not be proved with certainty. Attempts to culture the fungus on various media have also been unsuccessful. In a recent study pond water samples, from water in which patients had been bathing, were analysed and a unicellular prokaryotic cyanobacterium *Microcystis aeruginosa* was isolated. The same cyanobacterium was demonstrated in clinical samples from patients with rhinosporidiosis. Consequent to these findings, attempts were made to culture the causative organism of rhinosporidiosis under conditions that support growth of cyanobacteria.

This study describes a simple method for laboratory culture of this organism. Observations based on laser-scanning confocal microscopy, light and electron microscopy confirm that a cyanobacterium, *Microcystis* sp. is the causative agent of the disease. Rhinosporidiosis is the first human disease to be shown to be caused by a cyanobacterium. The findings have opened the way for development of therapy.

Key words: Rhinosporidiosis; Cyanobacteria; Culture

Introduction

A distinctive feature of the polypoidal masses of rhinosporidiosis is the presence of microscopic round bodies filled with spherules, that are considered by many as sporangia and spores of a fungus *Rhinosporidium seeberi* (Ashworth, 1923; Gaines *et al.*, 1996). The life history and taxonomic position of this fungus have remained unknown for many decades (Satyanarayana, 1960; Thianprasit and Thagerngpol, 1989). Since most patients have a history of bathing in natural pond and recreational waters (Vukovic *et al.*, 1995), the author collected pond water samples where patients were bathing in endemic states in India, for analysis. A prokaryotic cyanobacterium *Microcystis aeruginosa* could be isolated from all the water samples, and the same organism was demonstrated in human patients with rhinosporidiosis (Ahluwalia *et al.*, 1997). The transmitting agent of the disease seems to be a small cell stage of *Microcystis* referred to as a Nanocyte, that floats in apparently clean waters of ponds and lakes (Ahluwalia *et al.*, 1997). About six to eight nanocytes are produced from a single, much larger cell of *Microcystis* (Figure 1). Previous attempts to culture the causative organism of rhinosporidiosis had been unsuccessful (Kwon-Chung and Bennett, 1992). Two reports however, claim success in culture on agar and glucose (Krishnamoorthy *et al.*, 1989), and on a monolayer of human rectal tumour cells (Levy *et al.*, 1986). In *Bailey and Scott's Diagnostic Microbiology* (Baron *et al.*, 1994), rhinosporidiosis has been included in the chapter 'New controversial, difficult to cultivate, or non-cultivable etiological agents of disease'. Owing to lack of knowledge about a suitable method for culture and the disputed identity of the aetiological agent, effective therapy could not be developed.

This communication describes a simple method for laboratory culture of the organism causing rhinos-

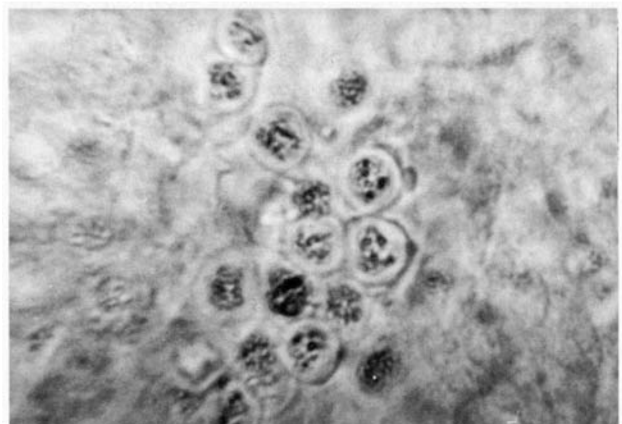

FIG. 1
Organisms lying on epithelial cells of human patient (H & E; ×1200)

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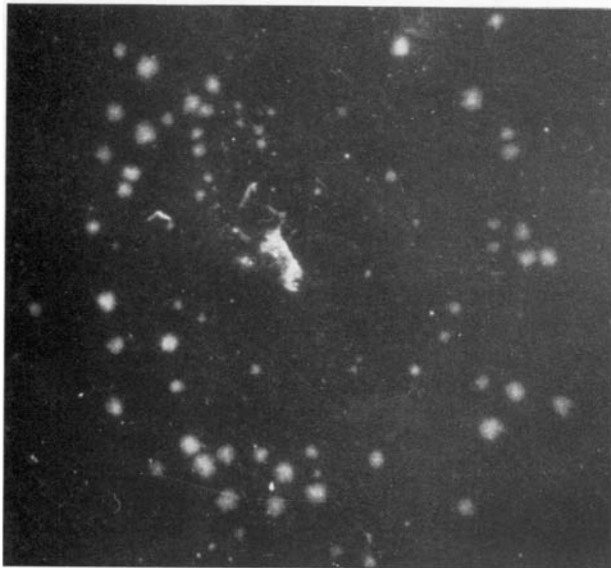

FIG. 2(a)
Colonies formed in petri dish culture by inoculum from human clinical sample.

