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Isolation of *Basidiobolus ranarum* from ectotherms in Antwerp zoo with special reference to characterization of the isolated strains

Charakterisierung von *Basidiobolus ranarum*—Isolaten aus wechselwarmen Tieren des Antwerpener Zoos

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Schlüsselwörter. *Basidiobolus ranarum*, Wechselwarme, Enzymaktivität, Pathogenität, Zoo.

Summary. Ten *Basidiobolus ranarum* (= *Basidiobolus haptosporus*) strains, isolated from faeces of 102 different lower vertebrates (ectotherms) exhibited in Antwerp Zoo, or from their environment were studied for their temperature requirements, haemolysis and other enzyme activities *in vitro*. All isolates grew well at 25 and 37 °C. Three strains that produced undulated zygospore walls were haemolytic and positive for hyaluronidase. All the isolates produced urease, N-acetyl-β-glucosaminidase, trypsin, lipase, lecithinase, gelatinase, collagenase and elastase, but failed to produce amylase, keratinase and β-glucosidase. Three isolates failed to produce phosphatase. Only one strain failed to produce DNase. Aesculin was not hydrolysed. Chitinase activity was inconclusive. The results of this study illustrate the importance of exotic animals kept in temperate regions as carriers of potentially pathogenic organisms. In addition to the morphological characteristics, the identification can be based on enzymatic profiles. Enzymatic activity detection may help to explain the pathogenic mechanism of the fungus.

Zusammenfassung: Zehn *Basidiobolus ranarum*-Stämme (=*Basidiobolus haptosporus*), isoliert aus Exkrementen oder Umweltproben, gesammelt von 102 verschiedenen niederen Wirbeltieren (Ectothermen), welche im Antwerpener Zoo ausgestellt sind, wurden auf Temperaturbedarf, Hämolyse und anderen enzymatische Aktivitäten *in vitro* untersucht. Alle Isolate wuchsen gut bei 25 °C und 37 °C. Drei Stämme mit gewellten Zygosporenwänden waren hämolytisch und positiv im Hyaluronidase-Test. Alle Isolate waren positiv für Urease, N-acetyl-β-glucosaminidase, Trypsin, Lipase, Lecithinase, Gelatinase, Collagenase und Elastase, jedoch negativ für Amylase, Keratinase und β-Glucosidase. Drei Isolate waren Phosphatase-negativ. Nur ein Stamm zeigte keine DNase-Aktivität. Asculin wurde nicht hydrolysiert. Chitinase-Aktivität war nicht eindeutig nachweisbar. Diese Ergebnisse veranschaulichen die Bedeutung exotischer Tiere in gemässigten Regionen als Träger möglicherweise pathogener Organismen. Zusätzlich zur morphologischen Charakterisierung kann auch das enzymatische Profil zur Identifizierung der Pilze dienen. Enzym-Analysen können auch zur Erklärung von Pathomechanismen beitragen.

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Introduction

Basidiobolus ranarum (=*Basidiobolus haptosporus*), a fungus belonging to the order *Entomophthorales* of the *Zygomycetes*, was first isolated from the intestinal contents of frogs and lizards by Eidam [1]. This saprophytic fungus has a world-wide distribution. It is commonly associated with plant detritus and may be present as a commensal in the intestinal tract of reptiles and amphibians [2–6].

Exceptionally cutaneous mycoses in frogs were ascribed to this fungus [7]. *Basidobolus ranarum* may cause basidiobolomycosis in humans. It is generally assumed that patients become infected through abrasions of the skin, but it is not clear why the fungus predominantly affects children [8–13]. Cases have also been observed in horses [14, 15] and may occasionally be seen in other animals [6, 16]. The majority of clinical reports concerning this fungus have been from tropical and subtropical regions. This is in contrast with the worldwide distribution of the species.

