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The Occurrence Of *Rhinosporidium seeberi* in a fresh water pond at Piranmalai (Sivaganga District, Tamil Nadu, India

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SUMMARY

Rhinosporidium seeberi the casual agent of Rhinosporidiosis is known for about hundred years and its association with stagnant water bodies has been known for as many years. Several reports of its aquatic associations have been published but so far there is no report of its occurrence in any of the water bodies that are associated with Rhinosporidiosis (6). We report for the first time the presence of *Rhinosporidium seeberi* in a pond, which is known to be associated with the disease. The sporangia isolated from the water samples showed typical structure of those isolated from the infected tissue. The pond water samples also yielded several developmental stages for the sporangia comparable to those obtained from the infected tissue. Since the organism cannot be cultured and there is no possibility of verifying Koch's postulates, an identical RAPD profile of the DNA from sporangia occurring in water and from those of infected tissue was considered confirmatory.

Key words: *Rhinosporidium seeberi;* Water Source; Developmental stages; RAPD Profile

INTRODUCTION

The disease Rhinosporidiosis has been known from 1900, when Seeber (1) first described it in his thesis. Comprehensive description of the manifestations of the disease in humans and animals and the developmental stages of the causal organism *Rhinosporidium seeberi* have been reported over the years by several investigators (2-9). Karunaratanae (5)

Correspondence: Prof. C. Rajamanickam, Tamil Nadu; Dr. Pran Nath Chuttani Oration delivered by Dr. S. Kameswaran at Chandigarh - 2005.

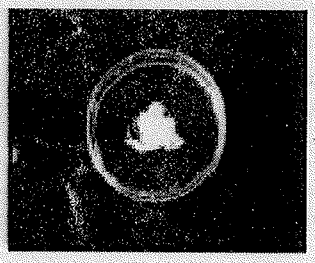
observed in his monograph that the disease was associated with agricultural workers and those who had occupational association with muddy water. In general, persons suffering from Rhinosporidiosis had a history of taking bath in a stagnant pool of water such as pools, ponds, lakes etc. Kameswaran (6) reported that patients of Rhinosporidiosis coming from a hyper endemic area (Rajapalayam-Tamil Nadu) had a history of taking bath in a specific pond. This association of Rhinosporidiosis with a stagnant pool of water has also been reported by Vukovic *et al* (10) who found that the only experience that the Yugoslavian patients had in common was that they all bathed in the same stagnant water called the "silver lake". The occurrence of Rhinosporidiosis in swans (9) also supported an aquatic environment as the reservoir for Rhinosporidiosis seeberi. However the presence of the pathogen Rhinosporidiosis seeberi in aquatic environment has not so far been reported. We reported here for the first time the occurrence of Rhinosporidiosis seeberi in a pond in Piranamlai (Sivaganga District-Tamil Nadu) which has been known to be associated with Rhinosporidiosis (6). Several Rhinosporidiosis patients coming from this area have a history of having taken bath in the specific pond. The samples of water collected from this tank at different time intervals over a year have been examined for the presence of Rhinosporidium and the observations are reported. It has not been possible hitherto either to culture the organism or to verify Koch's postulates in Rhinosporidiosis. The identity of the organism occurring in the pond as Rhinoporidium seeberi was suggested by the morphology and the RAPD profile of the sporangia obtained from water body, which was comparable to that of the sporangia, obtained from the human infected tissue.

MATERIALS AND METHODS

Separation of Rhinosporidium from infected tissue:

The infected tissue was cut into small pieces, teased in phosphate buffered saline (PBS) and treated with collagenase type I enzyme (0.9mg/ml) for 2 hours at 37°C with continuous stirring (see plate in Appendix 1). The sample was diluted ten fold with cold PBS to stop the reaction and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was suspended in 1ml of PBS, the number of sporangia counted and loaded on to a discontinuous (step) density

