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MEDICINE „ION IONESCU DE LA BRAD” IASSY
FACULTY OF VETERINARY MEDICINE
DOMAIN VETERINARY MEDICINE
SPECIALITY INFECTIOUS DISEASE**

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D O C T O R A L T H E S I S

**PhD coordinator,
Prof. univ. dr. Tudor PERIANU**

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Ph.D. THESIS

**INVESTIGATIONS REGARDING THE
SENSITIVITY AND SPECIFICITY OF SOME
DIAGNOSTIC TESTS IN BOVINE TUBERCULOSIS**

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A B S T R A C T

The bovine tuberculosis represents a serious obstacle in the development of animals' growing and a serious threat for the public health. Because of all this, the problem of surveillance and control of the animal tuberculosis in general, and bovine tuberculosis in particular become a more and more thorough preoccupation, both for the leading factors from the agricultural sector and for the veterinarian practitioners.

In the past, due to its spreading among the population and its virtually incurable character, the tuberculosis was considered for centuries as an important social danger, without adequate remedies.

In the last 70 years, even means of strategies of surveillance and control of the disease became available, tuberculosis continued to represent, on global level, a serious public health problem. Recent data released by the World Health Organization show that now almost one third of the world's population is infected with *Mycobacterium tuberculosis* and annually there are about nine million new registered cases of ill people.

Despite the assiduous research, a means with absolute efficacy in the prevention of the disease is still to be discovered. The Calmette Guérin (BCG) bacille, an attenuated strand of *Mycobacterium bovis*, was used for more than 60 years, and is capable of assuring a sensitivisation of the lymphocit T and macrophage activation, critical factor in antituberculous immunity.

The seric antibodies don't play an important role in the immune answer to this disease, the critical factor of the rezistance being the sensitivisation of the lymphocytes T and the activation of the macrophages.

The bovines are the main source of *Mycobacterium bovis* and they can transmit the infection to a big number of mammals, including the man. This become infected especially by raw milk and milk products consomption or by inhalation.

The infection with bovine bacili in humans is seen especially in the personnel living near the animals, and then in children fed with the tuberculous cows' milk. So, even from 1933 Gervois, in France discovered that from 17.045 cases of tuberculosis reported, 11,2% were of bovine origin, and Mulak (1962) in Poland finds the frequency of bovine tuberculosis being 17,5-19,2%. From these cases, in children, the frequency was between 16-36%, and even 41%.

Milk is an aliment with an essential role in the transmission of the disease, because it constitutes a medium for maintaining the bacilli alive. The concentration of the milk in *Mycobacterium bovis* is proportional with the seriousness of the animal infection. Equally dangerous as the milk, if not even more dangerous, are the milk products (from milk not pasteurized or boiled). Some of these, as whipped cream and cream, produce by a mechanism of flotation big contents of bacilli, constituting as well environments where the bacilli survive for a very long time. Because the milk products are not consumed immediately, but are put on sale, they become a means of spreading at distance the disease.

This situation determined that the problem of knowing and fighting animal tuberculosis, in general, and bovine tuberculosis in particular, to become become a more and more thorough preoccupation, both for the leading factors from the agricultural sector and for the veterinarian practitioners. The control of the transmissible diseases from animals to humans, including the tuberculosis produced by *Mycobacterium bovis* is essentially a matter of public hygiene, but also a problem proving at the same time that the hygiene is unique, because intertransmissibility and reciprocity of some zoonosis, as tuberculosis, do not allow separation from the point of view of the prophylaxy between the two medicines and impose the cooperation among them. „Una sanitas, una medicina!” (Goret și Joubert, 1966).

The growing global interest for the infection with *Mycobacterium bovis* was due to the risks of transmitting the disease in humans and the bad consequences on the animal health and productivity, and on the trade with animals for reproduction and meat, from areas where the pathogenic agent of tuberculosis is present (Rochat T., 1993).

