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Anatomical and Histopathological Researches in Experimental Infection with *Prototheca* Algae in Mice

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Abstract. In order to realize this study, three BALB mice groups were used: one inoculated with *P. zopfii*, one with *P. wickerhamii* suspension and one control group. Each infected group was constituted of twelve animals, 2 months old, with an approximate body mass of 20 g.

Both *Prototheca* species: one referent strain *Prototheca wickerhamii* (RE-4608014 ATCC 16529) and one *Prototheca zopfii* isolate (from cow mastitic milk sample), were cultured in glucose broth medium, 48 hours, at 37ºC.

Except control group, each animal was i.p. inoculated with 0,1 ml of live alga suspension (approximately 10^7 CFU/ml). Two control mice were i.p. inoculated with 0,1 ml of sterile broth media.

Although any clinical disease sign was not observed in 10, 15 and 20 days post – infestation, for both inoculated groups, four mice were slaughted. Macroscopic lesions caused by *Prototheca* algae were represented by granuloma and piogranuloma found in different internals as confirmed by identification of organism by staining with periodic acid-Schiff (PAS) and culture. So appreciated illness severity we utilized a score system in order to obtained a lesional systemic score for each animal. We appreciated: limph nodes reaction, the presence or absence of granuloma in skin, liver, pancreas, diaphragm, kidney, spleen and intestine. The data were statistically process using "t" test. In group inoculated with *P. zopfii* the lesions were more severs than those evidenciated in *P. wickerhamii* infected group.

Key words: strains, protothecosis, granuloma, histopathological, microscopic, cultural

INTRODUCTION

Protothecosis is a rare infection caused by members of the genus *Prototheca*. These organisms are generally considered to be achlorophyllic algae and are ubiquitous in nature (Huerre *et al.*, 1993). Genus *Prototheca* include many species but the most important are: *P. zopfii* and *P. wickerhamii* (Pore, 1985).

Recently, a novel thermotolerant strain of *P. zopfii* was isolated from a hot spring (Ueno *et al*., 2002). Its taxonomic characteristics coincided with those of *Prototheca zopfii* var. *hydrocarbonea*, and phylogenetic analysis based on a small-subunit (SSU) rRNA gene sequence also revealed a close relationship between the two strains.

Prototheca species are spherical unicellular organisms ranging from 3 to 30 µm in diameter. These organisms do not possess glucosamine, a specific fungal cell wall component, or muramic acid, a specific component found in bacterial cell walls (Kockova-Kratochvilova *et al.*, 1974; Lloyd *et al*., 1968; Vorisek *et al.*, 1975). *Prototheca* species are distinguished from other algae, such as *Chlorella*, by their lack of chloroplasts and the presence of a two-layered, instead of three-layered, cell wall on electron microscopy (El-Ani, 1967; Joshi *et al.*, 1975; Nelson *et. al.*, 1987).

Algae were previously not considered pathogens in humans; *Prototheca* species isolated from previously damaged skin, blood, or feces have been interpreted as contaminants in the majority of cases. Sonck *et al*., (1971) recovered *P. wickerhamii* from five patients with dermatologic diseases but suggested *Prototheca* to be a skin saprophyte. The pathogenesis of protothecosis is largely unknown. It is believed that *Prototheca* species may infect humans through contact with potential sources or by traumatic inoculation with the algae (Cho *et al*., 2002; Follador *et al*., 2001; Jones *et al*., 1983; Kuo *et al*., 1987; Leimann *et al*., 2004).

In general, *Prototheca* spp. also seem to have low virulence in animal experiments. Phair *et al*., (1981) were unable to induce *P. wickerhamii* infections in neutropenic guinea pigs or athymic mice. Animals displayed local reactions at the point of injection despite high inoculum doses, and only a few cases of protothecosis have been caused in laboratory animals. In contrast, *P. zopfii* is lethal for immunosuppressed mice when used as an inoculum of 10^6 CFU. Overall, pathogenicity and virulence are moderate, and *Prototheca* species are considered rare opportunistic pathogens (Huerre *et al*., 1993).