Infected Rhino Tissue



Collgenase treated

Appendix I

Sporangia at various stage

gradient made of fetal calf serum, ficoll, percoll and sucrose as given in Table I. The 10ml gradient was centrifuged at 1,000 rpm for 30 minutes at 4°C in a swing out bucket. After centrifugation, each fraction was isolated using a Pasteur pipette, washed by centrifugation and sporangia counted in a Neubaur Haemocytometer (Table I). Sporangia of different sizes were found to be distributed in different fractions/layers. There was also some contamination of host tissue debris. Hence sporangia from different fractions were pooled and subjected to a sucrose gradient centrifugation (2M, 1.5M, 1M, 0.5M and 0.25M) at 2500 rpm for 30minutes. Majority of the sporangia were recovered without host cells in the 1-1.5 M gradient interphase. These sporangia were washed and used for DNA extraction.

Table 1 : Density Gradient Separation of *Rhinosporidium* sporangia from infected human tissue in Ficoll-Percol gradiant

	Number of Sporangia				
	Debris Particles	Small (Juvenile) <100µ	Medium (Intermediate) 100µ–250µ	Large (Mature) >250µ	Total
Digested whole sample count in 50µl	Large nos	200	230	30	460
In total sample loaded		48000	55200	7200	110400
Yield on Gradient Fractions Fetal Calf Serum	Present	900	7200	300	8400
Interphase 1	90	7500	19500	3000	30000
Ficoll 1.07 g/ml	40	26250	6900	1200	34350
Interphase 2	20	8800	7000	1800	17600
Percol 1.13 g/ml	10	900	2230	330	3460
Interphase 3	0	0	0	0	0
Sucrose 2.5 M	0	0	0	0	0
Yield		44350	42830	6630	93810
Yield in percentage		94.4	77.59	92.08	84.97

Note : The sporangia from each fraction were separately collected and suspended in appopriate volume of PBS.

Collection of *Rhinosporidium* from pond water

Water samples were collected from the Piranmalai tank (78.2°E, 97°N) during May, August, September and December, 1999. Sporangia of different sizes were observed in every sample collected. The sporangia were collected by passing plankton net several times through the pond water and the samples transported to the laboratory in cold box. Samples were further concentrated by centrifugation and resuspended in 1ml of PBS. One drop of this suspension was placed on a small piece of filter paper (Whatman No.1, 2 cm x 1 cm) placed on a slide and viewed under a microscope. Sporangia were picked up with a sharp needle and transferred to phosphate buffered saline (50µl) in an eppendorf tube. Five hundred such sporangia picked up were washed thrice by centrifugation, and used for extraction of DNA.

Isolation of Genomic DNA

Total genomic DNA of sporangia from water sample and tissue sample was isolated employing "Lysis" method. The sample was treated with lysis buffer containing 0.32M Sucrose 10mM Tris pH 7.5, 5mM MgCl2, 1% Triton 100x in 1:1 ratio.

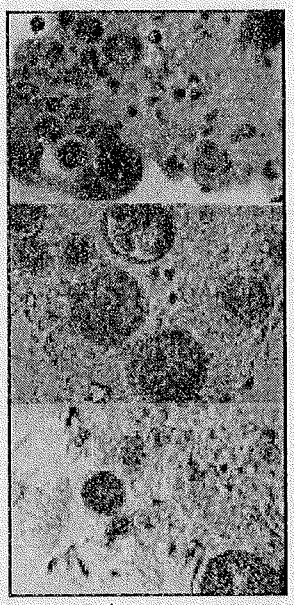


Plate I

Fig 1. Juvenile, intermediate and mature sporangia in the infected tissue. x120.

Fig 2. Infected tissue mass treated with collagenase showing sporangia in different stages of development. x120 (phase).

Fig 3. Collagenase treated infected tissue in advanced stage of digestion showing several juvenile sporangia. Some of them are equal to or smaller than the host cell nucleus. x120 (phase).

The sample and lysis buffer were mixed well using a vortex mixer and then spun at 10,000 rpm for 20 seconds to obtain the pellet. The pellet was suspended in 1ml of lysis buffer and the same step was repeated thrice. The pellet was finally resuspended in 0.5ml of sterile PCR buffer (5mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% T20) and stored frozen. The sample was thawed and digested with proteinase-K (0.6 µl of 10 mg/ml per 100µl) at 60°C for one hour and the

reaction terminated by incubating at 97°C for 10 minutes. This was spun at 10,000 rpm for 20 seconds and the pellet was resuspended in 10µl of PCR buffer.

