Key words: bovine tuberculosis, diagnosis of tuberculosis of living animals, diagnosis of tuberculosis of killed animals.

SUMMARY

The essay "COMPARATIVE RESEARCH IN CONCERN WITH USING SOME DIAGNOSIS TESTS WITHIN BOVINE TUBERCULOSIS" is scientifically and practically justified by the following viewpoints:

- ✓ nowadays tuberculosis is still one of the main animals' diseases; tuberculosis produces high economic losses and continue to be one of the big risks concerning people's health;
- ✓ despite the fact that with the scope of prevention and controlling, various medical veterinary measures were carried out and as a result, the bovine tuberculosis was considerably reduced, still the tuberculosis infection is not possible to be eficiently controlled due to lack of information on its real incidence;
- ✓ introducing certain complementary diagnosis procedures would help to get a more rational and accurate support, viewing the decision to be made in killing an animal which is reagent to allergic tests, instead of using allergic tests solely;
- ✓ making the difference of the peculiar and unpeculiar reactions of the specific sensitiveness to tuberculinoreaction, will became a necessity, keeping in mind that over the past decades, a higher number of units and households where tuberculosis was not confirmed at the reagent animals to tuberculinoreaction came out;

The essay consists of 274 pages and it is structured in two parts, in conformity with the recent legal stipulations: the first part is **''The knowledge status''** and consists of 92 pages representing 33,58 % of the essay. The second part "**Individual research''** represents 66,42 % of the essay. The whole paper work includes 35 tables and 43 graphs and photos, with the scope of having a better make up of the content. The bibliography used consist of 236 titles from national and international specific literature.

The first part - ***''The knowledge status''*** consists of two chapters, in which information from specific literature are shortly revealed on the paper work's topic. The information was used for interpreting and comparing the results which were acquired in the second part of the paper work.

Within the first chapter are presented data regarding the taxonomy of Mycobacterium type, as well as their morphological, cultural, biochemical and pathogenic features. Therefore, within mycobacterium type, there have been described a wide number of species, which can be rallied in three groups: pathogenic, opportunist and saprophyte mycobacterium. There have been described also the "L" forms of bacterium, which are particular aspects of them, featured by granular, spherical (spheroplast), with flaw or with no cell wall presence. The latter are featured by a relatively resilience toward environmental conditions. There have been related information concerning to the "rankness parameters" of mycobacterium, which are similar to various antigen structures. There have been presented data concerning the mycobacterium ,,rankness factors'', which are similar with the various antigens structure due to the mycobacterium get away from the immunitary mechanism capacity of the host. The Mycobacterium are able to survive and multiply especially at the place of entrance.

Chapter II *"The implications of Mycobacterium type in animals' pathology"*, based on the specific bibliography, describes Mycobacterium bovis infection. A special focus is dedicated to the contamination way of bovis Mycobacterium type from bovines to other tame species: conventional tame, wild species and to people. In the same time, there have been distinguished the diagnosis of bovine tuberculosis, taking into account that there is a principle difference between the way of diagnosing the bovine tuberculosis comparing to this procedure in other species' case. The above mentioned difference refers to the fact that the suspicion of bovine tuberculosis is compulsory to be evident based on the allergic examination. The allergic examination should be done to all bovines (the healthy ones), and afterwards only the positive results of the allergic are to be confirmed by other testing examinations. Concerning the other species, the tuberculosis suspicion come out first as a result of certain clinical or necropsy based procedures, followed by other testing examinations.

The second part ***"Individual research"*** consists of four chapters, conclusions and bibliography. There are presented and debated the results which were carried out as a follow up to the investigations.

Chapter IV describes the tuberculosis diagnosis of living animals. The epidemiological investigations with refer to bovine tuberculosis have been done between 2000 - 2005. There have been used and applied certain multi-annual data with regard to the

evolution of bovine tuberculosis in Vaslui county. Data applying has been made using various mathematic methods, such as the arithmetic mean used on multi-annual series, other recognized formula. The mathematic methods were used in order to estimate the inner value of the allergic test (sensitivity, specificity) and in order to establish certain epidemiological parameters: prevalence and incidence of the disease.