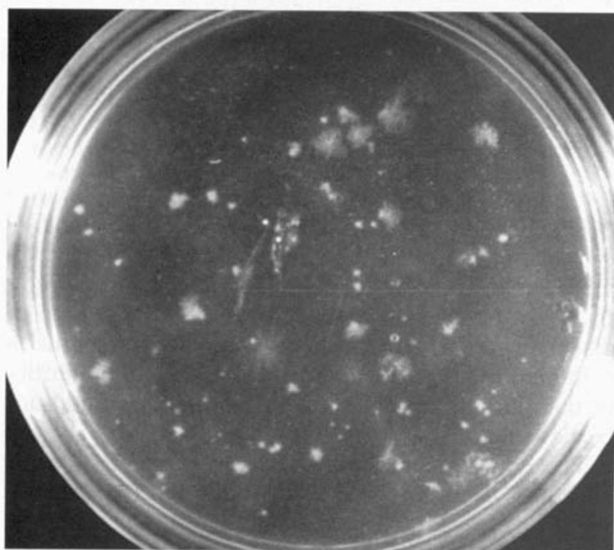

FIG. 2(b)
Colonies in petri dish culture produced by *Microcystis* from pond water.

poridiosis. Some of the observations *in vitro* confirm that the cyanobacterium isolated from pond water samples is the causative agent of rhinosporidiosis.

Materials and methods

Patients samples Surgically excised samples of nasal polypoidal masses from six patients of rhinosporidiosis were collected from the ENT department of this institute, and four samples were sent from Raipur. The processing for light and electron microscopy was done as described earlier (Ahluwalia and Bahadur, 1990; Ahluwalia, 1992). Laser scanning confocal imaging was carried out on the MRC–1024 BIO RAD Confocal imaging system in the Anatomy department of this institute.

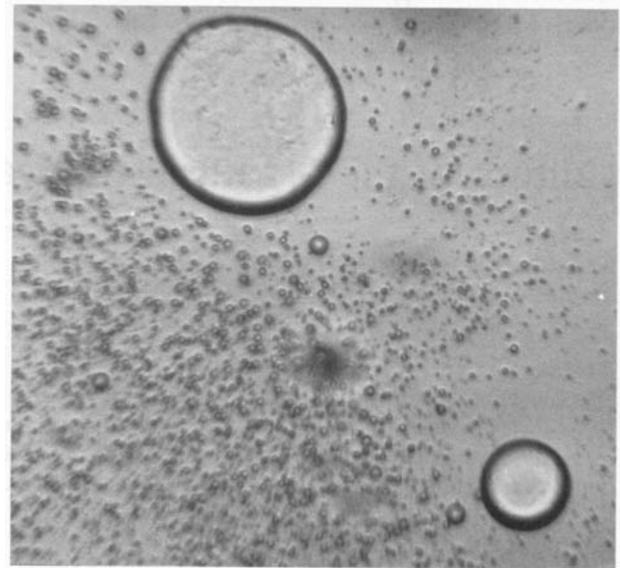

FIG. 3
Proliferation of nanocytes from clinical sample in slide culture; two stages of developing round bodies are visible (Unstained; ×1200).