Random Amplified Polymorphic DNA (RAPD) Analysis:

RAPD analysis is a technique employed to detect genomic polymorphism, utilizing oligonucleotide primer for an arbitrary sequence in a Polymerase Chain Reaction (PCR). 50ng of genomic DNA isolated from the sporangia from the water or from tissue was added to the tube containing the RAPD analysis bead (25 pmol of RAPD primer IV) and made up to 25µl with sterile distilled water. PCR was performed in a thermocycler (Perkin Elmer, Gene Amp 2400 USA) with the following cycle profile; 1 cycle at 95°C for 5 min followed by 45 cycles consisting of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min.

Results:

Almost all of developmental stages of R.seeberi similar to those obtained in the infected tissue were observed in the pond water samples (Plate I, Figs 1, 2 and 3). These are summarized below.

Several sporangia in juvenile stages of development in a spore mass (Plate II, Figs 1 and 8). However there were also individual occurring very small sporangia of 12 µm diameter (Plate II, Fig 3), which was probably the earliest stage in the

Different Stages of *Rhinosporidium seeberi*

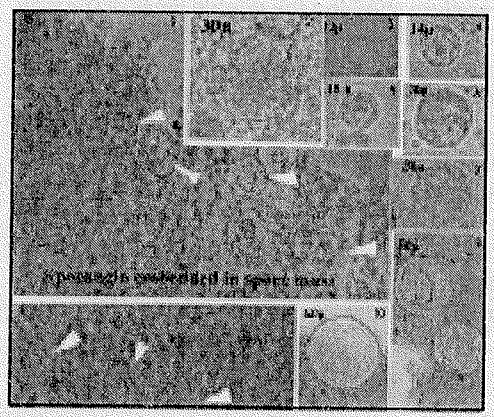


Plate II

Figs 1 and 8. Several sporangia embedded in spore mass. x120.
Fig 2. 30µ sporangium, outer sporangial wall more clearly visible than in the earlier stages as also the inner translucent layer, several nuclei are seen. x760.
Fig 3. 12µ spore probably showing the earliest stage in development nucleus not yet divided. x760.
Fig 4. 14µ sporangium nucleus enlarged, probably undergoing division x760.
Fig 5. 18µ sporangium enclosing a few nuclei. x760.
Fig 6. 20µ sporangium showing further nuclear division having a prominent translucent layer and not so prominent outer wall. x760.
Fig 7. 22µ sporangium showing the beginning of the cleavage of cytoplasmic. The outer wall is not prominent.
Fig 9. 50µ and 50 micro sporangia clearly showing nuclei and cytoplasmic cleavage the thick dark brown, outer wall is clearly visible. x120.
Fig 10. 60µ sporangium (x304) showing a thick dark outer wall and well defined, translucent inner wall exhibiting the initial stages of development of the pore and the annulus. Several nuclei, each surrounded by a small amount of cytoplasm are present inside the sporangium.

development to the sporangia where the nucleus had not yet divided. This was further enlarged to 14 µm (Plate II, Fig 4) where the nucleus was large and was probably undergoing division. The next stage of development was seen in the 18 µm diameter sporangia with a few nuclei inside the sporangium (Plate II, Fig 5). The nuclei underwent further division along with the enlargement of the sporangium to 20µm exhibiting a prominent translucent layer of sporangial wall and not so prominent outer wall (Plate II, Fig 6). The next stage of development namely the 22 µm sporangium showed the prominent nuclei and the beginning of cleavage of the cytoplasm. At this stage also the outer thick wall was not prominent and the translucent inner wall was clearly seen (Plate II, Fig 7). In the next developmental stage observed as 30 µm diameter sporangium (Plate II, Fig 2) the outer sporangial wall was more clearly visible than in the earlier stages as also the inner translucent layer. The sporangial content showed several nuclei. This pattern was more clear in the 50 µm diameter sporangium (Plate II, Fig 9), which clearly showed the nuclei and the

