National Center of Infectious and
Parasitic Diseases

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26 Yanko Sakazov Blvd.
Sofia 1504, tel. 02/9446999

Review of Doctoral Thesis

of Prof. Rossitza Ivanova Kurdova-Mintcheva, MD, PhD, member of Scientific Jury

Scientific specialty PARASITOLOGY AND HELMINTHOLOGY

Institution: National Center of Infectious and Parasitic Diseases, Sofia

Concerning PhD thesis for the award of educational and scientific degree "Doctor"

Thesis topic: ***"Development and application of serological methods for the diagnosis of toxocariasis and evaluation of their significance as markers for the disease stage in humans"***

Author: Eleonora Marinova Kaneva

PhD student - Self-training education form, NCIPD – Sofia

Scientific field: 4. Nature science, mathematics and information technologies, 4.3. Biological Sciences

Scientific specialty: "Parasitology and Helminthology"

Supervisor: Assoc. Prof. Iskra Georgieva Rainova, MD, PhD, NCIPD – Sofia

I declare, that there is no conflict of interests between me and the author of the dissertation, according to par.1, p. 3 and 5 of ADASRB.

Analysis of the applicant's career profile

Eleonora Kaneva was born on 03.09.1976 in Pleven. In 1999 she graduated Master program of "Molecular Biology" in SU "Sv. Kliment Ohridski", Sofia. After that she worked as a laboratory assistant in Veterinary office "Dr. Kanev"("Hristo Kanev – 92" Ltd.) and since 2006 till present she has been working as biologist at the Department of "Parasitology and Tropical Medicine", NCIPD, Sofia.

Participation of E. Kaneva in a number of continuing post graduate education courses in medical parasitology and tropical medicine at NCIPD-Sofia, real-time PCR analysis training (Biosystems Ltd., Sofia), training in medical statistics – work with SPSS20,

etc., that is documented by certificates, contributed to becoming a qualified specialist.

She has 16 publications, 3 of them are in Bulgarian scientific journals and the rest in foreign journals with impact factor and 4 implementations. She has participated in many scientific forums with posters and presentations. She has good knowledge in English (B2 level).

Relevance of the PhD thesis

Zoohelmintosis toxocariasis caused by the migration and location of the larvae of animal nematode *Toxocara canis* and *Toxocara cati* in the human tissues has a widespread distribution. Despite the fact that first description of the disease in humans dates at the middle of the last century, the researches' interest to the disease has not been reduced, and has even increased during the last couple of years.

The relevance of the PhD thesis, developed by E. Kaneva is determined by several facts:

- Relatively high seropositivity has been established, which varies in the west countries from 2-5% among the healthy urban population to 14-37% in the rural population. It reaches 63% in the tropics (Magnaval et al., 1994). The studies in Bulgaria revealed 8,6% seroprevalence in clinically healthy people (Raynova, 2008).
- Apart from the well known classic syndromes – visceral larva migrans, ocular toxocariasis and neurotoxocariasis , Covert toxocariasis, which has non-specific clinical symptoms, and asymptomatic form have been described. Their determination requires improvement of the laboratory diagnosis.
- The laboratory diagnosis of toxocariasis is based on indirect methods – serological, due to the inapplicability of the microscope methods, because parasites in human body do not develop to adult stage and don't produce eggs. Larvae identification is impossible, as well. Currently, ELISA IgG with larva secretory/excretory antigen and Western blot are widely used and

recommended tests for diagnosis. They are applied in Bulgaria, too. Due to the persistence of antibody production, caused by *Toxocara* larvae in the host, the applied routine serological methods don't give the ability to determine the stage of invasion – acute or chronic, as well as the period of invasion and the treatment efficiency. This imposes the necessity of the development of new additional confirmatory methods.

- The data of other researchers on the protective and diagnostic role of the specific IgG subclasses in toxocariasis are contradictory, which is an indicator for the necessity of further studies.
- The diagnostic value of the total serum IgE, which increases above 500 IU/ml, is regarded by Magnaval et al. (2001) as an additional marker for fresh invasion in patients with toxocariasis is still in a process of clarification.
- In other parasitoses, toxoplasmosis for example, the specific IgA antibodies are important immunological marker for the diagnosis of acute – fresh or reactivated infection. The examinations of patients with toxocariasis in this area are very few and the information – extremely scarce, but there is a hope, that specific IgA antibodies can be an indicator of acute invasion. Additional studies are needed for the confirmation of this primary data.