Even a century ago, Koch R. figured the vaccinal role of a previous contact of the organism with the tuberculous bacilli. Continuing this line of research and trying to find a cure for the people with tuberculosis, he prepared the tuberculin, which, even if it didn't produce the desired effects, proved to be a very efficient tool in the diagnosis of the infected people.

Today the standard method of detecting the tuberculosis is the tuberculin test, based on interpreting the local reaction, appeared on the animals infected after inoculation of the tuberculin. Initially the tuberculin prepared by Koch (now named: old tuberculin koch or old tuberculin) was used. After some time, Seibert F. produced the Purified Protein Derivative (PPD), accepted by the WHO as reference material. The intradermic inoculation of PPD and the reading at 72 hours (when the maximum intensity of the specific answer is recorded) is

considered the most accurate method of distinguish the late hypersensitivity, induced by the natural infection with *Mycobacterium bovis*. Even though the intradermic tuberculin test is used on a large scale to find the tuberculous bovines, there are firm data concerning the reduced sensitivity of this test, due to the false negative or false positive reactions. Because of that, in the last decades efforts have been made in order to perfect the means of diagnosis of the bovines infected with *Mycobacterium bovis*.

In this context, the immunological tests fit for knowing the changes produced after the infection and the diagnosis in due time of the tuberculosis, both in animals and in humans, which leads to a better surveillance and application of the surveillance and control measures. Also, the comparative evaluation of some diagnostic tests with immunologic substratum in bovines, and the thorough epidemiologic analysis may lead to the elaboration of the scientific base for the application of an efficient programme for the prophylaxy and cure of the disease. The acknowledgement of the immune system as a whole is a condition for the understanding of the normal function or of some alterations of one or more loops of the complex network of immunity.

Considering on the one hand the presence of the disease in the bovine flocks of economic and sanitary significance, and on the other hand the amount of the means of tuberculous infection detection, we tried to verify the evaluation of some diagnostic tests for bovines, in use in Romania (the intradermic tests, the Bovigam test), and the estimation of the efficiency of some techniques, such as the lizozyme and immunoglobuline G dosage, compared with the diagnostic tests accepted in the diagnosis of the infection with the tuberculous bacilli.

The thesis has 145 pages, being structured in two distinct parts, formed from X chapters. The first part (chapters I, II and III synthesises the main bibliographical data concerning the tuberculous infection from the literature and constitutes „**The present level of knowledge**”, and the second part (chapters IV, V, VI, VII, VIII and IX) refers to „**The personal findings**”. Each chapter from the second part of the thesis contains the material and methods, the obtained results with their discussion and partial conclusions.

In chapter X, in the 23 „**Final conclusions**”, the main aspects concluded from the research are synthesised.

In the study there are presented 45 tables, 18 figures, and the bibliography contains 156 titles.

The first part of the thesis represents a synthesis of the literature concerning the diagnosis methodology in bovine tuberculosis, emphasising the etiopathogenetic mechanism, reviewing aspects concerning the knowledge level of the infectin with *Mycobacterium bovis*.

In the first chapter, entitled „**Bibliographical data about bovine tuberculosis**” are reviewed aspects concerning the history of the knowledge on tuberculosis, the national and international spreading of the disease, and the economic and sanitary significance of the disease.

The second chapter, entitled „**Implications of the genus *Mycobacterium* in animal pathology**” synthesises the main data from the literature concerning the taxonomy, the morphology and the antigenic structure of the pathogenic agent. The structural particularities and the pathogenity elements of *Mycobacterium bovis* are presented. The sources of infection are presented as well, emphasising the way of contagion and the pathogenic mechanism of the *Mycobacterium bovis* infection.

The third chapter, entitled „**The present aspects in the diagnosis of bovine tuberculosis**” refers to aspects concerning the methods of establishing and confirming the diagnosis. It is important to note that for a firm diagnosis, the completion of a complex set of allergic, bacteriological, serological, histopathological and biological exams is needed.

