Fungal flora of the digestive tract of *Panstrongylus megistus* (Reduviidae) used for experimental xenodiagnosis of *Trypanosoma (Schizotripanum) cruzi* Chagas, 1909

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Summary

A survey of the fungi isolated from the digestive tract of *Panstrongylus megistus* (insects vectors from Chagas' disease) used on xenodiagnosis was carried out. Two hundred and fourteen fungal strains were isolated from 180 nymphs. *Aspergillus* and *Penicillium* were the most predominant genera and some of their species were new records concerning insects. A great reduction in the fungal population was observed in the material that was positive for *Trypanosoma cruzi.*

Key words Fungi, Triatomine, Xenodiagnosis, *Panstrongylus megistus*

Utilización de la flora fúngica del tracto digestivo de *Panstrongylus megistus* (Reduviidae) para el diagnóstico experimental de *Trypanosoma (Schizotripanum) cruzi* Chagas, 1909

Resumen

Se llevó a cabo un estudio de los hongos aislados del tracto digestivo de *Panstrongylus megistus* (insectos vectores de la enfermedad de Chagas) que se utilizan en el xenodiagnóstico. Se aislaron 214 cepas de hongos a partir de 180 ninfas. Los géneros más frecuentes fueron *Aspergillus* y *Penicillium* y algunas de sus especies fueron nuevos registros en insectos. Se observó una importante reducción de la población fúngica en el material que fue positivo para *Trypanosoma cruzi.*

Palabras clave Hongos, Triatomas, Xenodiagnóstico, *Panstrongylus megistus*

Triatomine are obligatory hematophagous insects and vectors of *Trypanosoma cruzi* the etiological agent of Chagas' disease or American trypanosomiasis. This infection affects approximately five million people in Brazil [1] and 16 to 18 million in Latin America [2]. Triatomine are widely distributed all over the Americas (North, Central and South), as well as in Africa, Asia and Oceania [3], living in association with wild animals (marsupials, rodents, primates, birds, etc) or in the surrounding or domicile areas of human habitations. Therefore, the diffusion spectrum of the disease is very complex.

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Brumpt, in 1914 [4], developed a diagnostic test, xenodiagnosis, that consisted of application of nymphs bred in the laboratory, free from protozoan, on the skin of the infected host, in order to make them infected with circulating forms of *T. cruzi* which, after multiplication in the vector's digestive tract, can be easily identified by examination of its feces. Since then, researchers have been made to increase the xenodiagnosis' credibility and efficacy for the human and animal chronic American trypanosomiasis [5].

The association with a microbial flora in the digestive tract of several insects groups, such as Lepidoptera, Coleoptera, Isoptera, among others, is widely exemplified by studies of insect-fungus interactions [6-9] so as a wide variety of bloodsucking arthropods has been reported to contain symbiotic microorganisms, among these are the triatomine. In 1926, Duncan [10] discovered a Gram-positive bacillus in the gut of *Rhodnius prolixus*, later this bacteria was named *Nocardia rhodnii* in 1943 by Waksman & Henrici [11] and since then researchers are trying to stablish a consistent bacterial flora in Triatomine [12]. But the association of Triatomine with fungi, has received little attention [13]. However, there are publications that focus on the role of fungi in the biological control of some triatomine species [14], but those studies were based on

the use of fungi that had been isolated from other insects or hosts.

The importance of the insect-fungus interaction in the development of *T. cruzi* in the digestive tube of the vector has not been investigated so far. However, Dias [15] and Jandin [16] suggested that the presence of bacteria in the digestive tract of adult triatomine and nymphs could reduce *T. cruzi* infection in the species *Panstrongylus megistus*, *Rhodnius prolixus* and *Triatoma infestans*.

The objectives of this study were to identify the microfungi present in the digestive tract of *P. megistus* used in xenodiagnosis, their distribution patterns and their possible role in the development of *T. cruzi.*

MATERIAL AND METHODS

Triatomine and xenodiagnosis. The nymphs of *P. megistus* were donated by the Laboratory of Triatomine of the Department of Tropical Medicine, Oswaldo Cruz Institute - FIOCRUZ (Rio de Janeiro - Brazil). The triatomine were kept at a temperature of 28 ˚C ± 1 ˚C and 75 % air humidity, and hens (*Gallus gallus*) were used to feed them once a week.

