SUMMARY OF DOCTORAL DISSERTATION

Biology of the parasite *Blastocystis hominis*, evaluation of its pathogenic potential, and anatomical and pathological aspects studied clinically

The studies realized focused on the parasite and its pathogenic potential, starting from the premise that this opportunist protozoan particularly exploits the receptiveness of the host, i.e. of the human host, and represents one of the etiological agents involved in colon pathology.

Blastocystis hominis is a common enteric zoonotic protozoan, frequently met all over the world, however very little studied in our country, and its systematic classification, biology and pathogenicity are not completely elucidated even at present. These considerations determined its choice as a subject for the study.

The objectives proposed were organized in the following research directions:

- Description of life forms using complex staining, allowing the evaluation of the structure of *in vivo* population, and highlighting the four main morphological forms.
- Evaluation of the prevalence of commensal protozoa, associated with the emergence of the species *Blastocystis hominis* in the general population of Iaşi County.
- Cultivation of the parasite with the purpose to isolate it and to study its *in vitro* morphology and behavior.
- Study of the pathogenic potential of the species *Blastocystis hominis* in patients with irritable colon syndrome (I.C.S.) and colitis.
- Identification of *Blastocystis hominis* strains using the PCR technique in order to establish the pathogenic potential of different genotypes.
- Experimental inoculation in laboratory rats, and highlighting histological and pathological modifications.
- Highlighting the anatomical and pathological aspects studied clinically in patients with colitis and colon cancer, parasitized with *Blastocystis hominis.*

In the scientific research work with the title "**Biology of the parasite *Blastocystis hominis*, evaluation of its pathogenic potential, and anatomical and pathological aspects studied clinically**", multiple aspects were approached concerning this parasite, which allowed the realization of studies based on personal and original ideas and contributions.

The first part of the dissertation - the general part - focused on studies and proofs concerning the morphology of the parasite, genetic and structural characteristics, biological

cycle, pathogenicity, representing the documentation which constituted the starting point for the personal research, and illustrating a synthesis of the research on the parasite *Blastocystis hominis.*

The first chapter, Research History, contains the systematic classification and generalities on the biology, speciation, clinical importance, transmission, and the reservoir of this species. The following chapter, Morphology and Structure, includes data from specialty literature, where the life forms (vacuolar, granular, amoeboid and cystic) of the parasite are presented, life cycle, obtaining and cultivation of *Blastocystis* species, as well as its unique genetic and structural characteristics. Consequently, this parasite presents an amazing genetic variability, containing at least seven zoonotic genotypes (I, II, III, IV, V, VI and VII), is submitted to programmed cellular death, contains a particular type of organelles morphologically similar to mitochondria, but with functions belonging to both mitochondria and hydrogenosomes, it has a variable resistance to treatment, and complex pathogenicity mechanisms. The chapter also contains epidemiological and prevalence data from numerous studies that indicate the fact that *Blastocystis hominis* is the most frequently isolated, and the most spread parasite in the world (cosmopolite parasite).

Chapter III, referring to Materials and Methods used in this scientific endeavor, contains methods of collection, concentration, preservation and fixation, as well as simple and complex staining, of which some are original or adapted, relevant for the study of the morphology and structure of this parasite.

The personal research had the purpose, in the first stage, to highlight the morphology of the parasite in feces by microscopic examination, using Lugol staining. The use of this staining proved the existence of the transitional form from the vacuolar to the granular one, proving clearly the transition from one form to the other in feces, aspect emphasized microscopically for the first time.

For the study of the biology of the parasite *Blastocystis hominis,* and for the differentiation of life forms, an original staining procedure was elaborated and experimented, adapted to feces, called "***Staining procedure for the protozoa to highlight cellular structures microscopically***". This procedure had the purpose to preserve all the parasitic forms existing in feces, the trophozoites being easily degradable, and to highlight the internal structure of this protozoon. This procedure allowed the study of the internal structure, of the stadial evolution of the parasite, and the emphasis of the cyst. Using this staining, it was possible to show that the cytoplasm of the parasite can be eosinophilic at the vacuolar forms, and

basophilic at the granular and avacuolar forms, aspect not quoted in the literature, which constitutes an element of novelty and a starting point for the elucidation of the causes involved in the differentiated affinity of the parasitic elements for acid or basic stains. Also, the presence of the inclusions from the central vacuole, which is stained differently, can offer information on their composition. They can be of metabolic nature, bacteria (Mătiu *et al.*, 2013c), daughter cells, or substances from the environment, which possibly penetrated by diffusion.

In this type of staining, the cyst wall appears like a large hallo, thicker than at the vegetative forms, the cytoplasm is compact, basophilic, and the dimensions are significantly smaller. These morphological evidences allow the evaluation of the presence of the cyst in feces, a very important aspect for the biology of the parasite studied by this original staining method (method accepted for patenting - OSIM 2012).

The approach of the morphology of the parasite was realized using several other staining methods, some for the first time in our country – its study by Acridine-orange staining. The use of this fluorochrome also allowed, due to its characteristics, to emphasize all the life forms of the parasite, its stadial *in vivo* evolution by specific staining of the trophozoites, pre-cyst, and cyst, relevant aspects for the evaluation of the structure on stadial age of the parasitic *in vivo* population. The emphasis of the parasitic morphology is the main diagnosis method used in parasitology. From this reason, the acknowledgement of all life forms at the optical microscope is essential for the laboratory diagnosis.

