THE OPTIMIZATION OF HISTOLOGICAL TEHNIQUES FOR ANATOMICAL PIECES GATHERED FROM BEES

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Abstract.

In order to identify structural modifications produced on the intestinal epithelium of the bee, consecutive with sporozoa Nosema spp parasitism, examination studies have been performed for fixing and histological coloration steps of anatomical pieces gathered from medium and posterior intestine of these species.

Keywords: intestine, histological techniques, medium and posterior bees, Nosema spp.

INTRODUCTION

The purpose of this study consists in optimization of fixing and coloration histological steps of anatomical pieces gathered from medium and posterior intestine of a bee and identification of structural modifications produced at the level of intestinal epithelium parasited by Nosema spp.

The Nosema is a parasitic disease, produced by Nosema spp. a protozoa located in Microsporidium order, which affects digestive tracts of bees.

The disease affects adult bees and is very contagious, having temporal character, mostly the end of the year, winter and spring. The Nosema is conditioned by adjuvant factors represented by: weak families, long winter without cleaning flies, mana honey, adding lot of flours in food and increased humidity in hive. Only the laboratory examinations certifify the presence of disease.

When life conditions are not favorable, when the parasite is eliminated on the external medium once with excrements of bees or when the parasite dies, it sporulates, the form in which he is stronger and resistent. From that moment, by different causes, spores comes at intestinal level of the bee, germinate and produces the active parasite, that enters in the intestinal cell

wall level, feeds, reproduces and produces toxins. The dissemination of disease from one family to another and from one hive to another is realised through the apicultor, bees, and parasites like polish moth.

MATERIALS AND METHODS

The probes have been sampled at the beginning of the active season, the period April – May, before applications with anti-parasitic treatments.
Probes (digestive system) have been sampled from healthy bees, unparasited by Nosema spp, from two Apiary noted with A and B (forming control group I experimental), with the purpose of optimization histological techniques.
For identification of existent modified structures at intestinal tissue level, lot II has been created with live bees, and parasitized with Nosema spp gathered from two Apiary studied.
The technique of intestine sampling consists in catching with mini pliers with a sharp head of the last abdominal tergit and easy shooting of this, through horizontal move.
Sampling of digestive system from live bees, euthanatized with chloroform in closed spaces and fixing anatomical pieces have been made in side the Reference Laboratory for Bees Diseases in the building of IDSA (fig. 1).

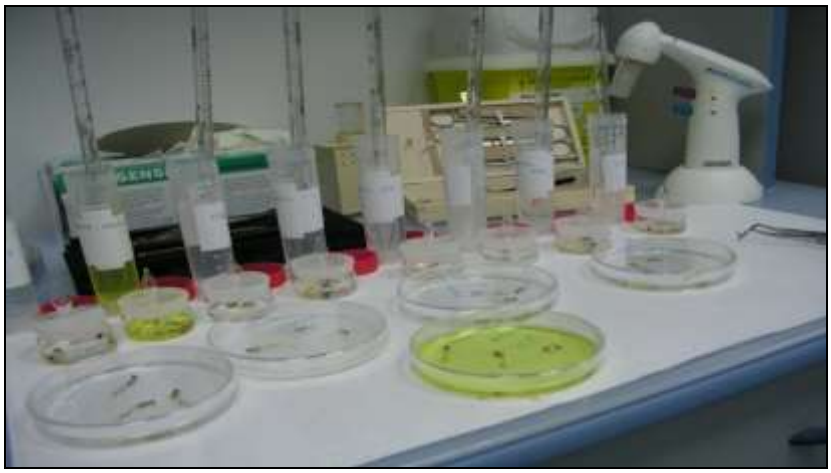

Fig. 1 Digestive system sampling and anatomical pieces fixation in different usual and specific liquids (original)

In obtaining methods for microscopical preparation more successive steps have been implicated: fixing (formalin – 5%, 10%, 20%, neutral formalin, acid formalin, mix Carnoy, mix Dubosq – Brasil, mix Lillie – for each lot studied), including them in paraffin, microtomia and pasting of sections, unparaffining and hydrating, and finally coloration (technique Masson modified, known as coloration tricromical), dehydrating, clarification and sections mounting. The tricromical coloration needs: latinium water, methyl blue (aqua sol. 0,5%), eosin (aqua sol. 1%), Mayer hematoxylin (alcoholic sol.).
The efficiency for every kind of fixating has been appreciated for volumes about ×100; ×200; ×1000 and ×1200.