Therefore, it has been established that the sensitiveness of the allergic test during the assesed period of time, represented 78.57% and specificity 99.74%, knowing that the prevalence was of 0.0019% and the incidence of 0.02%. The high specificity of the allergic test (99.74%) seems to be truth-like, knowing that on the assesed territory the real prevalence of the disease was very low.

The laboratory tuberculosis diagnosis on living animals, it has been done using the immunoenzymatic test, the sandwhich procedure for detecting gamma-interferon, the glutaraldehyde test and the complement fixation test.

The immunoenzymatic test EIA_s – γ IFN represents a possibility for making use of the diagnosis, which can help in eliminating an important percentage of the tubercular errors, with the scope of avoidance of killing non infected animals or alternatively maintaining in incipient stages of certain M. bovis tuberculosis infected bovines. The latter situation is to be done viewing that otherwise the infection can invade animals' bodies and the respective pathogenic micobacteries can widely spread within the environment.

The study has been effected on bovines of more than 6 months aged, which have been tested with the common intradermo-tuberculinic test (IDR-TU).

The reagent animals (positive and/or suspicious) to IDR-TU have been double controlled after 15-30 days using the following methods: immnoenzymatic for detecting gamma-interferon, sandwich procedure (EIA_s- □ IFN) using the standard "BOVIGAM - kit-EIA_s". From the above mentioned animals have been taken blood tests using the proof-coagulant (heparyne). The blood tests have been stimulated with bovine PPD and avian PPD. As a result of incubation of blood cultures at a degree of 37^0C, with the duration of 16-24 hours, the plasma has been getting in. For each sample it has been taken into account a non-stimulated control bench-mark.

The lymphocites separately stimulated with bovine PPD and avian PPD have been emitted gamma-interferon, with a level of detection which was done

spectrophotometrical. Its optical density was of DO 450nm. The object of study consisting in 743 blood samples was putting on coagulant-proof taken from reagent bovines at the allergic tests. As a result of the test, it has been concluded that 5.25% of the bovines presented positive reactions to the EIA_s- γ IFN. Over 26.51% of them have been ranked in the avian reactant category. The slightly increased percentage of positive reactions to aviar, suggests infections with non-typical mycobacterium, those from M.avium category or those contaminated with other agents of different etiology, which can cause other diseases (echinococosis, fascyolosis, brucelosis, leucosis etc). A number of 8 animals had a non-conclusive reaction (1,08 %).

Inside the underchapter 4.3.2. there are presented the results get as a follow up of making the glutaraldehyde test. This method is based on the capacity of the aldehyde to polymerise with the aminical groups from inside the structure of serum proteins. The diluted glutaraldehyde makes possible the intermolecular connections with Ig G and with fibrin. The timing for blood gelly process mixed with glutaraldehyde is proportionaly reversed with the concentration in blood Ig G and fibrin. The study has been effected on 168 reagent bovine to EIA_s- γ IFN test. The results were as follows: 37.5% of the bovines reacted positively and 17.26% have been inconclusive.

The animals with the positive tubercular reactivity had also a positive test reaction to glutaraldehyde. There is only a difference between these two categories - the timing of blood gelly process have been different. The glutaraldehyde test has been inconclusive) gelly process took 12-13 minutes) to a number of 29 bovines with tubercular positive reaction. It has been noticed that 45.24% of the tested, had a negative reaction to the glutaraldehyde test. As a conclusion, the glutaraldehyde test does not find out the bovines contamined with tuberculosis in the initial disease stages.

Due to the fact that the test is a simple one, it can be used in parallel with the tubercular test and used in the context of epidemiological data.

The complement fixation test was another test used for the living animals diagnosis. The study was effected on 160 reagent bovines to EIA_s- γ IFN test. The data assesment proves that 7.14% of the animals had a positive reaction and 45.24% have been inconclusive.

Out of the 76 animals with inconclusive reaction to RFC, 60 had positive reaction to glutaraldehyde test.