Nymphs of third and fourth instar were selected after fasting from two to three weeks and, then separated out into two groups. According to the xenodiagnosis technique described by Shenone [17] and Cerisola [18], mice and dogs were used to feed the first group of nymphs. Those animals were infected with the VLE 99 *T. cruzi* strain, isolated from a chronic human tripanosomiasis from an endemic area in Brazil. Hens (*G. gallus*) were used to feed the second group and it was kept as control group.

After 23 days from the first feeding, all the nymphs, including the control group, were fed using *G. gallus* in order to avoid a too long fasting. The dissection of the digestive tract was made after 22 days of the second feeding [19-21].

In a fume hood, the nymphs were killed with chloroform and consecutively washed for 2 min each with 1 % sodium hypochlorite, sterile distilled water, alcoholic iodine and 70 % alcohol. After this procedure, each group of insects was dissected according to Lacombe & Rangel [22] and the digestive tract removed. These digestive tracts were macerated in 0.4 ml phosphate buffered saline (PBS, Sigma, USA) and microslides containing macerated samples of each digestive tract were observed under light microscope to detect the presence of *T. cruzi.* The positive and negative digestive tracts were separated and, then, "pools" were carried out with 5 to 15 tracts each.

The same procedure was carried out with the nymphs that did not undergo xenodiagnosis.

Processing and isolation. The "pools" were processed according to Lachaise *et al.* [23] and 0.2 ml of each dilution were seeded on Petri dishes containing the following culture media (to which chloramphenicol was added): PDA (Potato Dextrose Agar, Difco, USA); MEYE [Malt extract, yeast extract (Difco) and agar (Merck, Germany)] and YEPGA [Yeast extract, peptone (Difco), glucose (Merck) and agar] and incubated at a room temperature ± 28 ˚C. During the first ten days, daily examinations were carried out in order to observe fungal growth and to count colonies, followed by examinations every three days until 21st day.

The isolated colonies were subcultured in PDA and Malt extract (Difco) medium for identification. The microscopic characteristics produced by the species were studied using the technique of culture on slide. The material was colored with 10 % KOH for the representatives with dark pigmentation and with Amann's lactophenol with cotton blue for the hyaline isolated representatives and observed under a Nikon model Labophot light microscope. Species identification was made according to Ellis [24,25]; Pitt [26,27], Raper & Fennell [28], Christensen & Raper [29-31] and Samson [32,33].

Representative cultures of the species studied were preserved in PDA under mineral oil and added to the Fungal Culture Collection of the Department of Mycology (IOC) - FIOCRUZ.

RESULTS

In this work, 180 fourth instar nymphs of *P. megistus* were selected. Six xenodiagnosis tests were made using 20 to 30 nymphs each one. Among 140 nymphs which were fed using the infected animals, 75 of them development the *T. cruzi* in their digestive tract (53 %) but 65 remained unable to colonize or multiply the *T. cruzi* (47 %). There were not *T. cruzi* in the digestive tract of the control group of 40 nymphs.

From 180 nymphs processed, 214 fungal strains were isolated, including 197 from the nymphs which were fed using the infected animals. One hundred eighty-five strains were isolated from nymphs which were negative for the protozoan, and only 12 were from the positive ones (Table 1). Seventeen strains were isolated from the control group (Table 2).

The isolated microfungi were classified into 13 genera and 37 species. The genera found and their respective species numbers were: *Aspergillus* 12, *Penicillium* 10, *Cladosporium* three, *Curvularia, Fusarium* and *Trichoderma* two for each, *Acremonium, Alternaria, Botrytis, Colletotrichum, Paecilomyces* and *Ulocladium* one species each. Some isolates ones remained sterile after several attempts made to induce sporulation and are here referred to as *Mycelia sterilia*. The most frequent species were *Penicillium corylophilum, Aspergillus fumigatus* and *Aspergillus awamori.*

DISCUSSION

The samples of *P. megistus* used in xenodiagnosis in naturally and experimentally infected mammals have presented a high density of parasites, as well as one of the highest positive indices in relation to other samples of other species. Based on this, their routine use in xenodiagnosis is recommended [34-36].