The thesis approaches a present theme, of especial interest for the bovine pathology, with aspects, particularly of diagnosis, less explained, reason why the investigations concerning the evaluation of some tests used in the diagnosis of bovine tuberculosis and the efficiency assessment of some other, compared with the methods used today, appear very interesting and necessary to the study.

The increase of the number of cases of tuberculous infection in animals and the apparition of the risk of transmission in humans, as the great number of false positive and false negative reactions determined the improvement of the methodology of surveillance and control of bovine tuberculosis.

The chosen theme is one of major interest, not only from a sanitary and sanitary-veterinary approach, but also from the point of view of the social and political factors, responsible for the population's health status, the incidence of tuberculosis growing.

So, the investigations concerning the bovine tuberculosis prevalence in the district Iași, between 2000-2008 (Chapter V), highlighted that from 1.412.694 bovines, 1.230.786 were submitted to the (UT), which represents 80,04%.

From those 1.203.870 bovines submitted to the unique tuberculin test 2.126 (0,18%) bovines reacted positive, and 2.997 (0,25%) bovines were registered in the re-control group.

The simultaneous comparative tuberculin test (TCS), made on 4.914 bovines with positive reactions and re-control at the unique tuberculin test, highlighted positive reactions in 18 (0,36%) bovines, re-control in 56 (1,14%) and negative in 4840 (98,5%) bovines.

The results of the investigations, obtained by intradermic allergic tests, concerning the situation of the bovine tuberculosis in the district between 2000-2008 were completed by the confirmation/invalidation of the disease after the laboratory exams after the slaughter. The obtained data permitted the assessment of a sensitivity of the allergic test of 78,37%, a specificity of 96,78% and a real prevalence of the disease of 0,0018%.

The serologic exam, performed on 205 samples using the Bovigam test, highlighted positive reactions in 17 (8,26%) bovines, re-control in 9 (4,37%) and negative in 182 (87,37%).

From the 25 bovines with positive reactions on the simultaneous comparative tuberculin test and on the Bovigam test, slaughtered in the slaughterhouse, in 4 (16%) the tuberculous infection was confirmed, and in 21 (84%) was invalidated, the exam being negative.

The results obtained from the intradermic tests (TU and TCS) demonstrated the classical oscillations, based on the one hand on the real hiper-sensitisation type IV reaction, and on the other hand, the possible errors of interpretation due to the para- or pseudo-allergic reactions, which sustain the necessity of the tuberculosis diagnosis based on a set of correlated tests, whose corroborate results facilitate the diagnosis, reducing the incidence of the false positive cases.

The values obtained after the “gamma-interferon dosage in different ranges of tuberculin-reagent bovines” (Chapter VI), classified according the intradermic test, are generally bigger for the TU and TCS positive categories, compared to the other categories. Also, there is a more intense answer for the stimulation with bovine PPD, compared to the stimulation with avian PPD, in the same categories, with an inversion of this situation for the dubious TU and re-control categories, respectively, where the answer for the stimulation with

avian PPD is only a little more intense than in that with bovine PPD, and a certain equality of the answer in both tuberculin for the TCS negative category.

Also, considering the obtained values, one can see that very big differences in the writing of the intradermic test in bovine tuberculosis, such as the means of more than 7 mm, may be or may be not reflected in the values of the Bovigam test. Of course the intervention of other causes producing these results, such as the chronic parasitosis (e.g. Linguatulosis) that not give way to antiparasitic treatments, is possible.

In all categories, other than positive, the value of the optic densities for the Bovigam test are maintained under a means of 0,150.

Examining the individual values and not the means, one can observe that in other categories than positive there are bigger values than the above mentioned means. Analyzing the data obtained with the Bovigam test, it turns out that the results are unsatisfactory. This conclusion is supported by the negative results with the Bovigam test on animals that reacted positive for the dermic test, or by the positive results with the Bovigam test on animals that reacted dubious in TU and negative in TCS.

