варіаційної пульсометрії з метою оцінки стану вегетативної регуляції / Московко С.П., Йолтухівський В.М., Московко Г.С. [та ін.] // Вісник ВДМУ. - 2000. - № 1. - Р. 238-239.

Шінкарук-Диковицька М.М. Особливості зв'зків між конституційними параметрами і показниками кардіоінтервалографії у підлітків з різними типами гемодинаміки : автореф. дис. на здобуття наук. ступеня канд. мед. наук : спец 14.03.03 "Нормальна фізіологія" / М. М. Шінкарук-Диковицька. - Вінниця, 2008. - 20 с.

Task Force of the European Society of Cardiology and North American Society of Pacing and Electrophysiology. Heart rate variability. Standards of measurement, physiological interpretation and clinical use // Circulation. - 1996. - Vol. 93, № 5. - P. 1043-1065.

Йолтуховский М.В., Ищенко Г.А.

ПОКАЗАТЕЛИ КАРДИОИНТЕРВАЛОГРАФИИ У ПРАКТИЧЕСКИ ЗДОРОВЫХ МУЖЧИН И ЖЕНЩИН ПОДОЛЬЯ ПЕРВОГО ЗРЕЛОГО ВОЗРАСТА

Резюме. *У практически здоровых мужчин и женщин Подолья первого зрелого возраста и при делении на две возрастные группы (до 25 лет и старше 25 лет) установлены возрастные и половые особенности показателей кардиоинтервалографии (КИГ). Полученные результаты указывают на относительное усиление влияния парасимпатической части автономной нервной системы (АНС) и активацию центров энергометаболического обмена у мужчин в возрасте от 22 до 25 лет и более выраженную активность симпатической части АНС у мужчин в возрасте от 26 до 35 лет. У женщин также установлена более выраженная активность парасимпатической части АНС в более молодом возрасте и симпатической части АНС у женщин старшего возраста. Анализ половых особенностей показателей КИГ указывает на более выраженную у мужчин (особенно общей группы и в возрасте от 22 до 25 лет), нежели у женщин активность механизмов саморегуляции парасимпатической части АНС и усиление у женщин общей группы и разного возраста, в сравнении с мужчинами, симпатикотонических влияний.*

Ключевые слова: *кардиоинтервалография, здоровые мужчины и женщины, возрастные и половые особенности.*

Yoltuhivs'kyi M.V., Ischenko G.O.

CARDIOINTERVALOGRAPHY INDICES IN PRACTICALLY HEALTHY MALES AND FEMALES OF FIRST MATURE AGE INHABITANTS OF PODILLYA

Summary. *Age and gender related peculiarities of cardiointervalography indices (CIG) established in practically healthy males and females of first mature age inhabitants of Podillya in general and after division of these persons into two age groups (under 25 and over 25 years old). The results indicate the relative increase of the influence of the parasympathetic part of the autonomic nervous system (ANS) and the activation of energy metabolic turnover centers in males aged from 22 to 25 years and more pronounced activity of the sympathetic part of the ANS in males aged from 26 to 35 years. Females also installed more pronounced activity of the parasympathetic part of the ANS at a younger age and the more pronounced activity of the sympathetic part of the ANS in older females. Analysis of the sexual peculiarities under the CIG indices indicates more pronounced activity of self-regulation mechanisms of parasympathetic part of the ANS in males (especially in the general group and in the persons from 22 to 25 years old) than in females and strengthening in females in general and of different age groups, in comparison with males, sympatheticotonic influences.*

Key words: *cardiointervalography, healthy males and females, age and gender peculiarities.*

Стаття надійшла до редакції 11.04.2013 р.

Йолтухівський Михайло Володимирович - д.мед.н., професор, кафедри нормальної фізіології Вінницького національного медичного університету імені М.І. Пирогова;
Іщенко Григорій Олександрович - аспірант кафедри нормальної фізіології Вінницького національного медичного університету імені М.І. Пирогова; (063) 2555460.

УДК: 616-002.5-078.7:576.8:576.852.2

Lysenko A.P.[1], Vlasenko V.V.[2], Broxmeyer L.[3], Lemish A.P.[1], Novik T.P.[4], Paliy G.K.[5], Paliy I. G.,[5] Sorokoumov V.P. [5]

Institute of The experimental Veterinary Science n. a. S.N. Wyshelesski (Belarus, Minsk)[1]; Podolsk Center of Tuberculosis (Ukraine, Vinnitsa)[2]; N. Y. Institute of Medical Research in Bayside, New York, USA[3]; The Minsk State Medical University (Belarus, Minsk)[4]; Vinnitsa National Pirogov Memorial Medical University (Ukraine, Vinnitsa)[5]