Among the fungi isolated from the digestive tract of *P. megistus*, a predominance of the genera *Aspergillus* and *Penicillium* could be observed in the nymphs fed using infected dogs and mice, but in the control group, the genera *Curvularia* and *Trichoderma* were the predominant. In the specialized literature, *Aspergillus* and *Penicillium* appear widely related to different types of insects such as bees [6,37], termites [38], mosquitoes [9,39], beetles [40] and Lepidoptera [8] as well as in triatomine [13,41]. The predominance of the genera *Curvularia* and *Trichoderma* in the control group specimens was due to the use of only one feeding source for those nymphs.

New records are cited for the first time in this work since *Penicillium lividum, Penicillium steckii, Aspergillus auratus, Aspergillus candidus, Aspergillus janus, Curvulatia clavata, Botrytis alli, Ulocladium botrytis* and *Paecilomyces variotii* have never been reported associated with insects in the literature. *Cladosporium cladosporioides, Fusarium oxysporum* and *Fusarium solani* are proven

Table 1. Isolated fungi from the digestive tract of *Panstrongylus megistus* nymphs used in the xenodiagnosis tests.

Xenodiagnosis tests	Isolated fungi from nymphs	
	Positives[a]	Negatives[b]
1	No fungal growth	*Paecilomyces variotii* (1)
2	*Botrytis alli* (1)	*Alternaria alternata* (1), *Penicillium corylophilum* (1)
3	*Cladosporium herbarum* (2), *Colletotrichum* sp. (1), *Ulocladium botytis* (1)	*Cladosporium cladosporioides* (1), *Cladosporium musae* (3), *Aspergillus fumigatus* (7), *Aspergillus niger* (4), *Mycelia sterilia* (2)
4	No fungal growth	*Aspergillus candidus* (1), *Aspergillus kanagawaensis* (2), *Cladosporium cladosporioides* (1), *Mycelia sterilia* (1), *Penicillium corylophilum* (23), *Penicillium decumbens* (5), *Penicillium fellutanum* (9), *Penicillium implicatum* (2), *Penicillium janthinellum* (23), *Penicillium waksmanii* (8), *Trichoderma pseudokoningi* (1)
5	No fungal growth	*Aspergillus awamori* (14), *Aspergillus fumigatus* (2), *Aspergillus janus* (5), *Aspergillus kanagawaensis* (1), *Aspergillus ochraceus* (1), *Fusarium oxysporum* (1), *Paecilomyces variotii* (5), *Penicillium corylophilum* (5), *Penicillium implicatum* (1), *Penicillium janthinellum* (6), *Penicillium purpurogenum* (1), *Penicillium waksmanii* (4)
6	*Aspergillus auratus* (1), *Aspergillus awamori* (2) *Aspergillus fumigatus* (2), *Aspergillus sydowi* (1) *Curvularia brachyspora* (1)	*Acremonium sordidulum* (1), *Aspergillus aureolatus* (1), *Aspergillus awamori* (2), *Aspergillus flavus* (4), *Aspergillus ochraceus* (2), *Aspergillus niger* (2),*Aspergillus terreus* (7), *Fusarium solani* (2) *Penicillium citrinum* (1), *Penicillium corylophilum* (20), *Penicillium steckii* (1)

a = 75 digestive tracts; b = 65 digestive tracts; ( ) = number of isolated strains.

Table 2. Isolated fungi from the control group.

Nymphs	Isolated Fungi
Pool 1[a]	*Curvularia clavata* (7), *Trichoderma harzianum* (5)
Pool 2[b]	*Aspergillus sydowi* (1), *Penicillium corylophilum* (2) *Penicillium lividum* (1)
Pool 3[c]	*Aspergillus aureolatus* (1)

a = 10 digestive tracts; b = 15 digestive tracts; c = 15 digestive tracts; ( ) = number of isolated strains.

entomopathogenic species for several insects groups such as Lepidoptera, Coleoptera, among others, according to Roberts & Humber [42], but none of the examined triatomine showed signs of fungal infections.