All above shows that the PhD student, based on the scientific facts, has correctly focused on the development and evaluation of additional confirmatory methods for the diagnosis of toxocariasis.

General characteristic of the PhD thesis

The PhD thesis follows the classical structure, which includes the following chapters:
Introduction - 1
Review of the literature - 35p.,
Goal and tasks - 1p.,
Materials and Methods - 13p.,
Results of the study - 74p.,
Discussion - 31p.,
Summary - 1p.,
Conclusions - 2p.,
Contributions - 2p.,

Bibliography - 24p.,
Publications and participations in scientific congresses and conferences, related to the PhD thesis - 2p.,
Summary - 3p.

The PhD thesis is written in 208 standard pages in good literary Bulgarian language. The ratio between the different chapters is well balanced and the attention is paid to two sections – Results and Discussion. I have to note, that the text is well illustrated with appropriate informative tables (36) and figures (54).

Review of the PhD thesis and evaluation of the results

Review of the literature. It is focused on the exact thesis theme and includes analysis of 306 scientific sources –71 in Cyrillic and 144 in Latin. It should be highlighted, that all Bulgarian publications on the topic are fully presented. It is obvious, that the PhD student has good knowledge and skills to make adequate analysis of the information known so far.

Based on the literary review and the necessity of improvement of *Toxocara*-diagnosis, the PhD student correctly formulated the goal and objectives of the study.

Goal and objectives. The aim of the dissertation is "to develop and evaluate confirmatory serological tests for the diagnosis of toxocariasis, as well as to determine the diagnostic value of total IgE and specific IgA antibodies for the detection toxocariasis stage in humans". The formulated 5 tasks specifically reflect the main directions in the work.

Materials and methods. Secretory/excretory antigen from *T. canis* larvae, produced by in vitro cultivated larvae through a method introduced at NCIPD (de Savigny, 1975; Rainova, 2006), was used for development of serological tests. The total of 130 patients with clinical symptoms, who had been serologically proved by ELISA-IgG and Western blot were included in the study. Serum samples of 46 healthy people, blood donors, negative for toxocariasis , were provided by the National Centre of Hematology and Transfusion. They were used for parameters determination of the developed methods.

Contemporary commercial kits were applied: RBiopharm (Germany) - Ridascreen *Toxocara* IgG, TestLine Clinical Diagnostic, CR for defining of anti-*Toxocara* IgA antibodies, Euroimmun, Germany for determination the concentration of the total serum IgE and Western blot IgG, LD BIO (France), which are a prerequisite for reliable results.

Statistical data processing with SPSS version 20.0. gives reason to accept the results and conclusions as objective.

Evaluation of the results, analysis and interpretation

Due to the prolonged persistence of anti-*Toxocara* IgG antibodies in the human organism and the existence of cross-reactivity with other helminthic diseases, the applicant investigated the value of other isotypes antibodies – total IgE and specific IgA antibodies, as well as anti-*Toxocara* IgG subclass antibodies (IgG1, IgG2, IgG3 и IgG4) for the diagnosis of toxocariasis.

The development, standardization and evaluation of ELISA, based on the secretory/excretory antigen of *in vitro* cultivated *T. canis* larvae for determination the level of specific subclass antibodies IgGl, IgG2, IgG3 и IgG4 in patients with clinically manifestation and serologically confirmed toxocariasis, is undoubtedly an excellent result. High specificity (92-95,7%) of the laboratory tests was reached.

I highly appreciate the results of the study of 130 *Toxocara* - patients with the developed tests, which show specific IgG1 subclass antibodies in 65% of them, IgG2 in 43%, IgG3 in 31% and IgG4- 42%. Differences in the specific IgG3 subgroup response are found by sex, age and clinical form (visceral or ocular). The presence of all subgroups in the same time was registered relatively rare – in 7,7% of the examined patients. Statistically significant relation between the production of anti-Toxocara IgG1 antibodies and each of the rest IgG subclasses (IgG2, IgG3 and IgG4) was established. Detecting the compulsory presence of IgG1 in combinations with other subclasses, the PhD student made a correct conclusion on the important role of this subclass in the immune response to *Toxocara*. The data on IgG2 clearance in a year

after the primary diagnosis allow her to identify these antibodies as immunologic marker for the detection of the stage of the invasion – acute or chronic.