THE TUBERCULINS CONTAIN UNKNOWN FORMS CAPABLE TO RESTORE VIABILITY OF CELL WALL DEFICIENT MYCOBACTRIA AND ACID-FAST CELLS

Summary. *In the research the existence of viable cell-wall-deficient forms of M. tuberculosis or M. bovis in "sterile" commercial tuberculins were studied. While using a mycobacterial growth-stimulating medium, it was found that tuberculin, been approved only as a diagnostic test, is an indication not without risk.*

Key words: *tuberculin, purified protein derivative, mycobacterium tuberculosis, mycobacterium bovis, cell-wall-deficient, sterilization of tuberculin.*

Introduction

Mycobacterium tuberculosis (MBT) and other species of mycobacteria remain the greatest infectious killers of the 21st century. According to World Health Organization (WHO), 1,7 million of people died from tuberculosis (TB) in 2009,

the equivalent of 4,700 deaths a day [WHO Report, 2010]. But many feel that the number of people that die annually from TB is closer to 2-3 million people per year. Besides such classic "tuberculosis", mycobacterial disease has been repeatedly implicated in other human disease such as cancer, diseases of the cardiovascular system, and other illnesses. [Livingston, Allen, 1948; Livingston, Alexander-Jackson, 1965; Livingston, 1972; Cantwell, 1982; Cantwell, Kelso, 1984; Cantwell, 1990; Guliang, Tefu, 1999; Anestad et al., 2001; Song et al., 2002; Broxmeyer, 2004; Centkowski et al., 2005; Nalbandian et al., 2009]. Mattman relates *that* the preferred configuration of MTB and the mycobacteria are its cell-wall-deficient (CWD) forms [Mattman, 2001]. In fact the success of MTB as the continuing scourge of mankind in many respects is tied to its unique ability to assume such resistant CWD forms as an adaptation and survival strategy when MBT faced *with* adverse conditions [Ibid]. CWD tuberculosis is stubbornly resistant to any kinds of physical insults, a fact which must always be kept in mind in the manufacture of products like tuberculin. For instance, recently it was established that such CWD forms of tuberculosis in the form of spores essentially had a greater survival rate at 60?C for 30 minutes than classical TB [Ghosh et al., 2009; Singh et al., 2010]. And the development of culture methods for MTB using certain growth stimulants has shown that the thermostability of classical forms of MTB can be made much, much higher by the CWD forms in such media stimulates [Vlasenko, 1998; Lysenko et al., 2007]. Yet somehow, thermal inactivation and sterilization are still relied upon in the production of tuberculins.

Certainly, in view of their extensive diagnostic use, there are strict rules for the manufacturing and standardization of tuberculin PPD, controlling not only its sterility, but the absence of mycobacterial cells within the tuberculins themselves [Vallat, 2010]. But a major pitfall in such attempts lies in the historic misunderstanding of the biological properties of CWD tubercular forms, and how their viral-size can escape through the finest filtration and most rigorous sterilization system, not only to survive, and which can, with time, revert back to classical MTB. The *purpose* of this study was to therefore probe into the possibility of the existence of such viable CWD forms of *M. tuberculosis* or *M. bovis* in "sterile"commercial tuberculins, using a mycobacterial growth-stimulating medium to facilitate this.

Materials and Methods

Tuberculins investigated

- PPD BOV AN5 - 1st International standard purified protein derivative *M.bovis* (Central Veterinary Laboratory, Lion), comes in 1 ampoule of 1,8 mg tuberculoproteins in accordance with contents of an ampoule are dissolved in 1,8 ml of sterile water for injection with 0,32 % phenol.

- PPD *M.bovis 8* (Kursk biofactory, Russia), a standard solution 0,86 mg / ml with 0,4 % phenol.

- PPD *M. avium* dry (Kursk biofactory, Russia), contents of a vial (10 mg) dissolved in 10 ml sterile solvent for mycobacterial allergens with 0,4 % phenol.

-PPD tuberculina mamifera AN5 (Rosenbusch, Argentina, 3412487 H, SENASA 12112/06), a standard solution with 0,4% phenol.

- PPD bovituberculin AN 5 (Biovet Pulawy, Polska, P-481/98, 01/2008), a standard solution (0,5 mg / ml with 0,4 % phenol.

- Tuberculine bovine HCSM AN 5 (Intravrac Poly Miffa Merieux, lot 4112) with 0,4 % phenol.

- PPD *M.bovis* Vallee dry (LenNIIVS, Russia).

- PPD *M.tuberculosis* T-3840 dry (LenNIIVS, Russia), dissolved in solvent for mycobacterial allergens with 0,4 %, phenol.