In the studies carried out by Friend & Smith [43] we can observe that triatomine physiology contributes to the possible colonization and establishment of the fungal population. The digestive process of triatomine is slow (± two weeks), which is sufficient time for the adhesion of the conidium. Furthermore, during feeding and some time after ingestion, the stomach and its wall undergo a "plasticization" which causes a greater hydration of the area, a factor that is also important for the fungi development. Another factor is the existence of only two enzymes found, up to now, in the whole digestive tract of those insects, ATPase in the salivary glands and proteinase in the stomach, which are not efficient in the digestion of the fungal wall, which is basically composed of chitin and glucans [44].

Some studies [15,16] indicated that triatomine perhaps have a mutual symbiotic association with microorganisms which synthesize vitamins of complex B which are deficient in the blood. These associations, however, would not be obligatory and could happen only when triatomine are under inadequate nutritional conditions. This is, possibly, the reason why several researchers failed to demonstrate the presence of a constant microbial flora in the digestive tract of triatomines [43,45,46].

Schlein *et al.* [9] observed, in several *Phlebotomus* species from endemic areas for leishmaniasis, that the insects that presented fungi in their digestive tract did not contain any protozoan form. Similarly, in our study a great reduction in the fungal population was observed in the material positive for *T. cruzi*. In three of the six-xenodiagnosis tests carried out there was no fungal growth in the positive material (Table 1). Besides the insects are different models, the Schlein's work was a point to leads us to speculate that perhaps the presence of fungi in the intestinal tract of triatomine has an influence on *T. cruzi* colonization. Various studies have already been done regarding bacterial flora in the digestive tract of triatomines [12], however none of these relate this flora with possible interference in the development of the protozoan, nor has any variation in the flora been observed in relation to the presence or absence of same. So the different qualitative and quantitative distributions of some fungus species in the digestive tract of the nymphs negative to the *T. cruzi* (Table 1) lead us to think of the possibility that those fungi may be inhibitors of the parasite growth.

Despite the variations noted in our results, various points should be taken into consideration. One of these is the competion that exists between the microorganisms for the nutrients, even though their survival in a hostile environment is already an indication of adaptation to or corelationship with the environment. Another important point is the immune system of the insect itself. Mello and collaborators [47], observed that the presence of *T. cruzi* in the hemolymph of *R. prolixus* activates various components of the insect's immune system, but that this activaion does not occur when *Escherichia coli* have been inoculated, with the system remaining de-activated. Thus, the presence of *T. cruzi* perhaps leads the insect's immune system to attack the fungi present as well, causing a reduction in the population of these fungi.

Further studies are needed, using *in vitro* and *in vivo* models with germ-free insects to verify the type of association between fungi and insects, and the possible role of the fungus as a barrier to protozoan colonization.