Petri dish cultures Culture media BG–11 used traditionally for cyanobacteria (Rippka *et al.*, 1979; Castenholz, 1988) was prepared, autoclaved, and mixed with 0.2 per cent agar that had been autoclaved separately as suggested in the published method, and layered in sterile petri dishes. Organisms in clinical samples were separated from extraneous material by treatment with lysozyme and inoculated aseptically onto petri dishes. Cells of *Microcystis* isolated from pond water were examined under the phase contrast microscope to ensure absence of contaminating bacteria, and subsequently inoculated on media in a petri dish. Petri dishes were kept at 37°C in an incubator fitted with a Philips 18W, 240V, 50 Hz bulb as source of white fluorescent

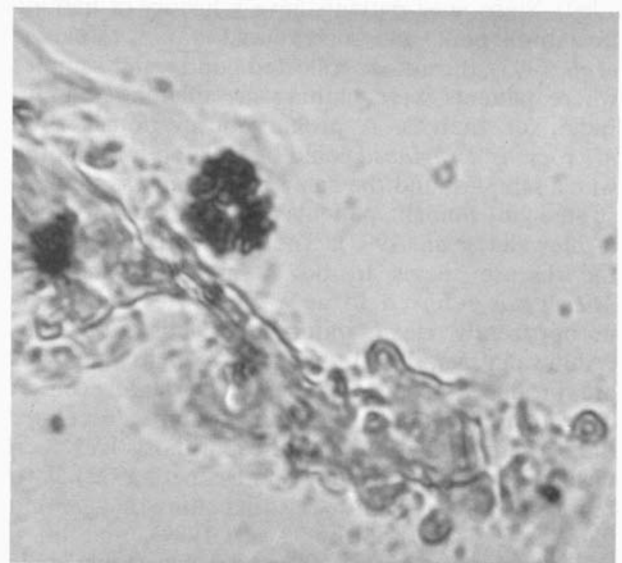

FIG. 4
Tubular structures produced by dividing nanocytes from clinical sample; a macrocolony is present. (Unstained, slide culture; ×1200)

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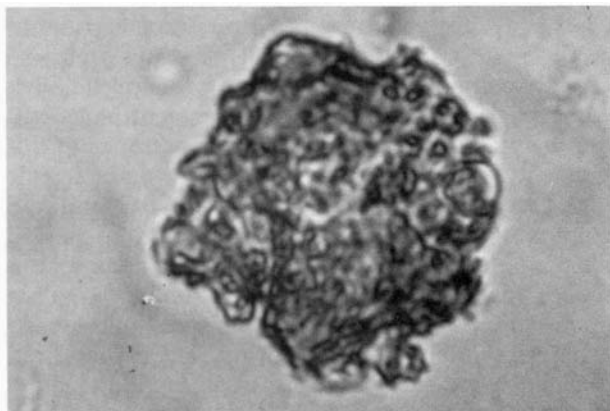

FIG. 5
Macrocolony in slide culture of nanocytes from human patient (Unstained; ×1200)

light of 2000 1× intensity. Petri dishes were exposed to a 16-hour dark and eight-hour light cycle. After colony formation had commenced after eight to 10 days, a single colony was isolated for subculture on BG–11 media in a fresh petri dish.

Slide culture Cleaned microscopic glass slides were provided with 50 per cent, 80 per cent and 95 per cent glycerol prepared either in Tris buffer at pH 7.0 or in double distilled water. A single colony cultured from the human clinical sample, and one from the pond water sample was inoculated aseptically into glycerol on glass slides and overlaid with a sterile cover slip. Slides were kept in the incubator under white light of 2000 1× intensity (16-hour dark and eight-hour light cycle), and examined periodically under the microscope. Control slides were prepared by mounting the organism in Tris buffer without glycerol.

Results

Petri dish cultures showed slow growth of the organism after four weeks. Colonies formed by organisms from both the human and pond water sources were similar (Figure 2). Single colonies from

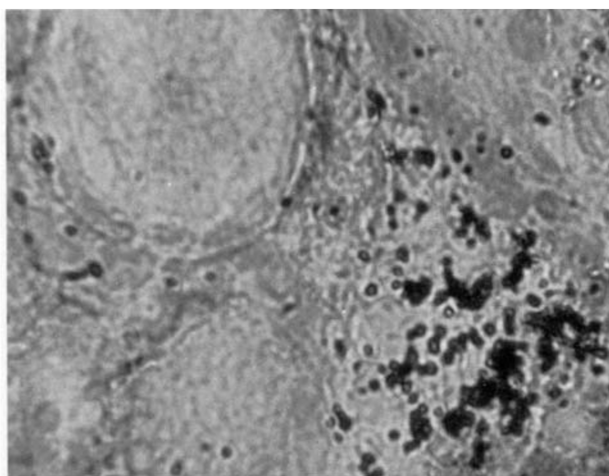

FIG. 6
Macrocolonies in 5 μm section of human polypoidal mass containing round bodies. (Feulgen stain; ×1200)