- Tuberculin purified *M. bovis 8* (Vitebsk biofactory, Belarus) - ultrafitrate of autoclaved culture filtrate (UFACF). The strain *M. bovis 8* was cultured on Soton medium for 8 weeks, autoclaved at 121°C for 30 minutes and filtered. 0,4% phenol was added to the culture filtrate before filtering through a 22MK Durapore® sterilizing filter with observance of sterile conditions. This was then separated with a polyethersulfone membrane (Biomax©® 300K), collecting an ultrafiltrate of 0,5-0,6mg/ml and 0,4% phenol was added.

Nutrient Medium and Growth stimulant

We used MycoCel DW (Doctor Vremia, Belarus), a mycobacterial growth enhancing substance which comes as a sterile, transparent liquid with 0,05% chorhexidine bigluconate as an antiseptic. Its accompanying medium is a powder which, after being mixed with deionized water, dissolves at 100°C, and can then be filtered and autoclaved for 15 minutes at 121°C, and then placed in sterile glass test tube slants for use after 48-hour control for sterility. This medium, when ready, appears as a transparent yellowish gel on an agar base. Subsequent time until actual use never exceeded 14 days.

Microscopic research of tuberculins

Sterile glass slides were rendered with 0,02 ml or one drop of each tuberculin solution. Sterile cover plates where applied and each slide was looked at through light microscopy (10x100).

Ten ml of each tuberculin were centrifuged at 10,000 g for 10 minutes. Supernatants were then discarded, leaving a prospective pellet which was suspended in 0,02 ml sterile 0,15M NaCl and which could then be put onto sterile glass slides, dried, fixed at 65°C and stained by Ziehl-Neelsen (Z-N). Results of microscopy were recorded using an Olympus 56BX digital microscope.

Preparation and inoculation of tuberculins

Tuberculin samples, under aseptic precautions, were mixed with growth stimulant ycoCel DW (1:2) and then incubated for 24-48 h at 37°C. After that 0.3ml of each mixture were placed in test tube slants with growth stimulating medium, and incubated for 10 days at 37°C. For control, growth stimulant was inoculated by itself on MycoCel DW growth-enhancing medium.

Research techniques engaged on isolates

Upon the appearance of colonies, smears were made and stained with acid-fast Ziehl-Neelsen (Z-N), as well as

probed by immunoenzyme technique (IET). In such Immunoenzyme Technique (IET), antibody (Ig) from the rabbit antiserum of *M. tuberculosis H37Rv* was purified on Affi-gel (Bio-Rad) with *M. tuberculosis H37Rv* antigen. Purified antibody was then conjugated (Nakane, 1977) with a peroxidase (Sigma TM). Smears of each culture were inactivated by heating at 65°C with 3% hydrogen peroxide for 30 minutes and then 95% methanol for 5 minutes. Both were subsequently washed-off with distilled water and a solution of peroxidase conjugate with 0,02 % Tween 80, and incubated for 1 h at 18-22°C. Smears were again flushed with distilled water with diaminobenzidine hydrochloride (DAB), and hydrogen peroxide, followed by flushing for 30 minutes with distilled water alone.

S*tudying the pathogenicity of tuberculin isolates*

Four healthy guinea pigs testing negative for mycobacterial antibodies or DNA in their blood were intradermally injected with 5 mg of isolate from UFACF *M. bovis* (I passage). Two healthy guinea pigs, not injected, and served as controls. On autopsy, two months later liver, spleens, and lungs were homogenated and decontaminated with 4% sulfuric acid and then that mixture hypodermically injected by a sterile 1 ml syringe into healthy guinea pigs as a second passage. In addition, a portion of these decontaminated homogenates were previously mixed (1:2) with growth stimulant, and incubated 24 hours at 37°C. These mixtures were then placed in test tube slants previously filled with growth stimulating medium and incubated for 7 days at 37°C. Two months later, autopsy was carried out on the animals receiving this second passage. Again homogenates of liver, spleen, and lungs were decontaminated with 4% sulfuric acid and planted on MycoCel DW medium.

Inoculation of tuberculins in pathogen free chicken embryos

Three groups of 9-day-old chicken embryos (Loman, Germany) were injected with either 0,2 ml (0,11 mg) of tuberculin UFACF *M. bovis*, 0,2 ml (0,2 mg) PPD tuberculin *M. avium*; or 0,2 ml of sterile 0,9 % solution of NaCl as a control, respectively. After nine days of incubation fluid was removed from the allantoic sac of each embryo which was mixed in a 1:2 ratio with mycobacterial growth stimulant and plated on MycoCel DW medium for 24 hour incubation.