RESULTS AND DISCUSSIONS

The appreciation of the best fixator has been made by:

a. Conserving capacity of general tissue structure and evidence of topography of digestive system in bees.
b. Cell integration keeping capacity for specifically analyzed tissues.
c. The capacity of cellular components evidence.
d. The capacity of chemical unalteration of the core and basal membrane.
e. The capacity of unalteration tissue reactivity opposite colorant solutions.

For this, a value scale has been made, with absolute natural numbers, from 0 to 5 in which every morphological section examined has been situated. Using large numbers of fixing liquids, *usual* and *specials*, have permitted the evaluation of quality efficiency in report with specifically tissue substrate of the bees, and on the other side selection of the most adequate indicate fixing to be used in terrain conditions for sampling and fixing probes necessary for histopathological diagnostic. In table number 1 histological sections examined have been situated through anterior criteria, and in table number 2 percentage values of chemical efficiency have been showed, specific at 8 usual and special fixating liquids, seeing the scale with absolute values from 0 to 5 (table 1 and 2).

Table nr. 1 Valorical fit of histological sections obtained from anatomical pieces fixated in different usual and specific liquids

Liquid fixating	**Total number of morphological sections**	**Scale 0 – 5 (absolute values)**					
		0	**1**	**2**	**3**	**4**	**5**
Formalin 5%	30	0	23	5	2	0	0
Formalin 10%	30	0	23	4	3	0	0
Formalin 20%	30	0	16	7	7	0	0
Neutaal formalin	30	0	17	8	5	0	0
Acid formalin	30	0	6	11	8	4	1
Carnoy	30	0	0	6	15	6	3
Dubosq - Brasil	30	0	0	0	0	9	21
Lillie	30	0	0	0	14	11	5

Table nr. 2 Percentage values of chemical efficiency specifically of 8 fixator liquids

Liquid fixating	**Total number of morphological sections**	**Fixating efficiency (in procents %)**					
		0	**1**	**2**	**3**	**4**	**5**
Formalin 5%	30	0	76,67%	16,67%	6,66%	0	0
Formalin 10%	30	0	76,67%	13,33%	10%	0	0
Formalin 20%	30	0	53,34%	23,33%	23,33%	0	0
Neutral formalin	30	0	56,66%	26,67%	16,67%	0	0
Acid formalin	30	0	20%	36,67%	26,67%	13,33%	3,34%
Carnoy	30	0	0	20%	50%	20%	10%
Dubosq - Brasil	30	0	0	0	0	30%	70%
Lillie	30	0	0	0	46,66%	36,67%	16,67%

From usual fixating liquids, chemically the best is acid formalin which has an efficiency for tissue fixing of 13,33% and 3,34%, in value scale from 4 and 5. The rest of the usual fixating liquids, formalin 5%, formalin 10%, formalin 20% and neutral formalin, didn't override the value step scale 3, for that it will be considered as inefficient.

The inefficiency of liquids presented, can be associated with a weak stable capacity of unalterative conservation for fixated tissues but also because of very brutal chemical activity resulting in tissue disruptions on the core, cytoplasm and/or basal membrane (fig. 2, 3, 4, 5)

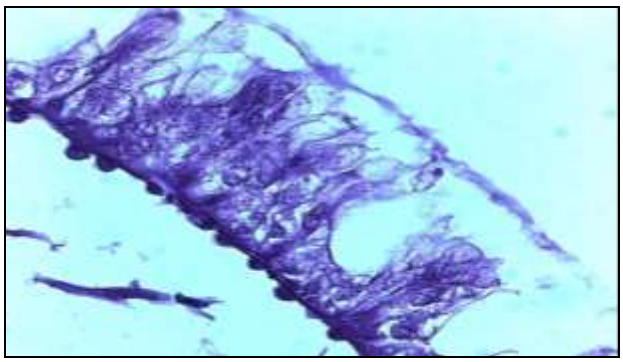

Fig. 2 Vacuolations, with core-cytoplasmic wrecks on epithelial cells of small intestine, adult bee; Method Masson modified, with fixing in neutral formalin x 900 (original)

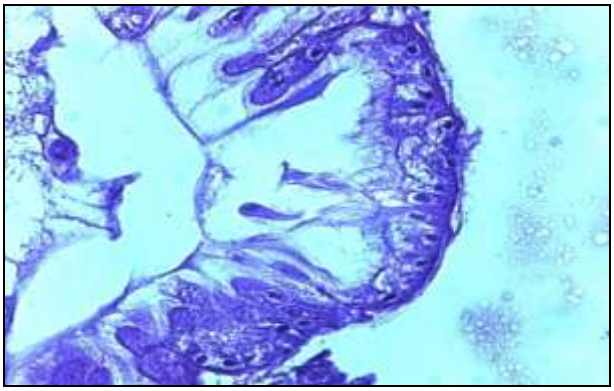

Fig. 3. Subtotal wrecks of epithelial intestine. Small intestine, adult bee. Method Masson modified, with fixing in neutral formalin x 1200 (original)