For this reason it seems that this test (Bovigam), expensive and laborious, seems to be more helpful where there are isolated cases of disease, and not for an indubitable diagnosis of the disease with the elimination of the reagent animals. Because of that, the Bovigam test has mainly a group diagnosis value than of individual diagnosis.

The analysis of the obtained correlation factors (Chapter VII) suggests a lack of correlation ($r=0,034$) between the **positive intradermic tuberculin** test and the results for the **Bovigam test**, if they are positive, and a weak correlation in a positive sense, respectively ($r = 0,14$) if they are negative, in case of stimulation with bovine tuberculin.

The correlations established between the positive intradermic tuberculin test and the Bovigam test, regardless if its results are positive or negative, show close values ($r = 0,68$; $r = 0,65$) for the stimulation with avian tuberculin. Their significance is $p < 0,001$, and the variation is intense directly proportional.

In case of the re-control TCS category for both tuberculin, the negative correlation shows a reverse proportionality ($p < 0,001$). For this category the two sets of values could reach low limits, not detectable with the respective tests (as an example, very big values in TCS and very small in Bovigam and vice versa), highlighted by the negative result for

Bovigam in the presence of TCS reaction. From the point of view of this category, to clarify the diagnosis, performing both tests is useful.

Between the two tests from the TCS-negative - Bovigam-negative category the correlation is negative, of weak to medium intensity for the avian type of tuberculin ($r = -0,29$) and positive, of medium intensity ($r = 0,44$) for the bovine type of tuberculin, respectively. The negative sense of the correlation in case of the couple TU-positive - Bovigam-negative reflects the inversely proportionality dependence of the results obtained by the two tests.

It seems that in case of the animals TU-dubious, the Bovigam test has a maximum accuracy for the positive animals ($r = 1$; $p < 0,001$).

As for the Bovigam test, it seems this is better correlated with the comparative simultaneous test in the animals with non-specific sensitivity, with reactions for avian tuberculin respectively, both for a positive and a negative result, even though in case of the tuberculin unique test the correlation for the hypersensitive animals is reversed.

As a conclusion, one could say that not for every pair of rows, r variation follows the same pattern and if a possibility of replacing the intradermic tests with the Bovigam test is needed, additional investigations on bigger flocks must be performed in order to eliminate the bias.

Also, one can assess that corroborating the results of the allergic tests with the results of the complementary tests, would constitute a more rational and accurate base concerning the decision of sending to the slaughterhouse a tuberculin reagent animal, then the allergic tests alone.

The comparative analysis of the values of the IgG concentrations in those two categories of analyzed animals (subchapter 8.1.) emphasises that the means of the lot that reacted at the cutaneous test is higher than that of the healthy lot, with no tuberculosis. Even so, the p value ($p < 0,228$) does not reflect the statistic significance of the differences.

Also, from the analysis of the obtained results, the conclusion is that in healthy bovines the circulant IgG concentration is smaller than in those with reactions for the tuberculin test. As it is known, the basis of the immune protection in tuberculosis is represented by the activity of the cellular system of specific protection. Still, the study of the specific humoral reactivity denotes an increase of the IgG concentration, respectively a stimulation of the protection

humoral reaction, with the specificity of these molecules not being defined. The values determined in case of IgG correlates with the results of the comparative simultaneous test for the positive and re-control categories, which suggests the use of the test for the alternative diagnosis.

From the research concerning “the estimation of the lizozim concentration value in hiper-sensitivised animals” (subchapter 8.2.) results that, for the tuberculin-reagent animals the concentration of the seric lizozime grows compared with the healthy, non-reactive animals, maybe due to the presence in the infected organism’s bloodstream of some secretive cells (neutrophils, macrophages) with more intense activity.

Even though the difference between the two categories of animals (healthy and tuberculin-reagent) is not significant, there is a notable deviation from the normal of the lizozim concentration for the bovines with a positive reaction at the tuberculin test.

The obtained results suggest the possibility of using the difusimetric test as a complementary test in the diagnosis of the disease and in finding the correlation between the values of the lizozim and of the tuberculin test.