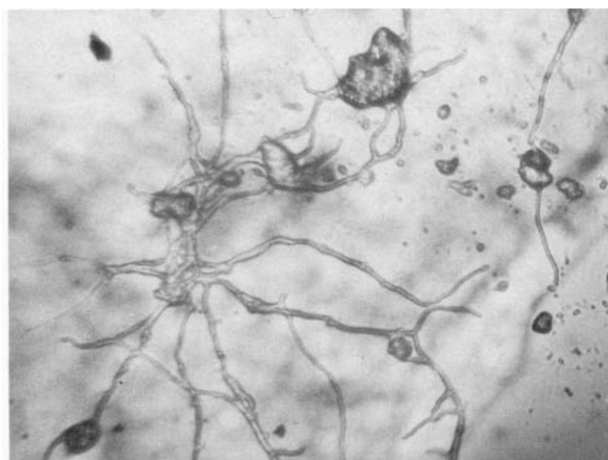

FIG. 7
Filaments produced in slide culture after three months showing absence of true nuclei. (Unstained; ×1200)

petri dishes that were transferred to higher concentrations of glycerol (80 per cent, 95 per cent) in slide culture showed extensive proliferation of nanocytes by binary fission. Some slides were stored in the refrigerator at 4°C. After 10 days of storage at 4°C, many of the nanocytes had increased in volume into round body-like structures some of which contained a few nanocytes (Figure 3). The round bodies induced in culture differed from those *in vivo* in two ways:

(a) Only a few nanocytes were present inside a round body that proliferated to a limited extent as against excessive multiplication seen in clinical samples;

(b) Absence of a thick polysaccharide envelope that is characteristic of round bodies in nasal polyps.

Many of the dividing nanocytes in culture produce linear tubular structures of various shapes that contain blue-green (BG) pigment. Several of these tubules aggregate and align into geometrical or irregular shapes (Figure 4) henceforth referred to as macrocolonies, which often become deep brown while still retaining recognizable BG pigment (Figure 5). Such macrocolonies are also encountered in

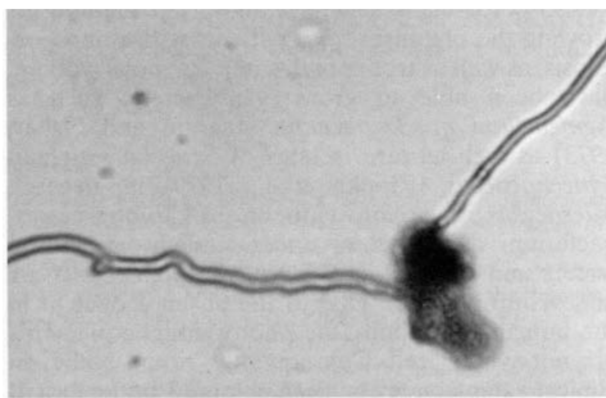

FIG. 8
Filaments in slide culture after two weeks and absence of true nuclei. (Unstained; ×1200)

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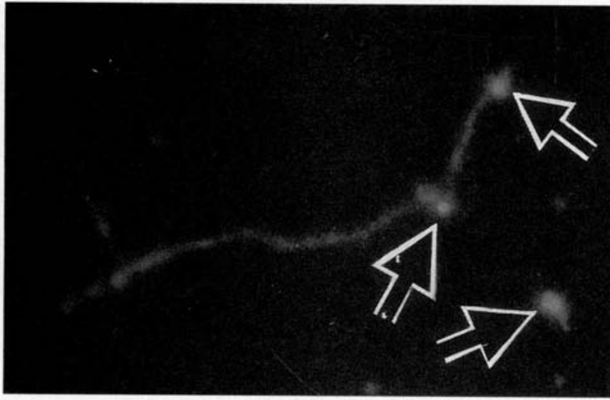

FIG. 9
Laser scanning confocal image of a filament; arrows indicate nanocytes in and outside the filament. (Acridine orange; ×1460)

cultures of organisms from pond water as well as *in vivo* in 5 μm sections of nasal polyps from rhinosporidiosis patients (Figure 6).