Polymerase Chain Reaction (PCR)

Isolates from all tuberculins placed on growth-enhancing MycoCel DW medium were then investigated with PCR real-time kits FAM for the *M.tuberculosis-M.bovis* complex, and JOE (SMU Narvak, Moscow) for *M. tuberculosis* The isolates from the embryos placed on the nutrient growth medium MycoCel DW were then investigated by PCR kits for the detection of *M. tuberculosis* and *M. bovis's* DNA MPB70 and 16S RNA (IBOCH, Minsk).

Financial support: The National Academy of Sciences of Belarus (grant No. 20065951). Travel grants for scientific presentations from Docktor Vremia, Inc. Belarus, Minsk 220131.

Conflict of Interest: Prof. Alexander Lysenko is the developer and manufacturer of the mycobacterial growth-enhancer Mycocel DW. No other authors' conflicts of interest exists.

Presented in part: This study was presented in part at the science conference "Ecology and Change of Mycobacterium bovis" 06/29-07/01/2011, held at the National University of Environment at Kiev, Ukraine.

Reprints or correspondence: prof. Alexander Lysenko Institute of the Experimental Veterinary Science n. S. N. Wyshelessk.i.

Results. Discussion

At light microscopy of the PPD tuberculin samples (10x100) were themselves, no specific bacterial forms were revealed. But in Ziehl-Neelsen (Z-N) acid-fast smears of tuberculin centrifugate, transparent fine spheres and larger spheres with dark blue centers and translucent peripheries became obvious (figure 1).

After 48 h incubation, mixtures of PPD with growth stimulant were observed under light microscopy.

Unusual broad, shoot-like filament forms were visualized, from which separated spiral forms, originating from the sides of each broad filament (figure 2A). Mycobacteria such as tuberculosis belong to the class Actinomycetes. Many Actinomycetes form true filaments that branch. Their filamentous forms produce spores that may, on occasion, appear to be spirals. Meirowsky reported the same thick filaments in certain stages of TB and other mycobacteria, along with refractile coccoid bodies or granules that sprouted from its sides - a phenomenon also evident in various forms of *Treponema pallidum*, the causative agent of syphilis [Meirowsky, 1914]. Eventually though, in all tuberculin samples, given a window of 2-7 days, colonies in the form of either "thin lawn" (fine, up to 1mm transparent colonies) or separate waxlike colonies, 2-3 mm in diameter, appeared.

All investigated samples of the tuberculins, including UFACF, bolstered with growth stimulant were then inoculated on MycoCel DW medium. At 48 hours other CWD mycobacterial forms were in evidence in each sample, such as non-acid-fast (NAF) honeycomb-like and ring forms (figure 2B). There

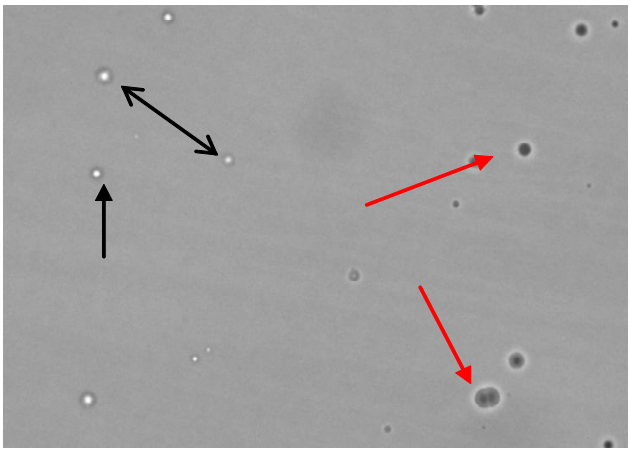

Figure. 1. The pellet from the centrifugate of tuberculin (UFACF) M.bovis. Transparent spheres (black arrows) and larger spherical forms, frequently dual, with dark blue centers and a transparent periphery (grey arrows) are visible (Z-N stain).