Those 12 animals with positive reaction to RFC, had a positive reaction to the glutaraldehyde test, in which case the blood gelly process took place in the first 6-7 minutes. The rest took place in the 7-10 minutes and more. The high percentage (45.24% of reagent tubercular with an inconclusive reaction to RFC suggest tubercular pseudeoallergic and para-allergic reactions, as well as an anti-complimentary activity of the tested serums.

Part of the animals above mentioned from which have been taken blood samples have been killed in the slaughter-house. Their carcasses and organs have been examined and samples for detecting the tuberculosis have been taken. Out of those 168 animals considered for the study, 11 were diagnosed with tuberculosis, as a result of complex laboratory diagnosis (6.55%).

In conclusion, in order to use same time the allergic test, glutaraldehyde test, RFC test and ELISA immunoenzymatic test, it is advisable to proceed with more judgement with regard to the decision of killing the animals with positive reaction and to take such a decision only in cases where the tubercular test is used as a detecting test.

Inside chapter V it is presented the tuberculosis diagnosis on killed animals. Between 2000-2005 there have been examined 69 samples viewing the anatomo-pathologic, bacteriologic, hystopathologic examinations and the bio test on guinea pig.

The anatomo-pathologic examination has been effected on samples taken from the bovines killed in the slaughter-house (57 tubercular reagent bovines and 12 tubercular negative bovines). From the respective animals have been taken the lymphatic lumps of the head, neck, chest, mediastinal, mesenteric, as well as the internal organs: heart, lungs, liver, spleen, kidney, mammal gland, genitals. All have been examined in order to detect macroscopic wounds. It have been discovered grey and slight yellow spherical wounds, ferm in consistency and a caseous centre, slightly yellow, dry, non-capsullated, less nuclea, of various sizes, with a diameter of 0.5 - 3 cm out of 11 cases (15.94%). It has been discovered as well the high consistency hard lumps, due to the calcification processes. On the other hand, to those three cases of negative tubercular animals, which have been confirmed due to laboratory tests, it has been noticed visible wounds specific for bovine tuberculosis. It was found on the lymphatic lumps and in the parenchima organs.

As well as, 15 lymphatic lumps and organs belonged to positively reaction bovines to ELISA test. At seven bovines, there were noticed alterations which are specific to bovine tuberculosis found in lymphatic lumps and organs (46.66%).

Concerning the samples taken from the positively reaction bovine to the EIAs-γ IFN test, it has been noticed at six of them, the Echinococcus granulosus cysts presence, of various stages and various sizes (40%). Other alterations noticed at the examined samples are as follows: anthracosis focuses found in bronchical lymphatic lumps and mediastinal at a number of 11 samples (15.94%), hepatic and renal distrophy at a number of 5 samples (7.25%).

Inside the underchapter 5.2. are presented the results got as a follow up to the bacterioscopic examination, which was done on the organs and tissues from each area where have been found congestions, infiltrations, calcification, densifications. All these were taken using pressed imprints on the slides.

The subjects of the examination have been dried, fixed and coloured using warm colouring technics (Ziehl - Neelsen method) or using the fluorescent colouring technic with auramine-rhodamine.

Speaking about microscopic examination of the subjects on which the Ziehl-Neelsen colouring technic was used, the mycobacterium (alcohool-acidproof bacillus) were noticed as straight stick or slightly bent forms in lenght of 1 - 4 μm and width of 0.3 - up to 0.6 μm. The Bacillus of bovines type have been coloured in striking red and were granular. Regarding the subjects got from tuberculosis wounds there have been identified other forms too: cocci, cocobacillus, pseudomycelium.

The fluorescent light microscopy has been used for selecting the slides where are the acid-proof mycobacterium. It served also to guarantee the presence of mycobacterium in the pathological products used for bio-sample and typifying. The bacterioscopic examination was effected to all those 69 samples analysed in the laboratory on 386 slides. The examination was positive in 14 cases (20.29%) out of 69 analyzed samples. All the samples were confirmed using the histopathologic examination. There is the possibility to record false negative results (due to the fact that the wounds are lack in bacillus) or false positive (in the wounds can be found other species of the Mycobacterium type, opportunistic or caused by surroundings).