Various species of *Basidiobolus* can be distinguished by their optimum growth temperature, by a smooth or undulated zygospore wall or by a *Streptomyces*-like odour. The determinative character of these features is doubtful. Only *Basidobolus microsporus* is recognized as a separate species by the production of exogenous microspores. Other species are considered to be synonyms of *B. ranarum* [17–19]. Immunodifusion techniques have been employed to provide a clearer understanding of the taxonomic relationships of *Basidiobolus* species [20, 21].

An accidental isolation of the fungus from the intestinal contents of a green iguana (*Iguana iguana*), newly acquired in Antwerp Zoo, prompted the examinination of the collection of reptiles and other cold-blooded animals. Special attention was paid to the *in vitro* production of enzymes by the isolated strains. The enzyme profiles can determine the biochemical homogeneity of the isolates and can assist in the identification of atypical, e.g. asporogenous strains. Within the order, *Entomophthorales*, *Basidiobolus* sp. can be differentiated from *Conidiobolus* sp. by the determination of trypsin and the absence of both β-glucosidase activity and aesculin hydrolysis [22].

Apart from breaking down fatty and proteinaceous material in nature, the extracellular enzyme production of *B. ranarum* probably plays an important role in the pathogenesis of human and animal infection. Collagenase and elastase act on the fibre matrix bonding epidermis to dermis and destroy the connective tissue, thereby promoting the spread of infection. The enzymes intervene in the formation of a granulomatous inflammation [23]. Hyaluronidase hydrolyses hyaluronic acid, a component of the intracellular ground substance of connective tissue, thereby facilitating the invasivenes of micro-organisms [24]. Lipase degenerates the fatty tissue [13]. Hydrolysis of lecithin to lysolecithin destroys cell membranes and is capable of causing tissue necrosis [25]. Invasion of muscle and internal viscera may be aided by enzymes [12, 15, 26]. The present report examines the enzymatic profile of the isolated strains in order to demonstrate their potential pathogenicity.

Materials and methods

Animals investigated

During a 3 month research period in Antwerp Zoo (April 1997–June 1997), a total of 140 samples were collected. Seventy different reptiles (36 lizards, 14 turtles and 20 snakes), 16 amphibians (seven frogs, two toads and seven salamanders) and 16 fresh-water aquaria fish were examined. In the case of the reptiles, faecal material or intestinal content were sampled. For amphibians and fresh-water fish, faecal material collected from the bottom of the aquarium or filter material were examined. In addition, grasshoppers, crickets and mealworms bred in the zoo to feed the reptiles, bloodworms and tubifex to feed the fish, and trapped free-living cockroaches, were ground and approximately 10 g of each was examined.

Isolation of fungi

Faecal material and food samples were diluted 1/10 in sterile distilled water. Pipetted aquarium deposits and liquid samples obtained by wringing out filter cotton-wool were used without further dilution. The isolation of *Basidiobolus* sp. was attempted by the procedure employed by Coremans-Pelseneer [2]. A 1 ml sample was poured onto a sterile moistened filter paper placed on a Petri dish lid. The bottom of the dish, containing Sabouraud glucose agar (SGA) supplemented with kanamycin and penicillin, was inverted over the lid and the plate incubated at 25 °C in daylight. In the case of positive samples, ballistosporic conidia were ejected onto the agar surface and small colonies developed after 2 to 3 days incubation.

Characterization of the isolates

Growth temperature

For comparison of temperature requirements the *Basidiobolus* isolates were transfered to SGA plates and incubated for 1 week at 25 °C. Inocula taken with a cork borer (6 mm diameter) were placed on SGA plates and incubated at 25, 37 and 42 °C for 3 days. The temperature preference was determined by comparing the colony diameter.

Haemolysis

Haemolysis was tested on Tryptone soy agar (TSA) (Lab M, Lancashire, UK) enriched with 5% horse blood.

Enzymatic detection

The enzymatic tests performed were essentially based on the methods described by Hankin and Anagnostakis and common microbiological identification methods [27, 28]. Each isolate was grown on SGA at 25 °C for 1 week. The pH of the test media was adjusted to be between 6 and 7. Growth and reactions on solid media were examined for 1 week. Liquid media were incubated for up to 1 month. All experiments were repeated once and incubation was carried out at 25 °C.