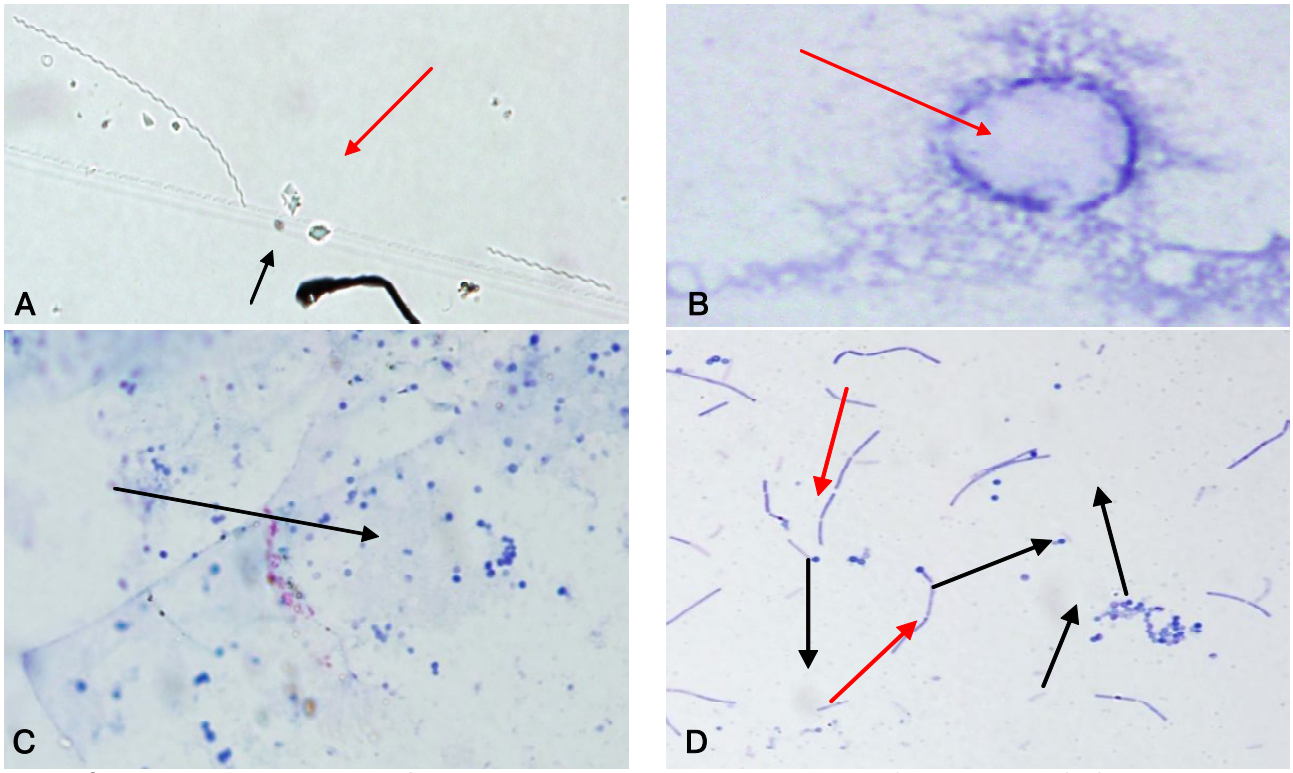

Figure. 2. A. Mixture of tuberculin (UFACF) M. bovis (lot 74) and growth stimulant after 48 h incubation at 37°C. Light microscopy (10x100). Formation of a shoot-like broad filaments (black arrow) from which separate spirochetel forms originate from (grey arrows). B. Growth from PPD BOV AN5 NIBSC two days after inoculation on MycoCelDW. Honeycomb-like structure (black arrow), and ring form (grey arrow) are visible. Z-N, 10X100. C. Growth of tuberculin (UFACF) *M. bovis 8* on growth enhancing MycoCel DW medium. A shoot-like "string" structure (black arrows), similar to that in figure 2A, this time in which NAF cocci (short black arrow) and AF rod-shaped forms (grey arrows) are visible. Z-N, 10x100. D. Growth of tuberculin (UFACF) *M.bovis 8* on MycoCel DW medium (day 5). Cocci connected to a series of rods are visible. Z-N, 10x100.

were variations within form as well, such as "shoot" formations similar to figure 2A, in which NAF cocci and rod shaped acid-fast (AF) tubercle bacilli were present (figure 2C). Within 3-5 days all tuberculin smears showed the familiar coccoid and rod-shaped forms of CWD tuberculosis, with rods of varying length containing granules. In some cases, these forms are visibly connected, demonstrating that they originated from and represent the same microorganism, simply evolving into different morphological forms (figure 2D).

All isolates from tuberculins *M. bovis* and *M. tuberculosis* contained common antigens with classic acid-fast (AF) forms of. MTB as they specifically stained by IET with the purified antibody to M. tuberculosis conjugated with peroxidase (figure 3).

Lengthily cultivation of isolates from tuberculins (1-3 months) on either MycoCel DW or classic Lowenstein-Jensen (L-J) mediums produced, in all cases, classic acid-fast (AF) tubercular rods (figure 4A, 4B, 4C).

Isolates from each tuberculin were subjected to PCR-real time. With FAM probe for the *M. tuberculosis-M. bovis* complex DNA all isolates got amplification from a 7-27 cycle. With JOE for *M. tuberculosis* alone, synthesis of specific sites coincided only with isolates from PPD *M. tuberculosis T-3840*, confirming it as its parent type. (table 1) Note that DNA samples of banal microflora did not react to either probe.