Cultivating the mycobacterium has been presented within chapter 5.3. - *"The bacteriologic examination"*. The cultural examination consisted of preparing the pathological material and the specific medium, their sowing, followed by their incubation. As a follow up to these processes the next steps are their lecturing and bacterioscopy. In order to isolate the mycobacterium, it has been used the Löwenstein- Jensen medium with

and without glycerine. For every single sample, there have been sowed 4 tubes with Löwenstein- Jensen medium (2 with glycerine and 2 without it).

In 78.57% cases, the M.bovis cultures became visible after a number of 50 days from sowing. The colonies revealed a dysgonic increase (small colonies, slightly convex, isolated), of type S (with neat and shining surface and margins), non pigmented.

The cultural examination has been effected to a number of 50 samples:

- 28 (56 %) samples came from lungs and afferent lymphatic lumps;

- 20 (40 %) samples came from lymphatic lumps of head, chest, mesenteric, hepatic, perch area and from the liver, spleen, kidney, lungs, genital apparatus;

- 2 (4 %) samples of the wounds found on the guinea pigs having the positive bio-test.

On certain mediums (21.43%) have been arised colonies with eugonic increase, more precisely 10 days after incubation, flat appearance, round, white-yellowish.

The mediums inoculated with samples with M. avium suspicion, were incubated at 42^0 C. It has been noticed that the stems with eugonic growth continued to evolve even at this temperature. As a result to the cultural examination made on 200 test-tubes, there have been isolated 14 bacterium stems (7%).

The bacteriologic examination offered additional positive results in comparison with the bacterioscopic examination. This way it was allowed to get a more workable form of the work subjects which were used for the identification tests: niacin, catalysis and nitrat-reductase.

The specific tests for mycobacterium identification have been effected to those 14 stems, which were isolated at the bacteriologic examination.

As a result of the bio-chemical parameters' detection, it has been noticed that 9 stems (64.29%) have been positive-catalase, 5 stems (35.71%) have been negative-catalase, all those 14 stems did not reduce nitrate and gave a negative result to the niacin test. From those stems which reacted positively to the catalysis test, 3 of them have been strongly positive.

On the other hand, a different scope which was approached within bovine tuberculosis diagnosis, it was the histopathologic examination. The samples taken were as follows: head' lymphospherical lumps, those of the respiratory tract, as well as other

lymphatic lumps and organs which appeared with macroscopic allterations. Soon after the prelevation, the samples have been introduced in formalin solution of 10%. The next steps for the samples were the following: washing, putting inside paraffin, microtomy, displaying and sticking on slides, taking out of the paraffin and hydrating sections.

The tissues sections' colouring methods have been displayed on the slides as follows: hematoxylin-eosine -blue methyl (HEA) and modified Ziehl-Neelsen (which allows to distinguish the histopathologic tissue structure and of acid-alcohoolproof bacillus.

All the 69 samples received at the laboratory with the scope of bovine tuberculosis diagnosis have been searched from the histopathologic point of view. For each sample it has been done two slides from each section.

Concerning those samples where the tuberculosis diagnosis was confirmed, there have been noticed patognomonical and morphological aspects of the disease: the tubercular granuloma was defined as a necrosis central area surrounded by epiteloid cells and multinucleus gigantic cells (Langhans type) situated at the margins of the epiteloid cells area.

At the histopathologic examination, has been noticed that in the initial stages of the tuberculosis infection there are visible concentrations of macrophage, lymphocites and fiber blasts.

The specific histopathologic allterations in case of bovine tuberculosis assure the certitude of diagnosis from the very beginning stages of the disease. In the future, due to the histopathologic examination diagnosis value using modified Ziehl-Neelsen colouring method, it is likely that this method to be considered a highly value one when the diagnosis decision should be taken. Thus, the bio-sample would not be needed and as a benefit the analysis certificate would be issued faster.

The bio-sample (underchapter 5.6.) done on guinea pigs represents the standard method for confirming the bovine tuberculosis diagnosis. The guinea pig inoculation shows only the bacillus belonging to M.tuberculosis and M.bovis species, taking into account that for M.bovis, the inoculation to the guinea pigs it is a more efficient method in comparison with the sowing. The bio-samples have been carried on from the pathologic material which was processed with the scope of enriching the sowing on Löwenstein-Jensen medium, as well as with the scope of colonies tuning up on that medium.