The five to seven-day-old slide cultures also showed a few linear filaments while older cultures had many more branched filaments originating from the dark brown macrocolonies (Figure 7). The filaments bear superficial resemblance to fungal hyphae (mycelium). But close and careful examination revealed absence of true nuclei in these filaments (as would be expected in fungal hyphae), and presence of BG pigment (Figure 8). In places, the filaments contained nanocytes (Figure 9) as well as larger, thick-walled brown spherical entities similar to the akinetes of filamentous cyanobacteria (Carr and Whitton, 1982). Nanocytes from clinical samples inoculated in Tris buffer without glycerol were rarely seen to be dividing.

Discussion

The medium BG–11 is widely used for growing cyanobacteria (blue green algae). BG–11 medium also supported growth of organisms from rhinosporidiosis patients and the colonies produced in petri dish cultures were similar to those reported for *Microcystis wesenbergi* (Shirai *et al.*, 1989). The slide culture method using high concentrations of glycerol (80–95 per cent) is a simpler and faster method for growing the organism from patients with rhinosporidiosis, as well as from pond water. Previous workers have been able to grow cyanobacteria such as *Agmenellum quadruplicatum* (Ingram and Fisher, 1973) as well as rare isolates of *Synechocystis* and *Synechococcus* (Rippka *et al.*, 1979) in glycerol. *Microcystis* is a photosynthetic prokaryotic cyanobacterium which grows under sunlight in natural waters and synthesizes BG and red pigments (Carr and Whitton, 1982). Due to the absence of light in the human epithelium, the photosynthetic pigments are not synthesized. Consequently, round bodies in clinical samples appear flesh-coloured under bright field microscope. Exposure of slide cultures in glycerol to white fluorescent light was found to induce synthesis of BG and red pigments in organisms and round bodies from human patients. According to Sidler (1994), all naturally occurring cyanobacteria have been found to produce the blue-coloured, deeply red-fluorescent pigment phycocyanin. As stated in Bryant (1994), the presence of this pigment 'gives cyanobacteria their characteristic blue-green colour'. To check if the BG and red colours observed in slide cultures could be optical illusions due to refraction in the Olympus microscope, the slides were examined under a Nikon microscope and in the laser confocal microscope using a krypton/argon laser beam. The BG and red pigments were seen to persist in all the illuminating systems. Vanbreuseghem (1973) had suggested that even though *R. seeberi* does not synthesize chloroplasts, it does produce precursors of chlorophyll, and therefore could not be excluded as a pathogenic alga.

As stated by Kwon-Chung and Bennett (1992) '*R. seeberi* has not been found anywhere other than in infected tissues of man and animals'. Attempts to find the round body in water samples collected from ponds were futile because it does not exist in nature. This study shows that the round body is organized inside human epithelium through transformation of single cells of *Microcystis*. When nanocytes from clinical samples were grown in glycerol in slide culture, many of the nanocytes increased in volume and displayed the developing stages of round bodies. Similarly, when *Microcystis* cells isolated from pond water were grown in organic media consisting of glycerol, some cells of *Microcystis* commenced enlargement to form round body-like structures (Ahluwalia *et al.*, 1997) (Figure 5). It is significant that when inocula from patients with rhinosporidiosis or organisms from pond water were grown on BG–11 media in petri dish cultures, developing stages of round bodies were not observed. The medium BG–11 which supports growth of many cyanobacteria consists only of inorganic salts (Rippka *et al.*, 1979). Thus it may be inferred that round bodies are produced only in slide culture in organic medium consisting of glycerol. The present *in vitro* findings therefore, suggest that when *Microcystis* cells in pond water enter the human nasal epithelium, they possibly utilize organic nutrients in the host environment and develop into round bodies.

The filaments observed in slide cultures at first glance do seem to resemble fungal hyphae. But close and critical examination of these filaments revealed three prominent features which prove that the filaments could not belong to a fungus; (a) absence of eukaryotic membrane-bound nuclei; (b) presence of BG pigment; (c) the thick-walled dark brown cells (akinetes) occur randomly in terminal or intercalary positions in filaments, whereas in fungi conidia become differentiated in specific locations. Similar filaments have been seen to develop in mutant strains of several cyanobacteria (Kunisawa and Cohen-Bazire, 1970; Ingram and Fisher, 1973; Ingram *et al.*, 1975; Carr and Whitton, 1982). In their study of culture of *Rhinosporidium seeberi*, Krishnamoorthy *et al.* (1989) also found filaments

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which the authors described as fungal mycelium 'bearing conidia as in fungi'. However, in the light of present findings it is unlikely that the filaments observed by Krishnamoorthy *et al.* (1989) could represent true fungal mycelium.