After the subcutaneous injection 5mg of isolate from UFACF *M. bovis 8* the guinea pigs remained alive for two months. On autopsy, typical visible lesions for tuberculosis were absent. Acid-fast (AF) stain of lung, liver or kidney tissue revealed no AF forms. But when each of these organ-tissues homogenates were planted on growth-enhancing MycoCel DW medium - although they showed mostly CWD NAF tubercular cocci, there were also some classical grey AF rods, growing within 3 to 4 days (figure 5A). This was as expected, since MycoCel DW is primarily CWD growth enhancer.

A second passage of decontaminated tissue homogenates from the animal of first passage was injected into the second group of guinea pigs, although they also remained alive for 2 months - this time autopsy revealed 1-2 mm in diameter liver granulomas in half of the animals. And plating upon MycoCel DW yielded many more classical AF rods of tuberculosis on smear then in the first passage (figure 5B).

Spontaneous appearance of viable forms after introduction of tuberculins into pathogen-free chicken embryos (PFE).

Nine days after the inoculation of chicken embryos with either 0,11 mg tuberculin (UFACF) *M. bovis 8* or 0,2 mg tuberculin PPD M. avium, the embryos remained alive. Nor, on a day 9 autopsy, were there any essential gross pathological

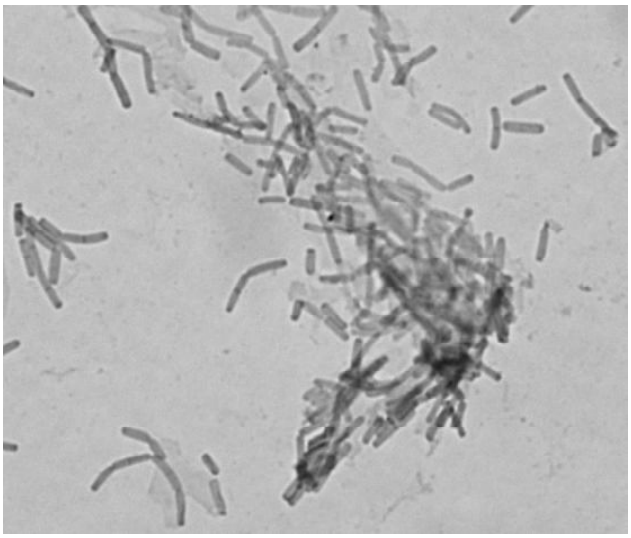

Figure. 3. Growth from PPD BOV AN5 NIBSC (6 day) on MycoCel DW medium. IET, DAB. 10x100.

changes showing. But when the fluid from each tuberculinized chicken embryo's allantoic sac was plated on growth-enhancing MycoCel DW medium both CWD and classic AF forms of the tubercle bacilli appeared. It was also validated that isolates from tuberculin treated embryos reacted to tubercular PCR primers 16S RNA and MPB70. This was true regardless of the two tuberculins tested. However, whereas DNA of isolate from tuberculin (UFAC) *M. bovis 8* reacted with both of the tubercular primers, DNA of isolate from PPD *M. avium* reacted positively only with primer 16S RNA (figure 6).

The US Department of Agriculture in its Code of Federal Regulations Title 9 which covers tuberculin specifies (9CFR 1113.409) that regarding all products like Tuberculin, each serial batch shall be tested for viable bacteria, mycobacteria and fungi. However, with regards to the TB that might be harbored in tuberculin, this in practice only includes investigating for red-staining AF tubercular forms, therefore ignoring the predominant form of tuberculosis, which, as a survival strategy, is cell-wall-deficient. Meanwhile, to this day, U.S. references contain the caution that Seibert's Tuberculin Purified Protein Derivative, although much preferred and much more refined than Koch's Old tuberculin (OT), still contains "soluble growth products from the tubercle bacillus". Yet at the same time, the U.S. pharmacopeia claims that tuberculin is "sterile". But of the commercial tuberculins tested here and previously considered "sterile", all grew tubercular microorganisms using a mycobacterial growth stimulant, while controls using this same stimulant in sterile Normal Saline, grew nothing.