Chitinase: Chitinase production was determined on TSA containing 2.4% purified chitin (ICN). Formation of clear zones around the colonies in the opaque agar was indicative of chitinase activity. Following Coremans-Pelseneer, the action of the isolates on chitin particles added to Sabouraud glucose broth was also examined [2].

Hyaluronidase: The rapid plate method for screening hyaluronidase-producing microorganisms described by Smith and Willett was used [29]. An agar medium that was based on brain heart infusion broth (Lab M) was enriched with 1% bovine albumin fraction (ICN Biomedicals, Asse-Regelem, Belgium) and an aqueous solution of umbilical sodium hyaluronidate (ICN) in a final concentration of 400 $\mu g\ ml^{-1}$. After incubation, the plates were flooded with 2N acetic acid for 10 min and examined for the formation of clear zones around the colonies.

Lipolytic activity: Lipolytic enzymes were tested on the medium of Sierra [30] supplemented with 1% polyoxyethylene sorbitan monolaurate (Tween 80 Merck-Belgolabo, Overijse, Belgium). Formation of a visible precipitate, composed of the calcium salts of lauric acid, around the colonies was indicative of lipolytic production.

Lecithinase production was determined by the formation of an opalescent zone around the colonies on TSA that was enriched with 1% sterile egg yolk.

Proteolytic activity: Proteolytic activity was either demonstrated by the production of clear zones beneath and around colonial growth on solid media, or the digestion of the substrate in liquid media. Different substrates were examined: TSA enriched with 0.3% bovine elastin (ICN), Sabouraud glucose broth in which a developed X-ray film strip was placed for gelatinase detection, Sabouraud glucose broth with 0.3% collagen from bovine achilles tendon (ICN) and TSA enriched with 0.3% bovine keratin (ICN). The keratinolytic properties of the isolated strains were also examined on a solid medium containing solubilized keratin. This medium was originally developed to test the keratinophilic properties of dermatophytes [31].

Amylolytic activity: The degradation of starch was examined on Mueller-Hinton agar (Lab M) containing 0.15% starch. After incubation the plates were flooded with a iodine solution (lugol) and checked for the formation of a yellow zone around the colonies in an otherwise blue medium.

Deoxyribonuclease: DNase agar (Lab M) was used. After incubation the colonies were flooded with 1 N HCl. The formation of clear zones in an otherwise opaque medium indicated degradation of deoxyribonucleic acid.

Urease: Christensen's urea agar base (Lab M) for the rapid detection of urease production was used. Urease-positive cultures produced an alkaline reaction evidenced by a red colour.

Phosphatase: Production of phosphatase was tested on TSA enriched with 2 ml of 0.01 M phenolphthalein diphosphate sodium salt (ICN). After incubation the plates were inverted over a bottle of ammonium hydroxide. Colonies that turned pink to red were presumed positive.

Additional tests: The enzymes tested were primarily of taxonomic importance. N-acetyl-β-glucosaminidase, β-glucosidase and trypsin were determined using diagnostic tablets (Rosco Diatabs, Rosco, Taastrup, Denmark). Readings were made after overnight incubation. Hydrolysis of aesculin was examined on Aesculin-Bile agar (Merck) and on aesculin agar with the same composition but without ox bile.

Results

Five *B. ranarum* strains were isolated from the faecal material of lizards and turtles. Five more strains were isolated from filter material or deposit samples of fresh water aquaria housing amphibia or fish (Table 1). During the 3 month research period, the fungus could be isolated weekly from the intestinal content of the green iguana (No. 1) and the Yemen chameleon (No. 3). No isolates