In this report, we describe the development and characterization of experimental *Prototheca zopfii* and *Prototheca wickerhamii* infection in mice in order to elucidate and compare the pathogenic capacity of two species. We also proposed to isolate from internal lesions the etiological agent using microscopic and cultural exams.

MATERIALS AND METHODS

♦ Infection protocol – animals.

The experiment took place between June-August 2008, within Faculty of Veterinary Medicine Cluj-Napoca, Microbiology and Pathology departments. In order to realize this study three BALB mice groups were used: one inoculated with *P. zopfii*, one with *P. wickerhamii* suspension and one control group. Each group was constituted of twelve animals 2 months old, with an approximate bady mass of 20 g. For all the period of the experiment, animals were maintained in different caging and were fed with bread and oat.

♦ Algal suspension.

Prototheca zopfii isolates from cow mastitic milk sample and one referent strain *Prototheca wickerhamii* (RE-4608014 ATCC 16529) were used. Culture used for mice inoculation were grown under aerophilic conditions for 24 to 48 hours, at 37ºC in broth media supplemented with 2-3 drops of glucose 40%. Broth cultures were microscopic examined in a drop of Lügol solution for purity, and than for each culture we determined optical density, this being 580 nm (approximately 10^7 CFU/ml).

♦ Inoculation protocol. Mice groups used in experiment as well as the inoculation protocol are presented in the table below:

Table 1

Inoculation protocol

Group	Animals number	Infested material	Administrated doze	The place of inoculation
1	12	*P. zopfii*	0,1 ml/animal	i.p.
2	12	*P. wickerhamii*	0,1 ml/animal	i.p.
3	2	steril broth media	0,1 ml/animal	i.p.

♦ The exams performed. Although any clinical sign diseases was registered in 10, 15 and 20 days post-infestation four mice for each infected group were slaughtered. Anatomic and pathological aspects following protothecosis induced in experimental conditions were described. The appreciation of lesions severity in affected organs after each slaughter (10, 15,

20 day post-infestation), a scoring system of lesions was used obtaining a systemic lesional score for each animal. The following lesions were considered: lymph-nodes reaction, presence or absence in skin, liver, pancreas, diaphragm, kidney, spleen and guts of granuloma.

In the final part of experiment, the average notes for each animals was calculated, statistical analysis of systemic lesional score was performed, using "t" test comparing the arithmetic average results obtained after experimental infections realized in two infected groups. To confirm the alga presence in the affected nodular internal lesions, microscopic (slides from internals lesions), cultural (embedment on glucose agar) and histo-pathological (PAS stained) exams were performed. From the slaughtered animals lesions tissue fragments were collected. For histo-pathological exam tissues samples were clamping in 20% formalin solution, for 24 h than in 10% formalin solution for 14 days. Than the samples were included in paraffin, divided into sections, and after that stained with periodic acid-Schiff (PAS).

RESULTS AND DISCUSSIONS

♦ Macroscopic anatomopathological exam.

Lesions caused by algae from *Prototheca* genus consisted of granuloma and piogranuloma on skin, liver, pancreas guts and diaphragm. The results obtained in experimental mice protothecosis, were different in accordance with *Prototheca* species used for infestation. In case of *P. zopfii* inoculated group, infestation achieved to 83,3% from group, while in *P. wickerhamii* group positively percentage was only 50%. Also in group inoculated with *P. zopfii* the lesions registered were more severs than those evidenciated in *P. wickerhamii* infected group.

Table 2

Internals lesional score, dinamically registred in mice group inoculated with *P. zopfii*

Animal number (mouse)	Examination day	Severity lesions	Lympho-nodes reaction	Granuloma lesions							Final score
				in the skin	hepatics	pancreatics	renals	in spleen	in diaphragm	in the gut	
1	10	2	1	1	0	0	0	0	1	0	5
2		1	0	0	0	0	0	0	0	1	2
3		0	0	0	0	0	0	0	0	0	0
4		3	1	2	0	0	0	0	2	1	9
5	15	4	2	3	1	1	0	0	2	2	15
6		0	0	0	0	0	0	0	0	0	0
7		4	3	3	2	1	0	0	2	2	17
8		3	2	2	1	0	0	0	2	2	12
9	20	5	4	5	2	2	0	0	4	3	25
10		4	3	4	3	2	0	0	5	3	24
11		5	3	4	2	3	0	0	4	4	25
12		5	5	3	3	1	0	0	4	3	24
Media		3	2	2,2	1,1	0,8	0	0	2,1	1,7	158

