Original Article

The Identification of the Natural Habitat of *Rhinosporidium seeberi* with *R. seeberi*-Specific in situ Hybridization Probes

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ABSTRACT

The disease rhinosporidiosis, caused by *Rhinosporidium seeberi*, has been described in humans and animals since 1892 in at least 70 countries including Europe, North America, South America, Asia, and Africa. South Asia is a known hyper-endemic region. Two persisting enigmas have been the inability to culture *R. seeberi* in vitro and to establish experimental rhinosporidiosis in animals. The natural habitat of *R. seeberi* remains unknown, although there is evidence for an aquatic habitat.

A *R. seeberi*-specific, fluorescein isothiocyanate (FITC)-labelled probe, and a complement of the *R. seeberi*-specific probe as a control probe, were used to investigate, by in situ hybridization, the putative ground-water habitat of *R. seeberi*. Sections of paraffin wax-embedded, purified rhinosporidial endospores, and human nasal, rhinosporidial tissue, served as positive controls. A human nasal, non-rhinosporidial polyp was used as a negative control.

Entities were identified, with 15-30 μm diameters, compatible morphologically with endospores and juvenile sporangia of *R. seeberi*. These entities were positively labelled with the FITC-labelled *R. seeberi*-specific probe, in four replicated experiments, in paraffin wax-embedded deposits of lake water. No labelling with the same *R. seeberi*-specific probe was seen on the human, non-rhinosporidial polyp, or on the background (non-rhinosporidial) areas of the rhinosporidial polyp. Spicules of sand particles in this water deposit were identified as possible causes of injury to the ocular and nasal mucosae that could initiate colonization by the aquatic *R. seeberi.*

This, as far as we are aware, is the first specific demonstration of the aquatic habitat of *Rhinosporidium seeberi*. (*J Infect Dis Antimicrob Agents 2008;25:25-32.*)

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INTRODUCTION

Rhinosporidiosis, first recognized in 1892, has now been reported from over 70 countries including Europe, North America, South America, Asia, and Africa. Rhinosporidiosis is hyper-endemic in India and Sri Lanka. The causative pathogen, *Rhinosporidium seeberi*, was identified in human tissues in 1900. Its taxonomy was under debate for many years until recently when molecular biological phylogenetic techniques classified it with frog and amphibian pathogens in a new Class, the Mesomycetozoea.[1,2] Independent confirmation of this taxonomy has been provided by Fredricks and colleagues.[3] However, there has been no demonstration of its natural habitat although there is some evidence that it is located in the ground water of natural lakes, reservoirs (artificial irrigation lakes), and in rivers.[4,5] Repeated polymerase chain reaction (PCR) analyses of ground-water deposits with *R. seeberi*-specific primers have failed to identify this organism in deposits of ground water (Malik Peiris 2004, R. P. V. J. Rajapakse, 2006, K. Kaluarachchi 2007, unpublished observations). Efforts using modified techniques for concentration of rhinosporidial elements and extraction of DNA are continuing.

This study used *R. seeberi*-specific in situ hybridization probes[3] on deposits of water from an artifical lake (reservoir) in Sri Lanka in which many patients with rhinosporidiosis had bathed, to investigate the putative natural habitat of *R. seeberi* in such waters. The advantage of in situ hybridization for the purpose of identifying *R. seeberi* in nature is that the morphology of the labelled entities could be compared with known developmental stages of the pathogen; and also that this technique is perhaps less affected by inhibitors or other factors that affect the PCR proceduce. However, PCR is more sensitive while also enabling the identification of the amplification products in relation to the defined genome of *R. seeberi*, and therefore is also more specific, especially in view of the genetic diversity that has been shown in strains of *R. seeberi*.[6,7]

MATERIALS AND METHODS

Human rhinosporidial tissue

A human rhinosporidial polyp, embedded in paraffin wax for conventional staining with Haematoxylin and Eosin (H & E) and the periodic acid-Schiff stain (PAS), was used as the first positive control.

Endospores of *R. seeberi*

Endospores were obtained from human nasal, rhinosporidial tissues after homogenization in phosphate buffered saline (PBS) (0.1M, pH 7.4) in an all-glass, hand-activated homogenizor, followed by partial purification through a 25-µm nylon sieve, also embedded in paraffin wax, were used as the second positive control.

Human non-rhinosporidial tissue

A human, nasal, non-rhinosporidial polyp embedded in paraffin wax, was used as the negative control.