Most of the cell-wall-deficient forms of MTB we found have been described by others [Lucksh, 1930; Mellon, Fisher, 1932]. This suggests that the autoclaved "products of growth" and lysis of mycobacteria in tuberculin manufacturing inadvertently keeps thermostable CWD forms in the final product, capable of springing back to life. The existence of such thermostable, filterable tubercular forms has been known for some time [Fontes, 1910]. Indeed, whether heated or not heated, filterable forms of MTB produce identical lesions in lymph nodes [Hababou-Sala, 1928; Morozova, 1929]. Nor does the addition of antiseptics such as phenol or chorhexidine to tuberculin assure its "sterility". Older literature assures that filterable forms of tuberculosis can grow in the presence of 0,5% phenol [Ramsine, 1926]. Mattman, in fact, spoke of a "phenol phenomenon" in which filterable CWD tuberculosis not only remains viable but flourishes and multiplies with one drop of 5% phenol [Mattman, 2001]. Such filterable forms of tuberculosis are not only often the size of a virus but because of the breech in their cell-wall, they become "cell-wall-deficient", with a plasticity that only adds to their filterability.

Although it is possible to assume contamination regarding the non-acid-fast cultures first isolated from the tuberculins, one would have to account for the subsequent appearance of classic acid-fast tubercle bacilli stains upon re-inoculation of these onto both MycoCel DW and L-J (Lowenstein-Jensen) mediums. Not only were tubercular forms validated by this

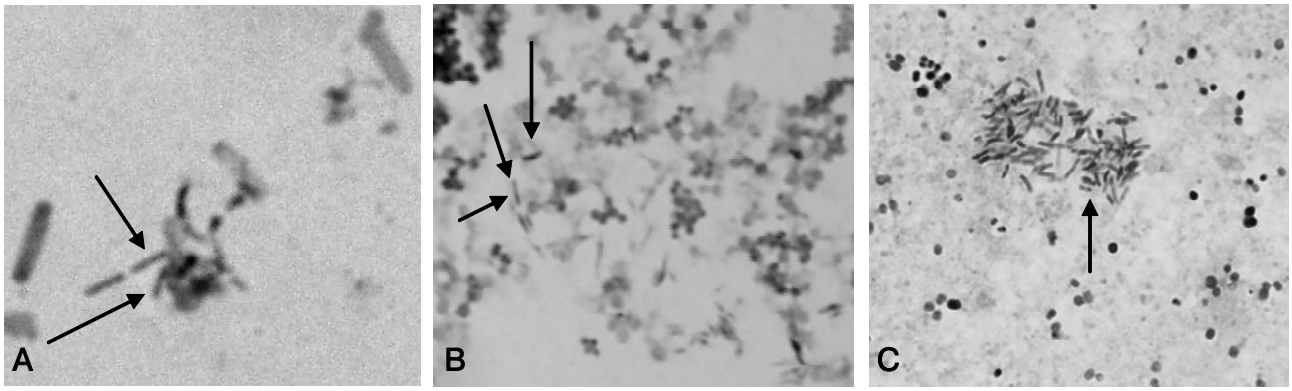

Figure 4. Lengthily growth-enhanced cultivation show classical AF tubercular rods in tuberculin. A. Growth from UK tuberculin BOV AN5 NIBSC on MycoCel DW medium (3 months). Formation AF rods (black arrow) is visible. Z-N, 10x100. B. Growth of tuberculin UFACF *M.bovis 8* (lot 66) on MycoCel DW medium (3 months). Here non-acid-fast (NAF) cocci as well as classic red AF rods (black arrows) become visible C. Growth of tuberculin UFACF *M.bovis 8* (lot 66) on Lowenstein-Jensen (L-J) medium (2 months). Both NAF coccoid forms and AF rods are visible.

Table 1. The results of PCR real-time with the DNA of tuberculin isolates.

Isolates from	Ct	
	FAM-M.tuberculosis-M.bovis complex	JOE-M.tuberculosis
PPD M. bovis Vallee	26,65	Not detected
PPD M. tuberculosis T-3840	12,88	2,93
PPD M. bovis 8	6,38	Not detected
UFACF M. bovis 8	25,98	Not detected
Controls		
M.bovis Vallee L-J medium	5,85	Not detected
M.tuberculosis H37Rv L-J medium	36,21	3,25
Staph.aureus	Not detected	Not detected
Strept. fecalis	Not detected	Not detected
Candida albicans	Not detected	Not detected
Proteus vulgaris	Not detected	Not detected

"gold standard", the Ziehl-Neelsen (Z-N) acid-fast stain, but this was reconfirmed by the fact that all tuberculin isolates had common antigens with reference strains of *M. tuberculosis/bovis* at IET staining. Add to this that upon PCR, all tuberculin isolates, using FAM and JOE probes, proved to have specific sites of DNA for *M. tuberculosis* and *M. bovis*.