Table 1. Origin of *Basidiobolus ranarum* strains isolated in Antwerp zoo

No.	
Reptiles	
1	Green iguana (*Iguana iguana*)
2	Brown basilisk (*Basiliscus basiliscus*)
3	Yemen chameleon (*Chamaeleo calyptratus*)
4	European chameleon (*Chamaeleo chamaeleon*)
5	Chinese softshell (*Trionyx sinensis*)
Amphibians	
6	Spanish newt (*Pleurodeles waltl*)
7	Axolotl (*Ambystoma mexicanum*)
8	Dendrobatid frog (*Dendrobates* sp.)
Fish	
9	Mexican cave fish (*Anoptichthys jordani*)
10	Mexican cave fish (*Anoptichthys jordani*)

1–5 *Basidiobolus ranarum* isolated from faecal material.
6–10 *B.ranarum* isolated from filter material or deposit in aquaria.

were obtained from the food samples examined. Colonies on SGA were greyish white, flat and radially furrowed, with a folded centre. The surface had a waxy aspect becoming velvety over time as a result of very short aerial mycelial growth. No *Streptomyces*-like odour was noticed. Microscopic examination revealed numerous thick-walled zygospores (20–45 μm) with characteristic copulatory beaks. Most isolates displayed zygospores with smooth walls. Two isolates formed undulated zygospore walls (No. 1 and No. 9) and one isolate produced both smooth and undulated zygospore walls (No. 10). After repeated subculturing, the colonies lost their ability to form zygospores. The temperature studies showed that all strains grew equally well at 25 and 37 °C. Four strains (No. 1, 5, 6 and 7) demonstrated reduced growth at 42 °C. Only three strains were haemolytic (No. 1, 9, 10). The production of enzymes on solid or in liquid media is shown in Table 2. All of the isolates produced urease, lipase, lecithinase, gelatinase, collagenase and elastase activity. Most strains were positive for DNase and phosphatase. The three strains that were haemolytic also produced hyaluronidase. Amylase and keratinase activity were not observed. Some chitinase activity was noticed after incubation for 1 week, but reactions were too faint to be conclusive. In liquid medium, the chitin particles were colonized by the fungus, in contrast to collagen that was totally digested after 1 month incubation. This digestion of collagen was accompanied by complete regression of fungal growth. All the isolated strains were positive for glucosaminidase activity but failed to produce β-glucosidase activity. The aminopeptidase test for trypsin was positive. Aesculin was not hydrolysed and bile tolerance was noted.

Discussion

The present work demonstrates the occurence of *B. ranarum* in the collection of reptiles, amphibians and fresh-water fish kept at Antwerp Zoo. It is noteworthy that of the infected animals, two lizards, one amphibian and a turtle were recently added to the zoological collection: the green iguana (No. 1) was newly imported from California, USA, the chameleon (No. 3) was obtained from a local merchant who asserted that the animal was locally bred offspring, the newt (No. 6) originated from Spain and the turtles (No. 5) were juveniles imported from Singapore. In 1973, Coremans-Pelseneer isolated *Basidiobolus* sp. from newly imported agamid lizards (*Uromastix acanthinurius*), but not from the reptile collection in Antwerp Zoo [2]. Presumably the fungus was introduced in the zoological collection by newly acquired animals originating from tropical regions. During a study in Nigeria, Gugnani and Okafor found 6.5% of the agamid lizards to be infected [3]. Obtaining isolates from the filter material of two fresh-water aquaria housing cave fish was an important finding, although it can not be proved that the fish were infected. The fungus may also have been derived from decaying plant material in the aquarium. Reports of isolates from fish are rare. Nickerson and Hutchinson mention the isolation of *B. ranarum* from fish [4]. Yang reported of *Basidiobolus* sp. as the cause of mortality in eggs and fry of the teleost fish *Cyprinus caprio* [32]. The insects used to feed the animals did not carry over the fungus. According to the literature insects are considered as transporters rather than reservoirs of *B. ranarum.* They can carry the fungus in their alimentary tract or on their cuticle without being parasitized [2, 33, 34]. Degeneration of chitin, the major substance of the insects cuticle, is considered an important feature for explaining the spread of the fungus by insects [2, 35]. The *Basidiobolus* isolates in the present study degraded chitin weakly or not at all. Following the observations of Coremans-Pelseneer, it was noticed that chitin particles in liquid medium were colonized [2].