Deposits of ground water

Forty litres of water, free of soil and mud, were collected from the edge of a reservoir in which patients with rhinosporidiosis had bathed. The sample area was away from the bathing area. This particular reservoir was chosen for sampling because a large number of our patients had bathed in it. The suspended matter was separated by centrifugation at 400 x g, at room temperature (ambient 25^{o}C). Coarse material in the deposit was allowed to settle after brief standing, and the supernatant was concentrated by further centrifugation; the deposit was embedded in paraffin wax for in situ hybridization, and smeared on

microscope slides for staining with the PAS which stains the rhinosporidial bodies, deep magenta.

Paraffin wax embedding

One mL of the reservoir water-deposit or the endospore suspension was added to an Eppendorf tube and centrifuged at 13,000 rpm for 5 min. The pellet was stirred in 1 mL of 4 percent paraformaldehyde and kept at 4°C overnight. The tube was centrifuged again at 13,000 rpm for 5 min; the supernatant was discarded and the pellet was washed with distilled water. The pellet was then mixed with 500 µL of 1 percent agarose in distilled water and heated to 80°C in a thermal block for 30 min. The tube was allowed to cool to room temperature and the solidified agar block was removed. The agar block was dehydrated through an alcohol gradient, cleared in xylene and embedded in paraffin wax. Six µm-thick sections were floated on to glass slides for in situ hybridization studies.

In situ hybridization probes

The *Rhinosporidium* probe, an anti-sense oligonucleotide of a unique region of *R. seeberi*'s 18S rRNA gene sequence ('Rhino FISH') and its complementary control probe ('Control FISH') described by Fredricks and colleagues[3] were received (without charge) from Prof. Malik Peiris (University of Hong Kong). The *Rhinosporidium*-specific probe and the control probe were biotinylated at both 5' and 3' ends.

Rhino FISH probe- BTGCTGATAGAGTCATT GAATTAACATCTACB

Control FISH probe- BACGACTATCTCAGTA ACTTAATTGTAGATGB

In situ hybridization probes

The paraffin sections on microscope slides were de-waxed, re-hydrated and immersed in Tris buffer (Tris-hydroxymethylamino methane, 0.1M, pH7.4). To enhance the penetration of the probe, the sections were treated with lysozyme [10 mg/mL in 50 mM EDTA (ethylene-diamino tetra-acetic acid) and 0.1 M Tris]. Sections were pre-hybridized with hybridization buffer for 30 min at 37°C. Approximately 50 ng of the biotinylated probe in 50 µl of hybridization buffer was added to each slide, covered with soft coverslips and hybridized overnight at 45°C. Post-hybridization stringency washes were carried out in 5 x SET (sodium chloride-EDTA-Tris) buffer at 40°C and 0.2 x SET buffer at 40°C and 20°C. After the stringency washes, the slides were brought to phosphate buffered saline pH 7.4, incubated with Vectastain Elite ABC complex (Vector laboratories, USA) and subjected to tyramide signal amplification (TSA-plus fluorescein system, Perkin-Elmer Life Sciences, USA).

RESULTS

The entities in the water deposits, positively labelled with the *R. seeberi*-specific FITC probe, are referable to the serial ontogenic stages of *R. seeberi*, shown in Figure 1.[5] The putative infective stage is a 10-15 µm thick-walled endospore (Figure 1a), with 10-20 spherical bodies including lipid bodies and the electron dense bodies (EDB). DNA has been demonstrated in the EDBs within the endospore.[5] Stage two (Figure 1b), the juvenile sporangium (previously termed a trophocyte) is a larger (15-20 µm), thin-walled, spherical or oval body with a small single, central nucleus. Stage three (Figure 1c), the vesicular juvenile sporangium (previously termed a pre-cleavage sporangium) is a spherical or oval body of approximately 20-50 µm diameter, with small multiple nuclei dispersed in the cytoplasm, derived from division of the single nucleus of stage 2. Walls that would later enclose the cytoplasm around the multiple nuclei have not yet formed at this late third stage (Figure 1c).