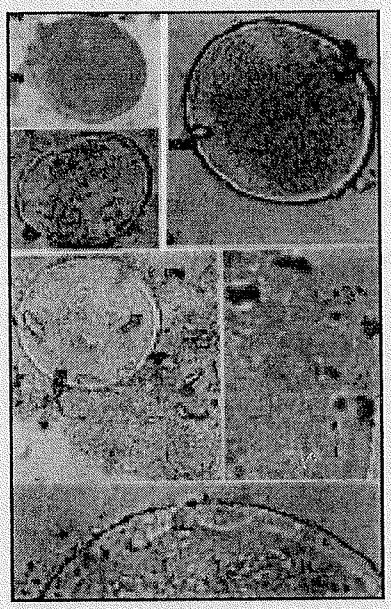


Plate III

Fig 1. 250µ sporangium showing bilamellar wall and a prominent pore large number of spores about to be released.

Fig 2. 200µ sporangium showing prominent dark outer wall and a translucent inner wall containing numerous developing spores (x304).

Fig 3. 135µ sporangium showing prominent wall layers. A few developing spores are clearly seen (x120).

Fig 4. 140µ sporangium showing the double layer of the wall and the formation of the annulus and the pore 35µ sporangium is present at the bottom and 60 micro sporangium is seen at the lower right corner (x760).

Fig 5. The pore and the annulus of a 165µ sporangium enlarged. Some spores are seen between the annulus and the pore region (x760).

Fig 6. 170µ sporangium showing the dark outer wall and a prominent and a prominent trunslucent innter wall forming the thick annulus near the pore region. Several developing spores are clearly visible. (x760).

cytoplasmic cleavage. Further development of the sporangium (60 µm diameter) showed a prominent, dark outer layer of the sporangial wall clearly defined translucent inner wall, which also exhibited initial stage of development on the pore and annulus, which is the thickening of the translucent wall layer surrounding the pore. Inside the sporangium several nuclei, each surrounded by a small amount of cytoplasm could be observed (Plate II, Fig 10). It may be mentioned here that the sporangia described above can be grouped under the category of "Juvenile" sporangia according

to the ontogenic nomenclature proposed by Kennedy *et al.* (9).

This juvenile stage is followed by further development of the sporangium to the intermediate stage where the sporangia attain 100 µm - 200 µm diameter exhibiting a prominent dark outer wall, a well developed, distinct and diffuse inner wall enclosing spores in different stages of development and maturity. In addition, we could also observe the gradual

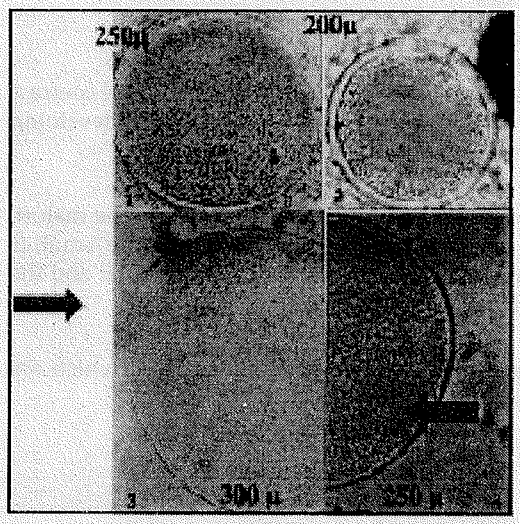


Fig 1. 250µ sporangium showing clearly the thick annulus and the pore, fully packed with numerous spores. (x304).

Fig 2. 200µ sporangium from infected tissue placed here to compare with figure 1.

Fig 3. 300µ sporangium with a thick outer wall but a thin translucent inner wall releasing the spore. The mature spore, are towards center of the sporangium and the small developing spore are towards a periphery. (x304).