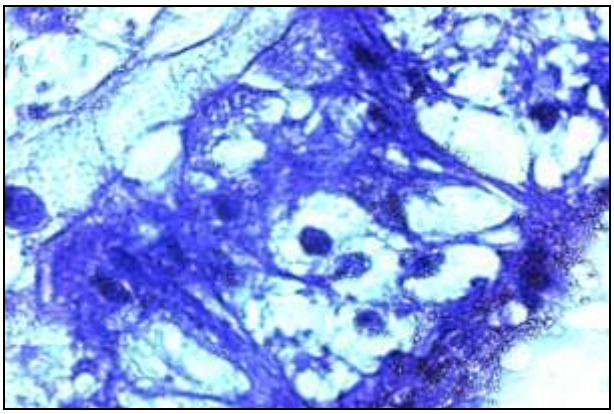

Fig. 4 Vacuolations and cytoplasmic aggregation, karyolysis with dilaceration on basale membrane. Small intestine, adult bee. Method Masson modified, with fixing in formalin 10%, x 1000 (original)

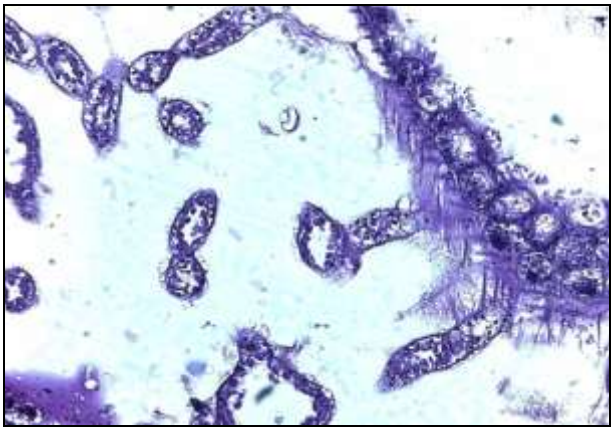

Fig. 5 Vacuolations and cells wrecks of intestine epithelium on Malpighi's tubes. Small intestine, adult bee. Method Masson modified, with fixing in licquid formalin 20% , x 150 (original)

In case of acid formalin the quality of fixing allowed us to observe one result at the limit of accessibility seeing evidence of topography medium intestine tissue at the place of insertion of Malpighi's tubes (fig. 6).

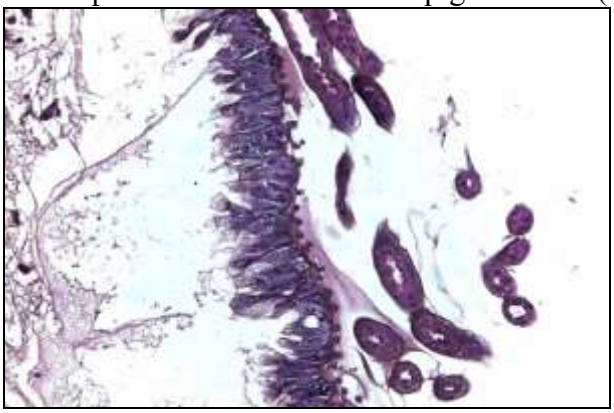

Fig. 6 Intracytoplasic modifications, vacuolation type of epithelial cells. Small intestine, adult bee. Modified Masson method, with fixing in formalin acid, x 400 (original)

From *special* fixating liquids: Carnoy, Dubosq-Brasil and Lillie, *Dubosq-Brasil* (fig. 7) have been more efficient by far: 70% maximum step 5 at values series 0 – 5, beside 16,67 % fixating Lillie (fig. 8) and only 10 % fixating Carnoy (fig. 9).

The fixating Dubosq-Brasil, with picric acid and one small proportion of glacial acethic acid – formaldehyde acid 37% by 1/60 in alcoholic solution has been proved to have a good and rapid permeability in digestive tissue of

adult bees. In the same time this complex fixing, conserved and chemically potentiated the tissues so that treated to realize a reaction "tissue substratum – basics coloring solutions", which allows structural details with an unnoticed grade of alterability.