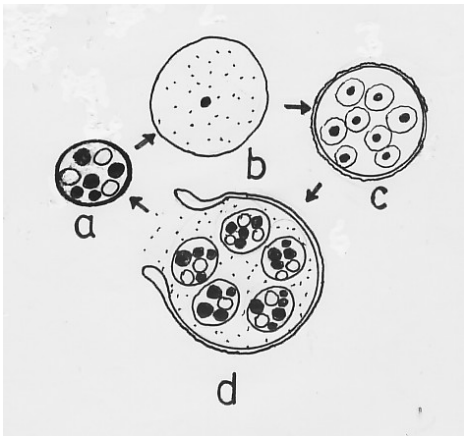

Figure 1. Diagrammatic representation of the serial ontogenic stages in the life cycle of *Rhinosporidium seeberi*, (a) endospore with electron dense bodies (EDB), (b) juvenile sporangium (trophocyte) with a small, single nucleus, (c) large juvenile sporangium (pre-cleavage sporangium) with small, multiple nuclei, and (d) mature sporangium liberating mature endospores. Not to scale.

Rhinosporidial endospores

The purified rhinosporidial endospore of approximately 8-µm diameter, contained spherical bodies, probably EDBs, which labelled positively with the *R. seeberi*-specific probe (Figure 2a). The thick endospore wall and the internal spherical bodies were visible under bright light (Figure 2b). No juvenile sporangia were seen; the homogenization procedure used in the isolation of the endospores could have disrupted the thin-walled juvenile sporangia.

Rhinosporidial tissue

The rhinosporidial tissue contained endospores that measured approximately 12 µm, and contained numerous spherical bodies. The majority of the latter, probably the DNA-containing EDBs, showed intense fluorescence with the *R. seeberi*-specific FITC probe (Figure 3a). The thick endospore-wall and the

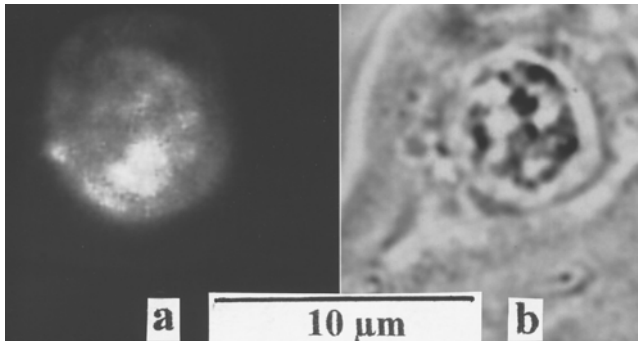

Figure 2. Paraffin-embedded section of rhinosporidial endospores, (a) stained with the *R. seeberi*-specific FITC *in situ* hybridization probe, showing an endospore with deeply fluorescent internal bodies, probably the DNA-containing electron dense bodies, and (b) same section as in (a), photographed under bright light, showing a thick-walled endospore with internal spherical bodies.

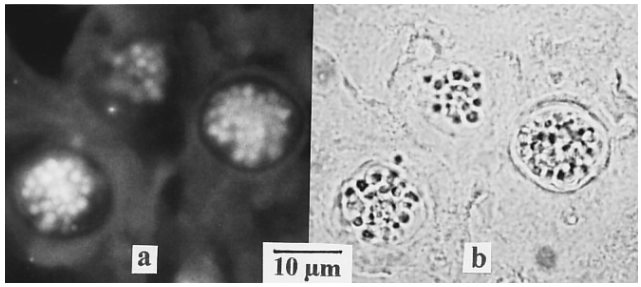

Figure 3. Paraffin-embedded section of rhinosporidial tissue, (a) treated with the *R. seeberi*-specific FITC in situ hybridization probe, photographed under ultraviolet light, showing intensely fluorescent intra-endosporial spherical bodies, probably the electron dense bodies, and (b) the same section containing numerous endospores with spherical bodies including the electron dense bodies, photographed under bright light.

spherical bodies were seen under bright light (Figure 3b).

The control probe did not label the rhinosporidial elements in the rhinosporidial tissue, positive control specimens.

Reservoir water-deposit

The spherical body (approximately 15 μm, Figure 4a), and the oval body (approximately 30 μm, Figure 4b) in the deposit of reservoir-water, which labelled positively with the fluorescent *R. seeberi*-specific probe, were similar in size and shape and were compatible with a rhinosporidial endospore (Figure 1a), and a thin-walled juvenile sporangium, with multiple nuclei, of stage three (Figure 1c) of *R. seeberi,* respectively. The 10-μm body (Figure 5a) also probably represents a juvenile sporangium. The relatively low intensity of labelling of the putative endospore in Figure 4a was similar to that of one of the endospores in the rhinosporidial tissues in Figure 3a. The intensity of the fluorescence of the juvenile sporangium in Figure 4b and 5a was less than that in the endospores in Figure 3a probably because the density of nuclear material in the juvenile sporangium is much less than in the EDB-laden endospore. The thin wall of the juvenile sporangium of Figure 5a was seen under bright light (Figure 5b).