Table 3

Internals lesional score, dinamically registred in mice group inoculated with *P. wickerhamii*

Animal number	Examination day	Severity lesions	Lympho-nodes reactio	Granuloma lesions							Final score
				in the skin	hepatics	pancreatics	renals	in spleen	in diaphragm	in the gut	

(mouse)			n								
1	10	0	0	0	0	0	0	0	0	0	0
2		0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0
5	15	2	1	1	0	0	0	0	0	0	4
6		0	0	0	0	0	0	0	0	0	0
7		2	1	1	1	0	0	0	0	1	6
8		3	2	2	2	0	0	0	0	0	9
9	20	3	2	2	3	0	0	0	0	1	11
10		0	0	0	0	0	0	0	0	0	0
11		3	2	2	3	0	0	0	0	1	11
12		4	2	3	3	0	0	0	0	2	14
Media		1,4	0,8	0,9	1	0	0	0	0	0,4	55

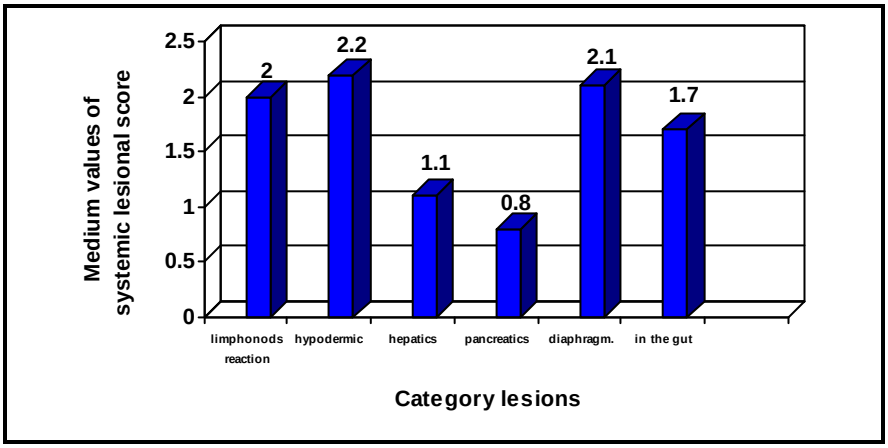

Fig.1, Internals lesional score medium values on lesions category in mice group infested with *P. zopfii*

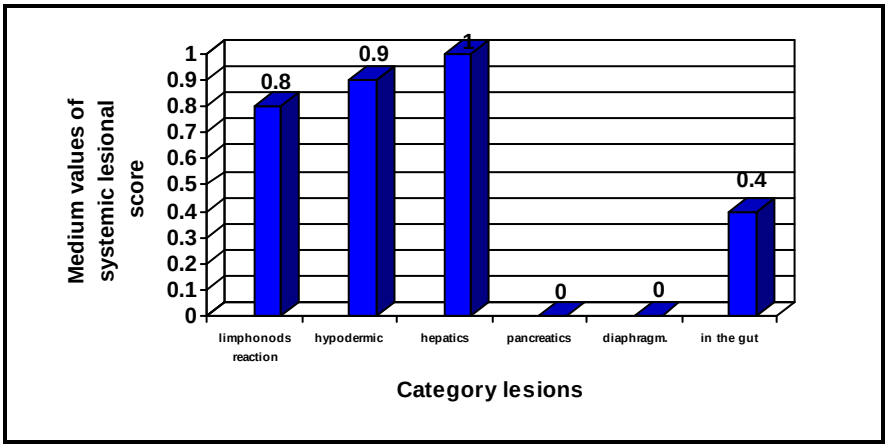

Fig. 2, Internals lesional score medium values on lesions category in mice group infested with *P. wickerhamii*

Medium lesional systemic score registered to the group infested with *P. zopfii* (Tab. 2, Fig. 1) was higher than those registered in group infected with *P. wickerhamii* (Tab. 3, Fig. 2).