The irregular macrocolonies formed in slide cultures compare with those in histological sections of nasal polypoidal masses, and are often dark brown in colour. Filaments often arise from macrocolonies. Strikingly similar macrocolonies are also reported in mutant strains of the cyanobacterium *Agmenellum quadruplicatum* (see Figure 1, B, C, D in Ingram and Fisher, 1973). Krishnamoorthy *et al.* (1989) also observed dark brown colonies in their cultures.

Molecular characterization of DNA isolated from organisms in clinical samples of rhinosporidiosis and DNA of *Microcystis* from pond water could provide the ultimate proof regarding the identity of the causative agent of rhinosporidiosis. Although the molecular biology of several cyanobacteria has been worked out to a considerable extent (Bryant, 1994), much less information is available on the genome of *Microcystis*. Asayama *et al.* (1996) reported multiple copies of the repeated sequences REP–A and REP–B in strain K–81 of *Microcystis aeruginosa*. A 41 to 42 mol per cent G+C content, resistance to several restriction endonucleases, and presence of two methylases have been demonstrated in nine strains of *Microcystis* (Sakamoto *et al.*, 1993). These authors also found multiple *rpoD* homologs (*rpoD* sequences code for the principal sigma factor of RNA polymerase) in *Microcystis*.

Therefore, a comparative analysis of DNA of *Microcystis* from pond water and DNA of organism from rhinosporidiosis patients has been initiated in the author's laboratory. It may be noted that for characterization of DNA, pure axenic cultures of organisms from the two sources (patients and pond water) are required. Since the method for culture described in this communication yields large populations of dividing cells, it has become possible to conduct DNA studies. Purification of DNA and digestion with restriction enzymes (*Hind* III, *Xba*I, *Bam* HI, *Eco* RV), Southern hybridization, as well as PCR using *Microcystis*-specific primers, fungal primers and universal prokaryotic 16S rRNA primers are included in this study.

The following observations on culture of organisms from patients provide supporting evidence in favour of a cyanobacterium as the causative agent of rhinosporidiosis:

(a) Organism formed colonies in petri dish cuture on BG–11 medium that is a standard medium for growing cyanobacteria.
(b) Under illumination with white light, organisms in slide culture start expressing the blue-green photosynthetic pigment found in all naturally-occurring cyanobacteria.
(c) Round bodies are produced only in patients with rhinosporidiosis; but when organisms from both pond water and clinical samples are grown in slide culture the organisms enlarge and display developing stages of round bodies.
(d) Filaments formed in slide culture resemble those produced by cyanobacteria, and are different from fungal hyphae.
(e) The irregular macrocolonies formed by organisms in culture and *in vivo* are very similar to those reported for the cyanobacterium *Agmenellum*.

Conclusions

(1) A slide culture method has been developed that uses organic medium (glycerol) for growing the causative agent of rhinosporidiosis.
(2) High concentrations of glycerol and illumination with white light promote extensive proliferation of the organism.
(3) Organisms isolated from human patients and growing in slide culture express blue-green pigment found in naturally occurring cyanobacteria.
(4) Many of the organisms in slide culture increase in volume and display developing stages of round bodies that are produced only *in vivo*. This explains why *R. seeberi* could never be found outside the human or animal host.
(5) Filaments formed in culture lack typical features of fungal hyphae; instead they resemble filaments produced by unicellular cyanobacteria after treatment with mutagenic agents.
(6) This *in vitro* study provides supporting evidence to our earlier findings that a prokaryotic cyanobacterium is the causative agent of this disease.
(7) Now that the identity of the causative agent as well as a method for its culture have been discovered, it should facilitate development of therapy.
(8) Rhinosporidiosis is the *first* human disease to be caused by a cyanobacterium.

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References

Ahluwalia, K. B. (1992) New interpretations in rhinosporidiosis, enigmatic disease of last nine decades. *Journal of Submicroscopic Cytology and Pathology* **24:** 109–114.
Ahluwalia, K. B., Bahadur, S. (1990) Rhinosporidiosis associated with squamous cell carcinoma in the tongue. *Journal of Laryngology and Otology* **104:** 648–650.
Ahluwalia, K. B., Maheshwari, N., Deka, R. C. (1997) Rhinosporidiosis: a study that resolves etiologic controversies. *American Journal of Rhinology* **11:** 479–483.
Asayama, M., Kabasawa, M., Takahashi, I., Aida, T., Shirai, M. (1996) Highly repetitive sequences and characteristics of genomic DNA in unicellular cyanobacterial strains. *FEMS Microbiology Letters* **137:** 175–181.

https://doi.org/10.1017/S0022215100144408 Published online by Cambridge University Press

Ashworth, J. H. (1923) On *Rhinosporidium Wernike* 1903, with special reference to its sporulation and affinities. *Transactions of the Royal Society, Edinburgh* **53:** 301–342.