Quadruplicate in situ tests on samples of this reservoir-water deposit gave similar findings.

Sections of the control-negative, non-rhinosporidial polyp, did not label with the *R. seeberi*-specific FITC probe.

The identification of the bodies in Figure 4b, 5a, and 5b as juvenile sporangia of stage 3 was aided by the appearance of similar, PAS positive bodies seen in smears from human nasal rhinosporidial polyps that showed juvenile sporangia with typical ridges on the thin sporangial wall representing collapsed regions,

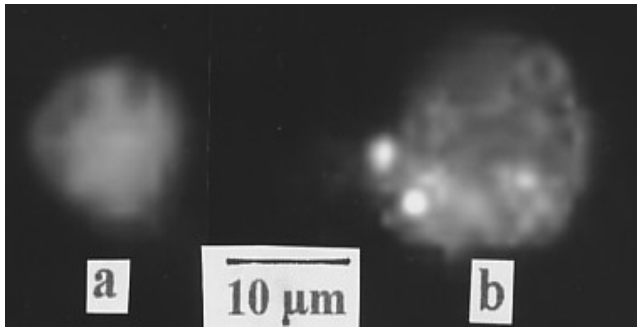

Figure 4. Paraffin-embedded section of reservoir-water deposit, photographed under ultraviolet light, (a) *R. seeberi*-specific FITC in situ hybridization probe-labelling of a 15-μm spherical body, probably an endospore with several internal spherical, fluorescent bodies that probably represent the electron dense bodies, and (b) *R. seeberi*-specific FITC in situ hybridization probe-labelling of multiple sites in an oval 30-μm body, probably a juvenile sporangium with multiple nuclei.

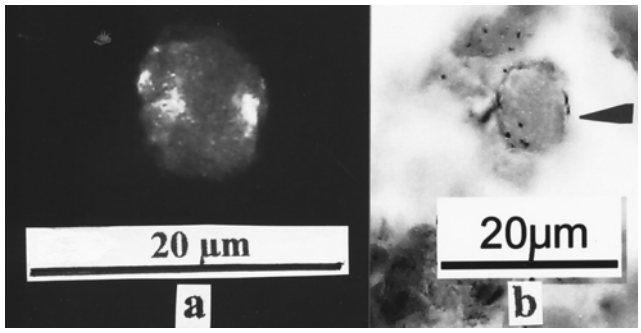

Figure 5. Paraffin-embedded section of lake-water deposit, photographed, (a) under UV light showing multiple foci of fluorescence representing nuclei, (b) under bright light showing small, thin-walled, oval-shaped juvenile sporangium.

and juvenile sporangia with ruptures of the thin wall (Figure 6).

Figure 7 shows sharp-edged spicules in the same sample of lake-water that contained the *R. seeberi*-

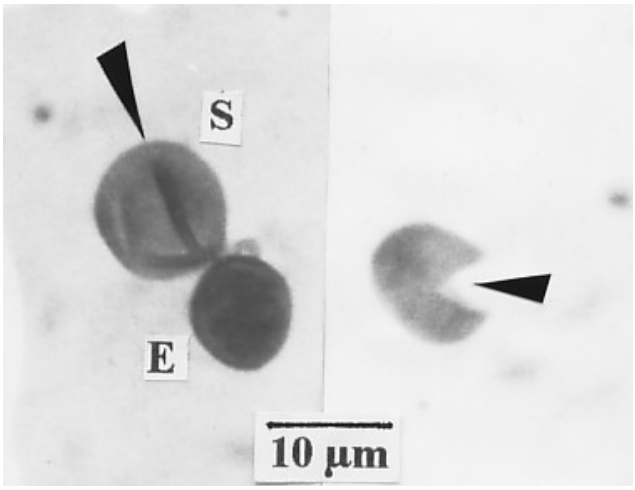

Figure 6. A smear from a nasal rhinosporidial polyp stained for the periodic acid-Schiff showing a small juvenile sporangium (S) with a ridge (arrow) representing a collapsed thin wall, adjacent to an endospore (E), and a small juvenile sporangium with a ruptured wall (arrow).