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References

1. Dias JCP. Epidemiology of Chagas disease. In: S Wendel *et al.* (Eds.) Chagas Disease (American Trypanosomiasis): It's impact on transfusion and clinical medicine. Sociedade Brasileira de Hematologia e Hemoterapia, São Paulo, 1992: 49-80.
2. Moncayo A. Chagas' Disease. UNDP World Bank WHO Special Programme for Research and Training in Tropical Disease (TDR). Tropical Disease; Progress in International Research, Geneva , 1993: 67-75 (Programme Report, 11)
3. Barretto MP. Epidemiologia. In: Brener Z, Andrade ZA (Eds.) *Trypanosoma cruzi* e Doença de Chagas. Rio de Janeiro, Guanabara Koogan, 1979: 89-151.
4. Brumpt E. O xenodiagnóstico. A aplicação ao diagnóstico de algumas infecções parasitárias e em particular à Trypanosomose de Chagas. An Paul Med Cir 1914; 13: 97-102.
5. Pereira JB. Xenodiagnóstico na Doença de Chagas Crônica. I - Sensibilidade de *Panstrongylus megistus* e *Triatoma infestans.* Rev Bras Med Trop1996; 29: 341-347.
6. Gilliam M, Prest DB. Fungi isolated from the contents of foraging worker honey bees, *Apis mellifera.* J Invertebr Pathol 1972; 20: 101-103.
7. Gilliam M, Prest DB. The mycoflora of selected organs of queen honey *Apis mellifera.* J Invertebr Pathol 1977; 29: 235-237.
8. Ismail MA, Abdel-Sater MA. Fungi associated with Egyptian cotton leafworm *Spodoptera littoralis* Boisdoval. Mycopathol 1993; 124: 79-86.
9. Schlein Y, Polacheck I, Yuval B. Mycoses, bacterial infections and antibacterial activity in sandflies and their possible role in the transmission of Leishmaniosis. Parasitol 1985; 90: 57-66.
10. Duncan JT. On bactericidal principle present in the alimentary canal of insects and arachnides. Parasitol 1926; 18: 238-252.
11. Waksman SA, Henrici AT. The nomeclature and classification of Actinomyces. J Bacteriol 1943; 46: 337-341.
12. Nyirady SA. The germfree culture of three species of Triatominae: *Triatoma protracta* (Uhler), *Triatoma rubida* (Uhler) and *Rhodnius prolixus* Stal. J Med Entomol 1973; 10: 417-448.
13. Cavanagh P, Marsden PD. Some bacterial symbionts of reduviid bugs. Trans R Soc Trop Med Hyg 1969; 63: 431.
14. Sherlock IA, Guitton N. Observações sobre a ação do fungo *Metharrhizium anisopliae* sobre algumas espécies de triatomíneos (Hemíptera, Reduviidae). Rev Inst Med Trop São Paulo 1982; 24: 234-239.
15. Dias E. Sobre a presença de symbiontes em Hemípteros hematófagos. Mem Inst Oswaldo Cruz 1937; 32: 165-168.
16. Jandin J. Du rôle des bactéries dans le tube digestif des insectes vecteurs des plasmodidae et des trypanosomidae. Ann Soc Belgium Med Trop 1967; 47: 331-342.
17. Schenone H. Valor del xenodiagnostico en la infección chagásica crónica. Bol Chil Parasitol 1968; 23: 149-154.
18. Cerisola JA. El xenodiagnóstico. Buenos Aires: Instituto Nacional de Diagnóstico e Investigación de la Enfermedad de Chagas "Dr. Mario Fatala Chaben". 1974; 111p. (Monography)
19. Dias E. Estudos sobre o *Schizotrypanum cruzi.* Rio de Janeiro: Faculdade de Medicina da Universidade de São Paulo. 1933; 115p. (Theses PhD)
20. Dias E. Técnica do xenodiagnóstico na moléstia de Chagas. Mem Inst Oswaldo Cruz 1940; 35: 156-168.
21. Salgado SAA. Consideraciones sobre metologia y sensibilidad del xenodiganóstico. Bol Chil Parasitol 1969; 24: 9-13.
22. Lacombe D, Rangel EF. Estudos de anatomia e histologia sobre a subfamilia Triatominae (Heteroptera, Reduvidae). XXIV: Anatomia comparada do aparelho digestivo de algumas espécies de Triatomíneos. Rev Bras Biol 1977; 37: 375-383.
23. Lachaise D, Pignal MC Ronault J. Yeast flora partitioning by Drosophilids species inhabiting a tropical African savanna of Ivory-coast. Ann Soc Entomol Fr 1979; 15: 659-680.