Reported to the lesions produced by *Prototheca* algae in both inoculated groups an ascendant increasing was registered; this was evidenciated as succession of medium lesional systemic score analised obtained in each slaughter day.

Using "t" test in order to compared the medium values of lesional systemic score on lesion category between two groups, "p" value was statistical significant ($p<0.05$) (Tab. 4).

Table 4

P values calculation based on the medium systemic lesional score on lesions category for the two infected groups

Lesions category	*P. zopfii* group	*P. wickerhamii* group
Limphonods reaction	2	0,8
Hypodermic	2,2	0,9
Hepatic	1,1	1
Pancreatic	0,8	0
Renals	0	0
Spleen	0	0
Diaphragm	2,1	0
In the gut	1,7	0,4
P value	0,0163	

Using "t"test in order to compared medium values of lesional systemic score between two groups at each slaughter,"p" value is significant only in 20 days post-infestation ($p>0.05$) (Tab. 5).

Table 5

P values calculation based on lesional score registred among two groups in each slaughter

T test	Day 10	Day 15	Day 20
P values	0,1336	0,1125	0,0138

♦ Microbiological exam

At microscopic exam spherical or ovoid cells in different development phases, apparent cells wall and the partition wall of the old cells, sporangiospors (morula), with endospors were distinguished in slides realized in Lügol solution (Fig. 3).

At cultural exam on glucose agar medium small, white - grey color colonies with light irregularities, granular surface and cauliflower aspect were developed (Fig. 4).

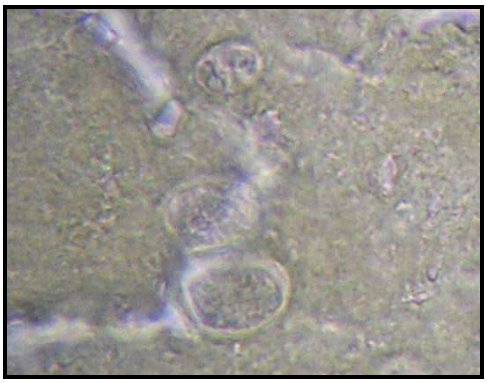

Fig. 3, *P. zopfii* cells - slide from hepatic granuloma

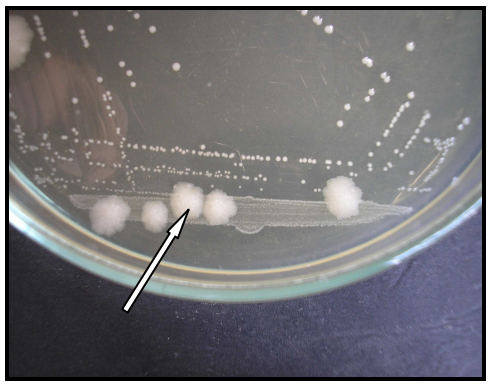

Fig. 4, *P. zopfii* colonies (arrow)-isolated from hepatic granuloma.

♦ Histopathological exam

In tissue fragments collected from both mice groups at each slaughter multiple granulomatous systemic lesions more frecvently at subcutaneous, hepatics and diaphragmatic level were evidenciated.

Variety and severity of lesions observed in mice group inoculated with *P. zopfii* were more abundant than that distinguish in *P. wickerhamii* infected group.

In early lesions at 10 days following *Prototheca* inoculation, focal granulomas composed of histiocytic cells and/or macrophages were observed. Mast cells were occasionally present among the histiocytic cell infiltrates.

In the granulomatous lesions at 15 days, scattered eosinophils and some lymphocytes were seen. Central necrosis, with numerous neutrophils and many endospores surrounded by the granuloma, was often observed.

In late stage lesions at 20 days, massive lymphocyte and plasma cell infiltration surrounding and/or intervening between vacuolated epithelioid cell clusters was evident. Histological reactions in epithelioid cell granuloma and the ultimate course of this disease can be staged from the histological point of view as follows: 1) diffuse inflammation, 2) cell proliferation leading to epithelioid cell formation, 3) hypertrophy of epithelioid cells with consequent formation of cell aggregates and/or organized granuloma and 4) degeneration of granuloma.