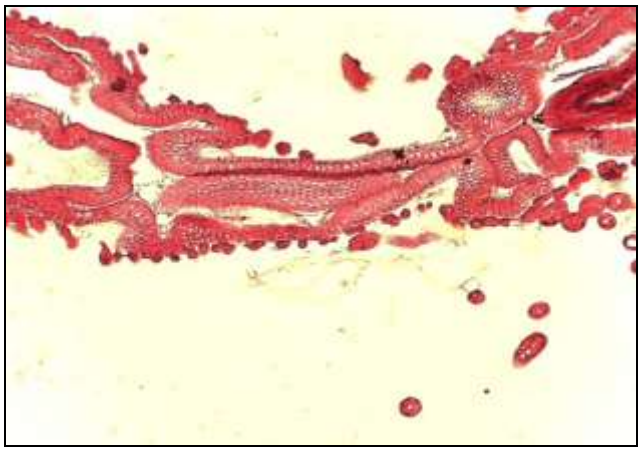

Fig. 7 Medium intestine, adult bee.
Modified Masson method, with fixing in liquid Dubosq-Brasil, x 100 (original)

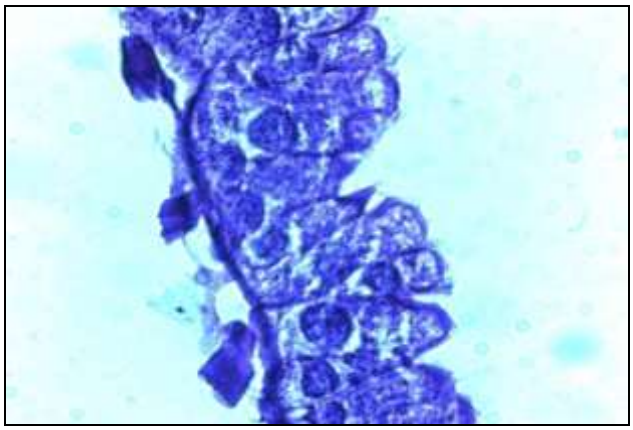

Fig. 8 Granulation modifications on intestine epithelium cytoplasm cells. Small intestine, adult bee. Method Masson modified, with fixing in licquid Lilie x l000 (original)

Fig. 9 Wrecks of parabase and supracores cytoplasm of epithelial cells. Small intestine, adult bee. Method Masson modified, with fixing in licquid Carnoy, x 500 (original)

In conclusion, it can be stated that fixating Dubosq-Brasil fixator is by far the most efficient, which can reveal normal aspects, but also modifications of tissues from the digestive system level of adult bees and others.
In histological samplings obtained from fixed pieces with this special liquid, maximum fidelity was obtained, at all volumes, celullarity of the specific tissue of medium intestine (who delineates through cardiac valve by anterior intestine and through pylorus by posterior intestine) (fig.10).

Fig. 10 Medium intestine (detail), adult bee.
Method Masson modified, with fixing in licquid Dubosq-Brasil, x 200 (original)

The fixating Dubosq-Brasil and general coloration Masson modified clearly revealed the structure of medium intestine and its celullarity (fig. 11).

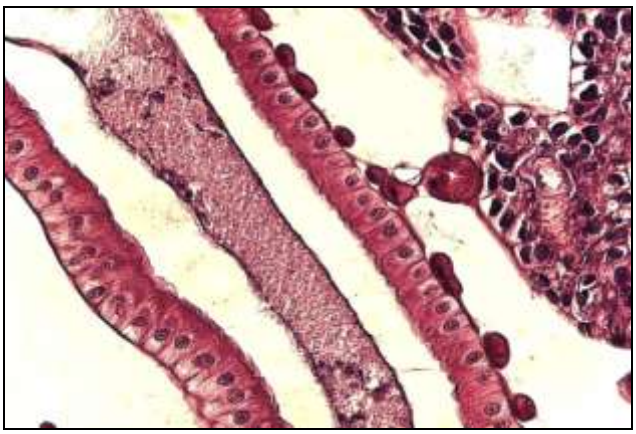

Fig. 11 Medium intestine, adult bee.
Method Masson modified, with fixing in licquid Dubosq-Brasil, x 1000 (original)

Histological preparations obtained from fixated pieces with Dubosq-Brasil, evidenced that the cells form median parts of intestine are cylindrical, have a large base, are strongly unified, and the cores are situated at different pitches of the cells, placed on the inferior half, near the basal membrane. The cores are circle shaped, and karyoplasm presents quick granulation. This structural detail can be observed using NG filter. If the filter is not used, the Masson coloration can't evidence the structural details of the cells.
The same fixator Dubosq-Brasil evidenced normal Malpighi's tubes and characteristics, originally from medium intestine, after passing in pylor at the first higher curbure.
At histological examination preparation, obtained from processed intestine sampled from bees in control group *II*, modifications have been observed in epithelial cell structure of medium intestine (fig. 12).
In one of the histological preparations, fixated in neutral formalin, , microvacuolations have been observed, in apical part of epithelial cells, consecutive with cytoplasm lysis. Acid formalin is one of the fixators with high efficiency grade used by many examiners for permeability of tissue in order to evidence Morrison's corpuscles, in case of using one tricromical colorations which differentiate and evidence cellular elements.