No difference in growth rate was noticed on

Table 2. Enzymatic reactions of *Basidiobolus ranarum*

Enzyme	Case number 1	2	3	4	5	6	7	8	9	10
Chitinase	(+)	−	(+)	(+)	−	−	(+)	(+)	(+)	(+)
Hyaluronidase	+	−	−	−	−	−	−	−	+	+
Lipase	+	+	+	+	+	+	+	+	+	+
Lecithinase	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+
Collagenase	+	+	+	+	+	+	+	+	+	+
Elastase	+	+	+	+	+	+	+	+	+	+
Keratinase	−	−	−	−	−	−	−	−	−	−
Amylase	−	−	−	−	−	−	−	−	−	−
DNase	+	+	+	−	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+
Phosphatase	−	+	+	−	−	+	+	+	+	+
N-acetyl-β-glucosaminidase	+	+	+	+	+	+	+	+	+	+
β-glucosidase	−	−	−	−	−	−	−	−	−	−
Trypsin	+	+	+	+	+	+	+	+	+	+

+, positive; −, negative; (+), delayed, weak positive.

SDA at 25 °C compared with 37 °C. According to Gugnani and Okafor, growth at 37 °C is an essential requirement for the isolates to be potentially pathogenic, but could be simply due to adaptation [3]. In addition, no *Streptomyces*-like odour was produced and zygospore walls were smooth, undulated or both in the same culture. These observations affirm the questionable validity of these features as taxonomic criteria.

Only three isolates were haemolytic and also produced hyaluronidase, an important finding regarding the possible pathogenicity of the strains. Haemolysis by *Basidiobolus* sp. was also reported from human isolates by Dasgupta [10]. To the authors's knowledge, there are no reports on hyaluronidase activity by the fungus. The fact that the three aforementioned strains also formed zygospores with undulated walls distinguishes them from the other isolates and may suggest that they constitute a separate variety. Production of extracellular lipases and proteinase by *Basidiobolus* sp. was thoroughly investigated [25, 36, 37]. Okafor *et al.* studied 64 strains. All produced protease and lipase on solid media but failed to produce amylase and deoxyribonuclease [38]. These results compare favourably with the present work, except that all but one of the strains in this study produced deoxyribonuclease using the same method. The lack of keratinase production explains why the infection is restricted to subcutaneous regions. Consequently, infection must be initiated by injuries of the epidermis. Reports on urease and phosphatase activity are scanty. Coremans-Pelseneer mentioned variable results for urease activity and Fromentin found that the tests for the presence of alkaline and acid phosphatase were positive for 16 *Basidiobolus* strains [2, 22]. No differentiation was made between alkaline and acid phosphatase reaction in the present study, which may explain why three of the strains reacted negative.

No differences were noticed in the tests used for the taxonomic differentiation of the isolates compared with the results obtained by Fromentin [22]. The use of aesculin bile agar for the determination of aesculin hydrolysis revealed the bile tolerance of the strains.

All of the strains in the present study grew sufficiently well despite the high bile content (4%) of the test medium. It is believed that the bile tolerance is beneficial to the colonization of the intestinal tract. Coremans-Pelseneer stated that the sporangia are not killed in the reptile's stomach but increase in number by a budding process during their passage through the digestive tract [2].

The isolation rate of *B. ranarum* is not consistent with that of the tropics where over 40% of the lower vertebrates are infected [3], but illustrates the occurrence of this potential pathogenic fungus in aquaria and terraria in which a subtropical microclimate exists. Likewise, the isolations demonstrate the importance of exotic animals as carriers of unfamiliar pathogenic micro-organisms, creating a possible public health hazard for zookeepers and amateurs. The fungus grows on ordinary laboratory media under a wide range of pH and temperature conditions, allowing it to be detected by routine procedures. Growth of moulds on microbiological media should be taken into account before they are dismissed as plate contaminants.

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