In order to obtain the cellular matter necessary for inoculation in rats, the parasite was cultivated in aerobic conditions on fluid culture medium, followed by separation using Ficoll Hypaque method. Though anaerobic, this parasite developed in xenic cultures, in aerobic conditions on fluid culture medium RPMI 1640, with added bovine fetal serum, and a mixture of antibiotics. The study of the parasites from the culture using Acridine-orange staining made possible the evaluation of the parasitic population, highlighting aspects of the biology of this parasite. The *in vitro* study on the diverse and spectacular division modalities developed by this parasite demonstrated that *Blastocystis hominis* is particularly exploiting the resources and the life environment, adapting morphologically and also by the division forms to the existing conditions. Consequently, the parasite develops multiple division modalities in the culture, like: budding, schizogony with formation of a division sack, symmetrical binary division, and sometimes at the same time burgeoning and binary division (Mătiu *et al.*, 2013b). Highlighting the presence of the division sack represents a world premiere for optical microscopy.

The cultivation on fluid culture medium made possible the analysis of *in vitro* behavior, including division modalities, which were compared to the ones emphasized *in vivo*, where binary division was dominant.

Also, for the *in vitro* study concerning the morphology of the parasite, the staining with methylene blue was modified by adding glucose solution, and by lowering the concentration of the stain. Consequently, we obtained a better penetrability of the stain in the vacuole, organelle which was not stained in the native preparation. The vacuole stained in shades of light blue, and the cytoplasm in darker blue, the same as the nuclei and the inclusions from the structure of the parasite.

Xenic cultures were made, leading to the separation of the parasite from the fecal matter and its identification by methods of molecular biology. In these cultures, the parasite either developed luxuriantly, or it did not cultivate, demonstrating that the diverse isolates can have different growth and life necessities, and not all the strains are cultivable.

The copro-parasitological evaluation of a number of 8300 patients (adults and children) emphasized the highest incidence of parasitosis with *Blastocystis hominis* in the general population of Iaşi city, as compared to the other commensal parasites (Mătiu *et al.*, 2013a).

A very high rate of the presence of the parasite was registered in patients with manifestations of irritable colon syndrome (I.C.S.) and colitis. Thus, from 63 patients investigated, *Blastocystis hominis* was registered in the feces of 49 (77.77%). Furthermore, in 11 patients the parasite was the only etiologic agent responsible for the intestinal manifestations registered. Consequently, we can state that *Blastocystis hominis* can cause intestinal manifestations, and it is involved in the emergence of the symptomatology of I.C.S. and colitis.

In order to evaluate the pathogenic potential of *Blastocystis hominis* strains isolated from patients with intestinal symptomatology of I.C.S. or colitis, also from patients with abdominal troubles, or from asymptomatic ones, the PCR technique was used, with primers specific to identify the circulating genotypes, in correlation with the clinical manifestations produced.

The genotypes II, III, IV were identified, as well as the genotype I, and their combinations. A correlation could be realized between the isolated genotype and the clinical manifestations, emphasizing the fact that their presence could be both due to the genotype and to the diverse local causes related to the host. The most frequent genotype identified was the zoonotic genotype II, of 650 pairs of bases.

Also, for the evaluation of the pathogenic potential, a strain belonging to the genotype II was inoculated in a lot of young immunocompetent rats, and we followed the emergence of the manifestations in the experimental infection of this species. The genotype II was chosen because this was highlighted with the highest frequency, and it is pig-human-rat zoonotic. We followed aspects related to local modifications, the presence of the inflammatory reaction induced by the existence of the parasite into the intestine, in order to establish possible mechanisms of pathogenicity. We determined that in the immunocompetent rats the elimination of the parasite is intermittent and short, the parasitosis being asymptomatic and self-limited.

The immunodepression of the animals due to the use of dexamethasone allowed the infection to manifest, and emphasized the possibility of intercellular superficial penetration of the parasite. In the bioptical pieces collected from the rats, the presence of the cyst was registered in the intercellular spaces.

The opportunist character of this parasite also derives from the fact that it is associated with other etiologic agents clearly involved in the intestinal pathology, or it is present with a high rate in the categories of patients with compromised immune status. Consequently, the study extended to a number of 104 patients with neoplastic disorders. The fact that this parasite was identified in feces in approximately 30% of these patients, as compared to the incidence in the general population of approximately 5% proves the characteristic of opportunism.

In the patients with neoplasm, in the feces of whom the parasite was found, we realized the microscopic analysis of the bioptical pieces from a parasitological point of view, and *Blastocystis hominis* species was identified intercellularly or superficially, with possible penetration through micro-lesions. We found that in the human bioptical pieces the location of the parasite was similar to the animal model.

As a result of this study, which approached both the parasite *Blastocystis hominis* and the human and animal host, we were able to elaborate some original and pertinent conclusions concerning its morphology, biology, and pathogenic potential. We proved the existence of diverse genotypes producing clinical manifestations and some anatomical and pathological aspects studied from a clinical point of view in the patients with digestive manifestations like colitis and neoplasm, the study accomplishing therefore its intended goal.