24. Ellis MB. Dematiaceous Hyphomycetes. London, CAB Press, 1971.
25. Ellis MB. More Dematiaceous Hyphomycetes. London, CAB Press, 1976.
26. Pitt JI. The genus *Penicillium.* Sydney, Academic Press, 1979.
27. Pitt JI. A laboratory guide to common *Penicillium* species. Sydney, Academic Press, 1985.
28. Raper KB, Fennell DI. The genus *Aspergillus.* Baltimore, The Willians & Wilkins Co. , 1965.
29. Christensen M, Raper KB. *Aspergillus robustus,* a new species in the *A. ochraceus* group. Mycologia 1978; 70: 200-205.
30. Christensen M. A synoptic key and evaluation of species in the *A. flavus* group. Mycologia 1981; 73: 1056-1084.
31. Christensen M. *A. ochraceus* group: Two new species from western soils and a synoptic key. Mycologia 1982; 74: 210-225.
32. Samson RA. *Paecilomyces* and some allied Hyphomycetes. Stud Mycol 1974; 6: 1-119.
33. Samson RA. A compilation of the aspergilli described since 1965. Stud Mycol 1979; 18: 1-38.
34. Perlowagora-Szumlewicz AS, Muller CA, Moreira CJC. Studies in search of a suitable experimental insect model for xenodiagnosis of hosts with Chagas'disease. 3 - On the interaction of vectors species and parasite strain in the reaction of bugs to infection by *Trypanosoma cruzi.* Rev Saúde Pub São Paulo 1988; 22: 390-400.
35. Perlowagora-Szumlewicz AS, Muller CA, Moreira CJC. Studies in search of a suitable experimental insect model for xenodiagnosis of hosts with Chagas'disease. 4 - The reflection of parasite stock in the responsivenes of different vector species to chronic infections with different *Trypanosoma cruzi* stocks. Rev Saúde Pub São Paulo 1990; 24: 165-177.
36. Pereira JB. Xenodiagnóstico na Doença de Chagas Crônica. I - Sensibilidade de *Panstrongylus megistus* e *Triatoma infestans.* Rev Bras Med Trop 1996; 29: 341-347.
37. Shoreit MN, Bagy MMK. Mycoflora associated with stonebrood disease in honey bee colonies in Egypt. Microbiol Res 1995; 150: 207-211.
38. Zoberi MH, Grace K. Fungi associated with subterranean termite *R. flavipes* in Ontario. Mycologia 1990; 82: 289-294.
39. Costa GL, Oliveira PC de. *Penicillium* species in mosquitoes from two Brazilian regions. J Basic Microbiol 1998; 38: 343-347.
40. Moore GE. Mortality factors caused by pathogenic bacteria and fungi of the southern Pine beetle in North Carolina. J Invertebr Pathol 1971; 17: 28-37.
41. Moraes AML, Junqueira ACV, Giordano CM. *Aspergilli* from the digestive tract of Brazilian Triatomids. Mycotaxon 1988; 66: 231-241.
42. Roberts DW, Humber, R. A. Entomogenous fungi. In: Barron GL (Ed.) Biology of conidial fungi. Vol. 2. New York, Academic Press, 1981: 201-236.
43. Friend WG, Smith JJB. La fisiología de los triatomínos con especial referencia a la alimentacion por sangue. In: Carcavalho RV, *et al.* (Eds.) Factores biológicos y ecológicos en la enfermedad de Chagas. Argentina, Organización Panamericana de la Salud, 1978: 55-72.
44. Bartnicki-Garcia S. Cell wall chemistry, morphogenisis and taxonomy of fungi. Annu Rev Microbiol 1968; 22: 87-108.
45. Moraes AML, Junqueira ACV, Giordano CM, Oliveira PC de. Microrganismos de Triatomíneos vetores do *T. cruzi.* II - Isolamento e caracterização de fungos do trato digestivo de triatomíneos. Mem Inst Oswaldo Cruz 1990; 85 Suppl. I: 114.
46. Moraes AML, Junqueira ACV, Giordano CM, Oliveira PC de. Micobiota do trato digestivo de triatomíneos silvestres vetores do *Trypanosoma cruzi.* Rev Bras Parasitol Vet 1993; 2 Suppl. I: 45-46.
47. Mello CB, Garcia ES, Ratcliffe NA, Azambuja P. *Trypanosoma cruzi* and *Trypanosoma rangeli:* Interplay with hemolymph components of *Rhodnius prolixus.* J Invertebr Pathol 1995; 65: 261-268.