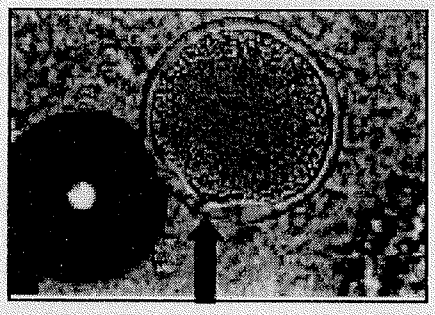
Fig 4. 250µ sporangium showing similar features as figure 3 except the pore is also clearly seen. (x304).

The arrow indicates the annulus opening and release of spores from it.

Plate IV

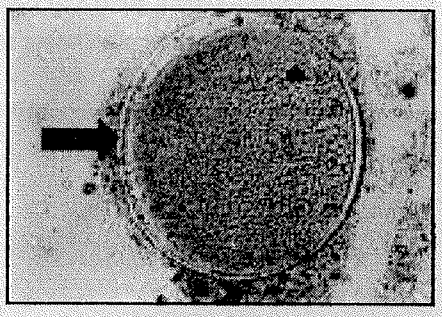
Sporangia isolated from

Infected Tissue



Mature sporangia 200µ thinner bilamellar wall. The pore is also visible in several sporangia. Mature sporangium contains about 16,000 endospores which are released usually through the pore.

Pond Water



Mature sporangia 250µ diameter show comparatively thinner wall of sporangium. The sporangium contains several thousands of spores, which are released through the pore.

Plate IV A

development of the pore and annulus in tissue intermediate stages (Plate III, Figs. 2, 3, 4, 5 and 6).

The intermediate sporangia gradually developed into mature sporangia enclosing progressively maturing process (Plate IV, Fig 1 and 2), which were released through the pore at the appropriate time (Plate III, Fig 1; Plate IV, Fig 3 and 4; Plate V, Fig 3). The morphological similarity of the sporangia during development in the infected tissue and in the water of the Piranmalai tank was remarkable (Table II).

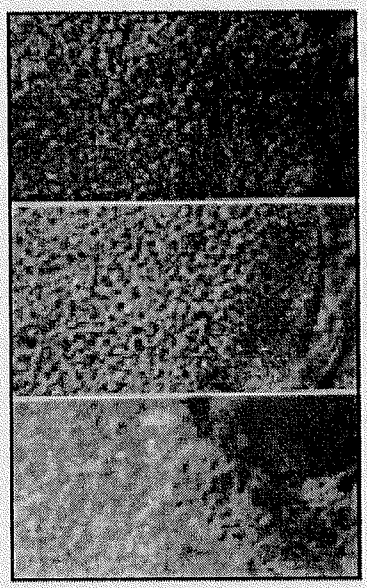


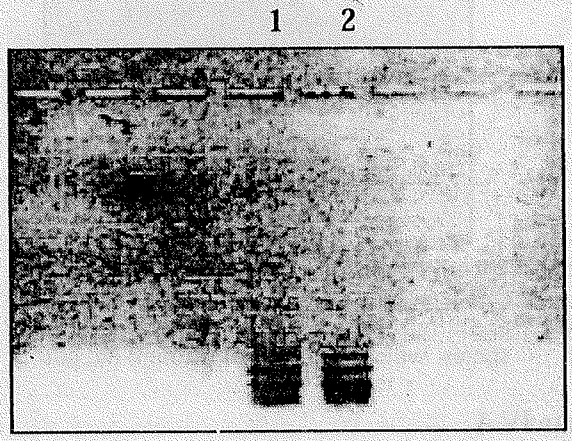
Plate V

Fig 1. A part of 200μ sporangium enlarged under phase contrast to show the mature spores in the center and the develoing spores towards the periphery (x760).

Fig 2. A part of 250μ sporangium showing clearly the mature spores in the center of the sporangium and the smaller developing spores towards the thick translucent wall and the dark outer wall are also clearly seen. (x760).

Fig 3. 250μ sporangium showing mature spores which are about to release.