Microscopical lesions evidentiated in mice group infested with *P. wickerhamii* were less expanded and with a moderate severity compared with those founded in *P. zopfii* infected group. The lesions were less abundant and the affected organs were less numerous. Therefore anafected tissue were evidentiated.

These prolutions in mice group inoculated with *P. wickerhamii* sugest a less complex pathological process than in case of experimental protothecosis in mice group infested with *P. zopfii*. The aspects can be also correlated with clinical evolution more insignificant in systemic experimental protothecosis in mice group inoculated with *P. wickerhamii*.

Histopathological exams performed allawed algal cells evidentiation, as we can observed in the pictures bellow:

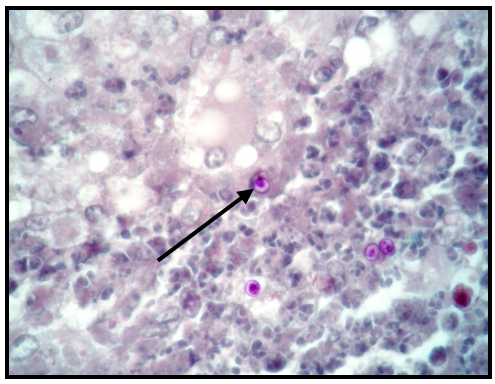

Fig. 5, Piogranuloma with polynucleated giant cells; alga (arrow) in the purulent necrosis matter, PAS x 100.

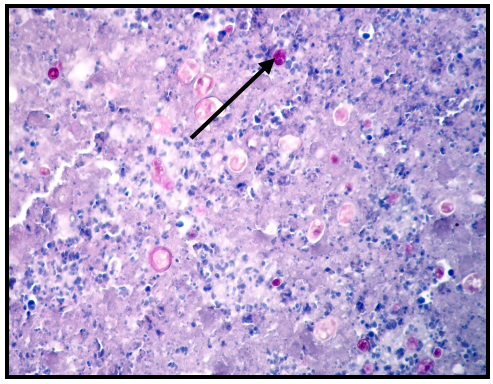

Fig. 6, *Prototheca* cells (arrow) dispersed in necrotic central mass of the granuloma, PAS x 40.

In accordance with Di Persio (2001), Roesler *et al.* (2001), Roesler *et al*. (2003), Linares *et al.* (2005) data we can sey that usually *P. wickerhamii* is etiological agent in human protothecosis while *P. zopfii* present a high pathogenity for animals.

Microorganism isolation following microscopic and cultural exams, as well as histopathological lesions observed, are a pathogenity proof of these algae.

CONCLUSIONS

1. Pathological lesions in experimental protothecosis in mice consisted of subcutaneous, hepetics, pancreatics, diaphragmatics and intestinals granuloma and piogranuloma.
2. Medium lesional systemic score registered in mice group infested with *P. zopfii* was higher than those from the group inoculated with *P. wickerhamii*.
3. Using "t" test in order to compared medium values of lesional systemic score on lesion category between two groups, p value (probability) was statistic significant (0,0163 <0.05).
4. Experimental sistemic protothecosis development was more rigorous in mice inoculated with *P. zopfii.*
5. Using "t" test in order to compared the values of lesional systemic score between two groups at each slaughter p value was significant only in 20 days post inoculation (p<0,05). In 10 and 15 days, differences between lesional systemic score in two varantes (groups) was not registered (p>0.05).
6. *Prototheca* algae were evidentiated folowing microscopic (slides from tisse lesions) cultural (embedment on glucose agar medium) and pathological (PAS staining) exams.
7. Even all the subjects used in experiment were mentioned in identicaly conditions (food, hygiene), the researches performed, established that in group inoculated with *P. zopfii* the lesion were more severs, comparatively with the group infested with *P. wickerhamii.*This findings we allawed to conclude that comparatively with *P. wickerhamii, P. zopfii* present a higher pathogenity for animals.

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