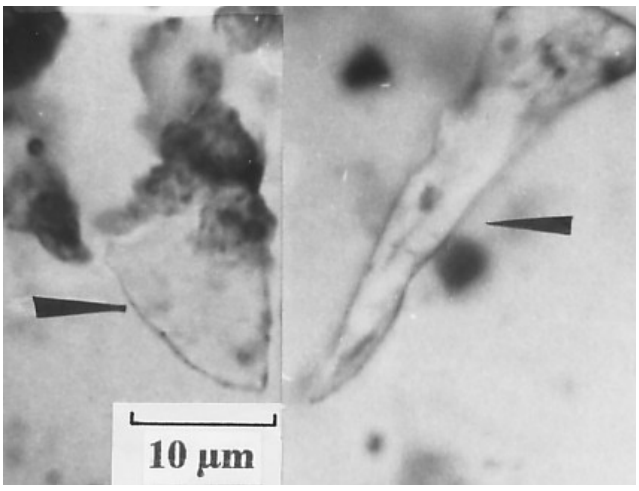

Figure 7. Suspended, sharp-edged spicules (arrows), probably derived from sand, in the lake-water that contained the rhinosporidial entities shown in Figures 4 and 5.

specific probe-labelled entities of Figure 4a, 4b, 5a, and 5b; these spicules probably originated from sand, and it could theoretically be responsible for injuries on the delicate nasal and ocular mucosae that could lead to colonization by the aquatic *R. seeberi* during vigorous immersion in the water while bathing.

DISCUSSION

The *R. seeberi*-specific probe used in this study was designed by Fredricks and colleagues,[3] who found it to be specific in labelling rhinosporidial elements in tissue from human nasal polyps. This *R. seeberi*-specific probe did not bind to three negative controls including *Coccidioidomyces immitis* in coccidioidomycotic tissue, samples of a cell line infected with the rosette agent (that is in the same DRIP clade as *R. seeberi*), and *Candida albicans* on smears.[3] Their control probe[3] was also used in this study, and did not bind to the rhinosporidial elements in the infected tissue.[3]

There has hitherto been no unequivocal identification of the natural habitat of *R. seeberi,* partly due to inability to culture the organism in vitro, and the inability to induce rhinosporidiosis in experimental animals with deposits from ground water, or with rhinosporidial tissue homogenates. The inability to establish experimental rhinosporidiosis with inocula of human or animal rhinosporidial tissues could be due to host-specificity of *R. seeberi*[7] that prevents strains from humans, from establishing disease in animals. Cogent circumstantial epidemiological evidence exists, in reports from India[8,9], on rhinosporidiosis in river-sand workers,[10,11] and also from Nepal[12] that the natural habitat of *R. seeberi* is ground water in lakes or rivers. The evidence was strengthened by reports of an outbreak of rhinosporidiosis in swans in Florida, USA[13] and an outbreak in humans who had bathed in a lake in Serbia.[14] Further support for an

aquatic habitat for *R. seeberi* is the recent re-classification using molecular biological techniques in a new Class the Mesomycetozoea[1] with amphibian and fish pathogens. This Class includes microorganisms that are also aquatic in habitat. A frog-pathogen *Amphibiocystidium ranae* has been identified as belonging to the Class Mesomycetozoea[15], adding support to the aquatic affinities of *R. seeberi*. In our series of 84 cases of rhinosporidiosis with a history of having bathed outdoors, 77 percent had bathed in lakes, 14 percent in rivers, and 8 percent in wells. These rhinosporidiosis patients and asymptomatic individuals in Sri Lanka showed appreciable titres of anti-rhinosporidial antibodies[16] (both groups having a history of regular bathing in lakes including that from which the water deposit examined in this study was obtained). In the latter group, exposure and sub-clinical immunization to the aquatic *R. seeberi* is thought to be the probable cause of their humoral immune response.

A wider epidemiological survey with characterization of ground waters is planned.

Preventive measures against infection by *R. seeberi*.

With this definitive identification of *R. seeberi* in a deposit of ground water, preventive measures against the acquisition of rhinosporidial infection by bathers could include the avoidance of trauma from vigorous immersion in the water. It is surmised that spicules, derived from sand particles, could act as a predisposing factor for colonization. Figure 7 illustrates the microscopic, sharp-edged spicules in the reservoir-water deposit that was examined in this study, that could cause mucosal injuries. The use instead of container-borne water for bathing would be preferable. Sand-workers, who are among those predisposed to nasal rhinosporidiosis, could be advised to avoid collection of river-bed sand by diving, and to use a sand-scoop attached to a long handle, actuated from above the surface of the water, instead.

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