Fig 1. Comparison of RAPD profile of Rhinosporidium sporangia from human tissue and from using primer 1V



Lane 1. Sporangia from tissue;
Lane 2. Sporangia from water

Almost all the stages that were observed in the surgically removed mass could be observed in the water samples also (compare figures Plate I and II). There was also striking similarity in the development of pore and annulus between the sporangia from water and the one obtained from the infected tissue (Plate IV, Fig 1 and 2; enlarged in Plate IV A).

The results of RAPD analysis of *R.seeberi* obtained from pond water and human infected tissues are presented in Figure I. The two profiles were markedly similar.

DISCUSSION

Ajello (13) attributes an aquatic habitat for *Rhinosporidium* and has given a compendious review of available literature

Table 2 : Comparison of principal morphological characters of Rhinosporidial sporangia occurring in the infected tissue and sporangia occurring in pond water

Sporangia from infected tissue	Sporangia from pond water
Juvenile (9) 12-100 μm diameter also termed "trophocyte" with variable intracellular appearances, chromatin either organized into a single nucleus (with nucleoli) (11) or may be fragmented and diffuse (12). At about 50-60μm diameter nuclear division results in the appearance of chromatin within vesicles.	Several juvenile sporangia with variable intracellular appearances. Chromatin organized into a single nucleus at about 12μ stage, starts dividing very early and appears fragmented. At about 50μ cytoplasmic cleavage is noticeable and at 60μ diameter nuclear division results in the appearance of chromatin with in vesicles.
Intermediate (9), immature sporangium 100-200μ diameter, spherical thick bilamellar wall, outer dark (Chitinous) and inner translucent, contains immature endospores in cytoplasm that is granular or fibrillar. Several thousand endospores in immature sporangium of about 200μ diameter.	Immature, intermediate sporangia 140-170μ diameter stage shows clearly thick bilamellar wall, outer dark and inner translucent and contains immature spores.
The annulus is prominent.	The annulus is prominent
Mature sporangia 250-400μ thinner bilamellar wall (4,13). The annulus (15μ) is prominent. The pore is also visible in several sporangia. Mature sporangium contains about 16,000 endospores (4), which are released usually through the pore.	Mature sporangia 200-300μ diameter show comparatively thinner wall of sporangium. The sporangium contains several thousands of spores, which are released through the pore.

in support of his consideration. However, none of the papers so far published have given evidence for the presence of Rhinosporidium in an aquatic habitat that is associated with the disease. For the first time in the history of Rhinosporidiosis we have identified Rhinosporidium in an aquatic habitat which is implicated in the disease. The spore appears to be the starting point of the life cycle since we have not observed any structure (trophocyte) smaller than 10-12μ in diameter in any of our samples collected. The developmental stages and the mature sporangia obtained from the water sample were markedly similar in structure to the sporangia obtained from the infected tissue. Further, the RAPD profile of DNA from the sporangia occurring in the pond and that obtained from the sporangia isolated from the infected tissue were identical (Fig. I) indicating that the organism occurring in the aquatic habitat is *Rhinosporidium seeberi.*

The absence of evidence hitherto on the occurrence of *Rhinosporidium seeberi* in nature, particularly in ponds or other stagnant water associated with the disease had led to various speculations (14,15). *Microcystis aeruginosa* a unicellar, colonial cyanobacterium was considered as a causal agent of Rhinosporidiosis (15). However this does not appear to be the case since the structure (16) and the ultra structure (17) of M *aeruginosa* are entirely at variance with the structure and the ultra structure of *R. seeberi* (18). *M. aeruginosa* is known to occur in the blooms in several ponds of Tamil Nadu including Piranmalai tank (16), nonetheless not at all these water bodies have been implicated with Rhinosporidiosis. Further the DNA sequence of an RAPD fragment of *R. seeberi* did not show any homology with the published sequences of M *aeruginosa* (unpublished observation). The identification of an organism identical to R *seeberi* in the Piranmalai tank implicated in the disease (6) and its confirmation by RAPD analysis establishes the transmission of Rhinosporidiosis through water bodies. Developing an animal model and culturing of *R. seeberi* will lead to further understanding of the development and transmission.

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