There have been inoculated two young male guinea pigs, weighing over 300 gr. Up to 7-10 days before inoculation, the guinea pigs were having their temperature taken

(normal values; 37.8 - 39.2 ^{0}C).

The experimental infection has been carried on by inoculating the guinea pigs using the under-tegument way, in the perch area, with 1 ml of the substance. This procedure was done after in the preliminary phase the guinea pigs have been depilated and disinfected with iodine tincture. The inoculated animals have been kept under observation 60 days and have been watched daily.

After 21 days since the inoculation took place, the clinical examination have been done. This consist of palpeting the lymphatic area where the inoculation was made. It has been noticed that the respective area suffered a hypertrophy up to aprox. 50% of the guinea pig size. The guinea pigs were having an allergic examination based on a single test, consisting of the under-tegument inoculation of PPD of a 0.1 ml dose, 1/5 dilution. The observation was made after a 24-48 hours, have been noticing the size and the parameters of the edema. Those guinea pigs which had specific tuberculosis wounds, have responded positively at the end of the experiment. This percentage was of 100% to the allergic reaction. The wounds which were found consisted of an edema and erythema on the inoculated area.

There haven't been found non-specific allergic reactions to the guinea pigs which presented the negative bio-sample.

The bio-sample has been effected for a number of 48 samples received from the laboratory during the period of time choosed for the experiment. It has been noticed that at the samples received from the laboratory, which were taken from the negative-tubercular animals, the bio-sample on the guinea pigs was done at 3 from the all samples, more specifically to those which presented anatomo-pathological and histo-pathological alterations, which lead to the suspicious of the tuberculosis diagnosis.

Regarding the guinea pigs examined after the inoculation of pathological materials, it has been noticed that the most dangerous organ, it was the spleen. The spleen was found extremely high in volume, with a granulated parenchyma, with white-grey necrosis areas, of various sizes, with non regular margins.

As incidence and wound spreading degree, as the second damaged organ was the liver. Here there have been found necrosis focuses coloured in white-grey or white-yellowish, of various sizes, with non regular margins.

The local lymphatic lumps have been found with higher volumes and in sections have been noticed white-grey or white- yellowish caseous lumps very well limited. The consistency of the lymphatic lumps was ferm in most of the cases. It has been noticed

that sometimes the consistency was pretty reduced, with a caseous-pus appearance due to the existance inside the inoculated area of some contaminated bacterium.

The guinea pigs' specificity of the wounds have been checked by doing the microscopic examination in order to find the acid- alcohoolproof bacillus, with the specific morfollogy for M.bovis.

By carrying out the frothius of the guineas pigs' wounds, it has been noticed that there are present a bigger number of acid-proofalcohool bacillus than in the lymphatic lumps wounds of the respective bovines.

The underchapter 5.7. consist of the statistical study of the bovine supervision activity, done based on the laboratory examinations on killed animals.

Therefore, with the scope of stating the diagnosis, during 2000-2005, have been killed a number of 57 tubercular-reagent bovines (29.28%) of the total of bovines found positive at the allergic test. Out of those 69 samples analysed, the disease was found with 11 animals, representing 15.94% average of contamination in the entire researched period of time. There has been confirmed a confirmation rate of 0.11% (2/18) in 2001, of 0.36% (5/14) in 2002, of 0.40% (2/5) in 2003 and of 0.22% (2/9) in 2004.

Distribution on structures of ownership was as follows:

1. 50.72 % of the samples become from colectivities. The confirmation rate was of 0.20% (7/35 animals);
2. 49.28 % of the samples become from the animals held in the households of the population, with a confirmation rate of 0.12% (4/34).

Chapter VI consists of an extended diagnosis scheme used in the tuberculosis diagnosis for a number of 33 bovines. This refers to both infection diagnosis on living animals and the diagnosis on killed animals. The used tests were the following; allergic tests (exclusive, and simultaneous comparative), immunoenzymatic test EIA_s- γ IFN, glutaraldehyde test, complement fixation test, anatomo-pathologic test, histo-pathologic test, bacterioscopic test, bacteriologic test, guinea pigs experimental infection.