Baron, E. J., Peterson, L. R., Finegold, S. M. (1994) New, controversial, difficult-to-cultivate, or non-cultivable etiological agents of disease. In *Bailey and Scott's Diagnostic Microbiology*, 9th Edition. Mosby, St Louis, Baltimore, Boston, p. 585.

Bryant, D. A. (1994) *The Molecular Biology of Cyanobacteria*. Kluwer Academic Publishers, Dordrecht, Boston, p 533.

Carr, N. G., Whitton, B. A. (1982) *The Biology of Cyanobacteria*. Blackwell Scientific Publications, Oxford, London. pp. 143-189; 387–412.

Castenholz, R. W. (1988) Culturing methods for cyanobacteria. *Methods in Enzymology* **167:** 68–95.

Gaines, J. J., Clay, J. R., Chandler, F. W., Powell, M. E., Sheffield, P. A. (1996) Rhinosporidiosis: three domestic cases. *South Medical Journal* **89:** 65–67.

Ingram, L. O., Fisher, W. D. (1973) Novel mutant impaired in division: evidence for a positive regulating factor. *Journal of Bacteriology* **113:** 999–1005.

Ingram, L. O., Olson, G. J., Blackwell, M. M. (1975) Isolation of a small-cell mutant in the blue-green bacterium *Agmenellum quadruplicatum*. *Journal of Bacteriology* **123:** 743–746.

Krishnamoorthy, S., Sreedharan, V. P., Koshy, P., Kumar, S., Anilakumari, C. K. (1989) Culture of Rhinosporidium seeberi: preliminary report. *Journal of Laryngology and Otology* **103:** 178–180.

Kunisawa, R, Cohen-Bazire, G. (1970) Mutants of Anacystis nidulans that affect cell division. *Archives of Microbiology* **71:** 49–59.

Kwon-Chung, K. J., Bennett, J. E. (1992) *Medical Mycology*. Lea & Febiger, Philadelphia, London, pp 695–706.

Levy, M. G., Meuten, D. J., Breitschwerdt, E. B. (1986) Cultivation of *Rhinosporidium seeberi in vitro*: interaction with epithelial cells. *Science* **234:** 474–476.

Rippka, R., Druelles, J., Waterbury, J. B., Herdman, M., Stanier, R.Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111:** 1–61.

Sakamoto, T., Shirai, M., Asayama, M., Aida, T., Sato, A., Tanaka, K. (1993) Characteristics of DNA and multiple rpoD homologs of *Microcystis* strains. *International Journal of Systematic Bacteriology* **43:** 844–847.

Satyanarayana, C. (1960) Rhinosporidiosis with a record of 255 cases. *Acta Otolaryngologica* **51:** 348–366.

Shirai, M., Matumaru, K., Ohotake, A., Takamura, Y., Aida, T., Nakano, M. (1989) Development of a solid medium for growth and isolation of axenic *Microcystis* strain (Cyanobacteria). *Applied and Environmental Microbiology* **55:** 2569–2572.

Sidler, W. A. (1994) Phycobilisome and phycobiliprotein structures. In *Molecular Biology of Cyanobacteria*, (Bryant, D.A. ed.) Kluwer Academic Publishers, Dordrecht, Boston. pp. 139–216.

Thianprasit, M., Thagerngpol, K. (1989) Rhinosporidiosis. *Current Topics in Medical Mycology* **3:** 64–85.

Vanbreuseghem, R. (1973) Ultrastructure of *Rhinosporidium seeberi*. *International Journal of Dermatology* **12:** 20–28.

Vukovic, Z., Bobic-Radovanovic, A., Latkovic, Z., Radovanovic, Z. (1995) An epidemiological investigation of the first outbreak of rhinosporidiosis in Europe. *Journal of Tropical Medicine and Hygiene* **98:** 333–337.

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