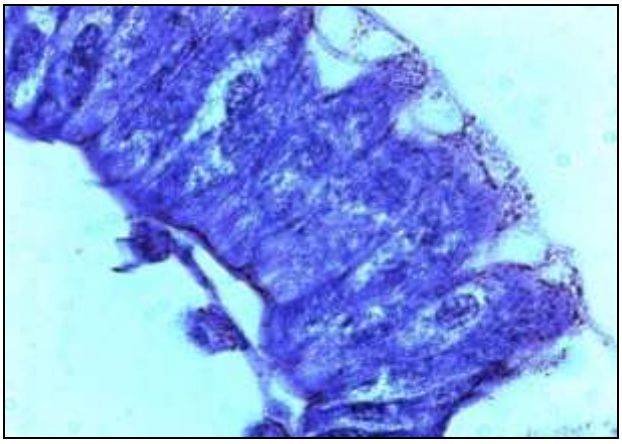

Fig. 12 Microvacuolations, apical lysis cytoplasmic in epithelial cells of small intestine. Method Masson modified, with fixing in acid formalin, x 1000 (original)

Partial wreck on small zones of cytoplasm from basal membrane zone can be easily observed, but also the apical zone from the core. Also on the level of bee's epithelium from the same control group intra-core and intra-cytoplasmic microvacuolizations can be observed, at the same time with apical lysis of epithelial cells.

The fixator with the highest efficiency Dubosq-Brasil and Masson modified coloration evidenced in bees from this lot another kind of histological modification which can be translated through appearance of one proliferative process observed only in medium intestine level of epithelium, where physiologically, the most intense metabolic reactions exist.

In fact, this proliferative process appearss like hyperplasia of intestine epithelium, associated with the appearance of microvacuols in enterocyte level. The cores have an abnormal disposition and karyolysis can be observed. Although epithelium hyperplasia can be observed as a proliferation by conjunctive tissue (fig. 13).

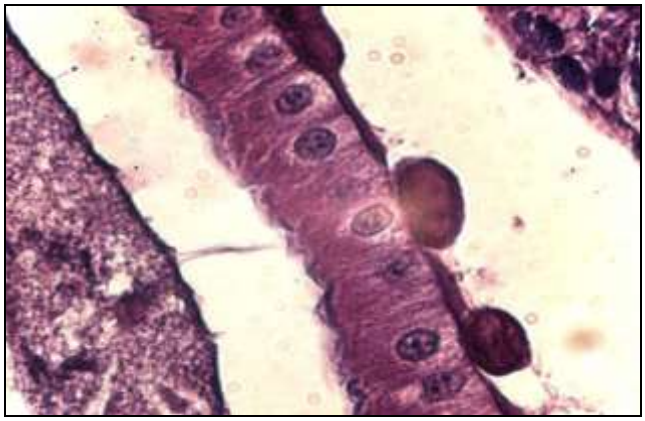

Fig. 13 Medium intestine, cytoplasmic microvacuolations, core base, adult bee (detail). Metoda Masson modificată, cu fixare în lichid Dubosq-Brasil, x 1200, filtru NG (original)

CONCLUSIONS

From usual fixating liquids chemically the best is acid formalin whose fixing efficiency of tissues was 13,33% and 3, 34%, in numerical scale steps 4 and respective 5.

From special fixating liquids, Dubosq-Brasil is the most efficient: 70% at maximum step 5 of valoric series 0 – 5, opposite by 16,67 % fixating Lillie and only 10 % fixating Carnoy.

The fixing Dubosq-Brasil and general modified coloration Masson clearly evidenced the medium intestine structure and cellularity, and also normal structure and characteristics of Malpighi's tubes.

In the process of examination of histological preparations obtained from processed intestine sampled from bees in control group *II* and fixed with usual solutions a lot of modifications have been observed (microvacuolisations, lisys, epithelial cells, etc.) in epithelial cells structure of medium and posterior intestine.

The fixator with the highest efficiency Dubosq-Brasil and modified Masson coloration evidenced in bees from control group another kind of histological modifications which can be translated to existence of a proliferative process observed only in medium intestine epithelium level, where physiologically intense metabolic reactions occurr.

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