In accordance with the results as a follow up of the tests, it has been considered the sensitiveness and specificity of each diagnosis tests done for the living animals:

- the allergic test:
  - sensitiveness - 71.43%
  - specificity - 60.00%

- the immunoenzymatic EIA_s -y IFN
    - sensitiveness - 80.00%
    - specificity - 62.50%
- the glutaraldehyde test
    - sensitiveness - 80.00%
    - specificity - 50.50%
- the complement fixation test
    - sensitiveness - 77.77%
    - specificity - 77.77%

In order to set up the value of the tests used in the tuberculosis diagnosis, in the present paper work it has been calculated the specificity, sensitiveness, the positive predictive value, and the negative predictive value of the test. With the aim of comparing the tests it has been statistically calculated, the kappa Cohen concordance parameter , which was estimated using a statistical programme.

Also for aiming the tests comparison, it has been calculated the frecquence of those four results categories and the distribution mean of suspicious results got as a follow up of the five tests, compared with the laboratory diagnosis result.

The ***exclusive test and the simultaneous comparative test,*** to all 33 examined animals, gave three categories of results: 9 (27.27%) positive results, 8 (24.24%) negative results and 16 (48.48%) suspicious results. Out of these, as a follow up of the diagnosis test, 11 (33.33%) have been confirmed as positive and 22 (66.66%) have been negative. There have been noticed a 4 false positive results and 2 false negative results. Out of the 16 suspicious results, 4 have been positive and 12 have been negative. The positive predictive value was between 0.56 and respectively 0.75. The K Cohen concordance parameter value when the results of the allergic tests were compared with the final results, it was of 0.30. There is a slightly positive correlation between the two sets of results.

Also in the case of ***imunoenzymatic test***, it has been noticed that there are a number of 15 (45.45%) suspicious results (it is about the samples which had the avian reaction). There have been also identified three (9.09%) false positive results and two (6.06%) false negative results. The predictive positive and negative values have been of 0.72 and respectively of 0.71. In the case of the suspicious samples, the proportion of the positive results has been lower than in case of the allergic tests. The value of the concordance parameter has been of 0.43 suggesting a moderated positive correlation

between the results of the laboratory diagnosis and the immunoenzymatic test results.

The complement fixation test used with the scope of tuberculosis diagnosis revealed for the 33 bovines which were examined , 9 (27.27%) positive results, 9 (27.27%) negative results and 15 (45.45%) suspicious results. Out of the 15 suspicious results only two (13.33%) have been positive. A special attention is drown to the small proportion of the false positive results (2) and false negative results (2). This status is reflected also with the values of the epidemiologic and statistical parameters, which were used for characterising the tests.

Regarding the ***glutaraldehyde test*** , out of the 33 cases, 15 (45.45%) have been positive, 9 (27.27%) have been negative and 9 (27.27%) have been suspicious. Within this test have been record a high number of false positive results (7 out of 15) which lead to getting of only 50% specificity. The acquired results compared with the laboratory diagnosis results lead to a K Cohen rate of 0.28 specific to a non important correlation.

Making the comparison of the acquired results, the conclusion to be made is that keeping in mind that the study was based on the value of the calculated parameters, the best tests were the immunoenzymatic test, the complement fixation test and glutaraldehyde test. They distinguished due to the small proportion of false results, small number of positive results belonging to the suspicious results category, specificity and high sensitiveness.

The specificity and sensitiveness of the allergic tests shows that those delivered comparable results with the other tests, but there is still a high number of suspicious tests, out of which as a result of the laboratory diagnosis 25% have been positively proven.

With regard to the predictive negative and positive results, the low values acquired are normal if it is kept in mind the low level of tuberculosis prevalence within the bovine investigated population.

The results of the present study concerning the sensitiveness and specificity of the tuberculosis diagnosis tests, suggests that using same time the allergic test, glutaraldehyde test, RFC and immunoenzymatic ELISA test, there is the possibility to take proper decisions with regarding to kill the positive reacted animals instead using the tubercular process only, as a detecting test.