The author directed her study to the examination of other classes of specific antibodies. For the first time in Bulgaria, she studied and found anti-*Toxocara* serum IgA antibodies in 26,2% of the *Toxocara*-patients. The detected highest values of IgA in the primary examination and their clearance 9-12 months after that, despite the prolonged IgG positivity, permitted the author to assume that they were related to the early stage of the disease, that is of great interest.

Based on the international experience, the PhD student studied for the first time in Bulgaria the total serum IgE in patients with toxocariasis and found, that in 75% of them the average concentration exceeded 4 times the reference values compared to healthy people. Moreover, the values were higher in affected children, than in the adults. Convincing data, for significant depletion (below the reference limit) of total serum IgE in the period from the 6^{th} to the 9^{th} month after the primary infection, were presented. This gives the opportunity for the application of this marker for the determination of the disease's activity, as well as to make some assessment of the efficiency of the conducted treatment.

Through comparative evaluation of the received results, E. Kaneva obtained an evidence of the relation in the production of specific IgG, specific IgA and total IgE antibodies, which is an important characteristic of the humoral immune response to toxocariasis.

The conclusion of E. Kaneva that the detection of anti-*Toxocara* IgA, IgG2 subclass antibodies and total IgE could facilitate the diagnosis of toxocariasis and be an useful tool for determining acute infection with *Toxocara sp.*, due to their early depletion has a special value for the practice.

Evaluation of the conclusions and contributions

The author formulates 10 conclusions, which correspond to the assigned tasks and obtained results.

I accept the contributions from the self-assessment of the PhD student and I think that they objectively reflect the real achievements.

I assess the following contributions as more significant and original:

1. Standard laboratory tests, based on secretory/excretory antigen from *in vitro* cultivated *T. canis* larvae for detection of specific IgG subclass antibodies - IgGl, IgG2, IgG3 и IgG4 for the diagnosis of toxocariasis, were developed and evaluated for the first time in Bulgaria.
2. Differences in the specific IgG subclass response were established in patients, divided by sex, age and clinical form of toxocariasis.
3. The data for correlation between the IgG subclasses are original. The important role of the IgG1 subclass in the immune response in toxocariasis has been demonstrated and IgG2 depletion has been established up to one year after initial diagnosis.
4. The data on the diagnostic value of the specific IgA antibodies for determining the early stage of toxocariasis are the first of their kind in Bulgaria.
5. Original is the information obtained about the relationship between the production of specific IgG subclasses, specific IgA and total IgE antibodies, which provide important information about the humoral immune response in toxocariasis.

Some contributions of applied science nature deserve certain attention, such as:

1. The data on the diagnostic significance of elevated values of total serum IgE in patients with toxocariasis are the first of their kind in Bulgaria.
2. The diagnostic approach proposed by the author for tracking the specific IgG subclasses (especially IgG2), the specific IgA and the total IgE antibodies in dynamics, through which the stage of the disease can be determined with high probability and the effect of the treatment can be judged, is original.

Reflection of the candidate's scientific results in the literature

The abstract is written on 73 pages and includes a summary in English. Its content corresponds to the content in the thesis.

Four publications were presented in connection with the topic of the thesis - 2 in Bulgarian editions and 2 - in foreign editions with IF 2.143 and - 0.210. E. Kaneva is the first author of three works, which unequivocally speaks of her active role in their development. She has participated with posters and presentations in three national scientific forums and in one international one, which is documented with abstracts.

Critical notes

The dissertation is shaped precisely and I have no significant remarks. In the final version, the author has taken into account all the comments and suggestions made during the internal defense.

Conclusion

The reviewed PhD thesis is up-to-date, well-planned and competently performed with modern laboratory methods under the responsible guidance of the supervisor and has a significant contribution in the field of contemporary diagnosis of parasitic diseases.

I believe that the implementation of the developed tests to determine specific IgG subclasses in practice, as well as the inclusion of specific IgA and total IgE in the combination of immunological diagnostic markers will improve the diagnosis of toxocariasis in humans and help formulate an adequate therapeutic approach.

The publication activity in connection with the dissertation is sufficient for this scientific degree.

The PhD thesis fully meets the scientific criteria in ADASRB and the Regulations of NCIPD for the educational and scientific degree "Doctor". This gives me a reason to vote positively for the award of the educational and scientific degree "Doctor" in the scientific specialty "Parasitology and Helminthology" to Eleonora Marinova Kaneva.

15.12.2019 Signature:

Prof. Rossitza Kurdova-Mintcheva, MD, PhD