As predicted by Mattman and others, cell-wall-deficient (CWD) cultures isolated from tuberculins did not immediately produce tubercular lesions in guinea pigs, but were capable of persisting as typical CWD tuberculosis [Fontes, 1910; Centkowski et al., 2005]. This was true from the very first guinea pig passage. But with repeated passage both the degree of pathogenicity and the ability to isolate acid-fast forms were increased. The application of finding latent forms CWD of tuberculosis through growth enhancing techniques has recently become a topic of interest and besides Myco Cel DW is found elsewhere [Mukamolova et al., 2002; Ghosh et al., 2009].

In conclusion, we maintain that tuberculins are potentially dangerous. In the name of better diagnostics, and with every tuberculin skin test administered, recipients are essentially being exposed to viable cell-wall-deficient (CWD) tuberculosis which can, with time, revert back to classical virulent TB. Chandrasekhar and others have shown that such CWD tubercular forms can easily produce typical disease in the immunodeficient [Chandrasekhar, Ratnam, 1992; Ma, 1995]. And such immunodeficiency can result from the mere childhood acquisition of a flu-like tubercular involvement, or as little as exposure to dust aerosol which can also tie up the immune system as well [Rosenzweig, 1979, Rosenzweig, 1980].

Furthermore, the synthetic intradermal diffusion of dormant cell-wall-deficient tuberculosis into the system known as the tuberculin skin test can also contribute to the establishment of other illness not presently linked with MTB, but suspected by some as being connected. For example, the honeycomb-like pattern and capillary-like networks seen in our Figures 3 and 4 are eerily similar to those formed by microorganisms isolated from certain malignant tumors [Robinson, 2005]. Seibert herself would later conclude that cell-wall-deficient tubercular rods with an acid-fast stage were behind malignant tumors and leukemic blood [Seibert et al., 1970]. At the same time tuberculins have never been tested for their carcinogenic or mutagenic potential.

The whole subject of the extreme heat-resistance of cell-wall-deficient tubercular forms brings into question the effectiveness of thermal inactivation of tubercular biologicals like tuberculin. In 2010, Singh [Singh et al., 2010] asserted that highly filterable CWD tubercular spores could be transmitted, even in the very food we eat, in spite of present sterilization protocols. Foddai, in that same year showed data which essentially confirmed the same for M. avium subsp. paratuberculosis, despite pasteurization. [Foddai et al., 2010].

It has been documented that repeated intradermal injections of tuberculin results in more tolerance and less proliferative responses in animals subsequently exposed to pathogenic strains of MTB [Thom et al., 2004]. This, in effect, possibly creates the same phenomena as the introduction of an extremely diluted

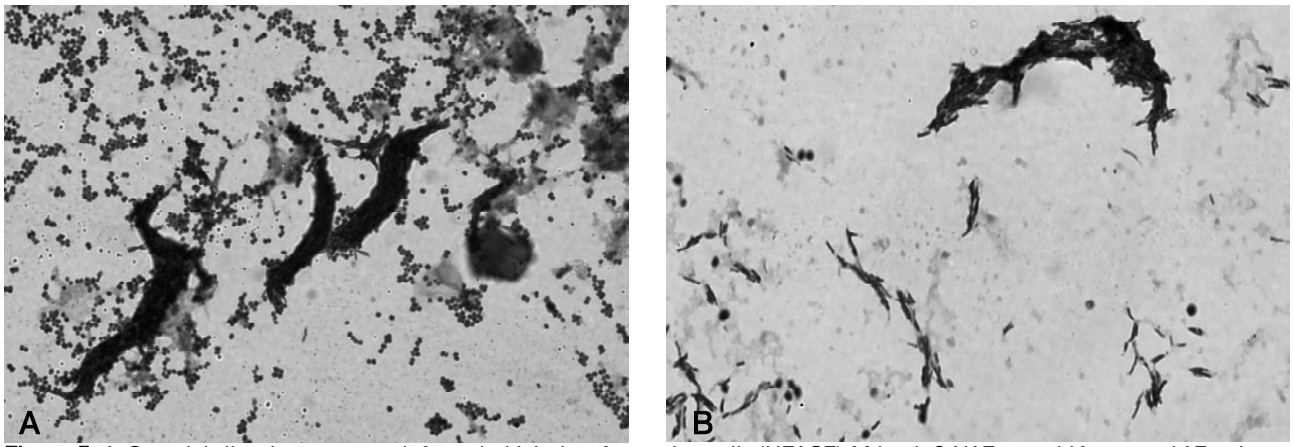

Figure 5. A. Growth in liver homogenate infected with isolate from tuberculin (UFACF) *M.bovis 8*. NAF coccoid forms and AF rods are visible. First passage. MycoCel DW, Z-N, 10x100 B. Growth of liver homogenate in second group of first passage guinea pigs. Note NAF coccoid and rod forms, with many AF rods visible from this second passage. MycoCel DW, Z-N, 10x100.

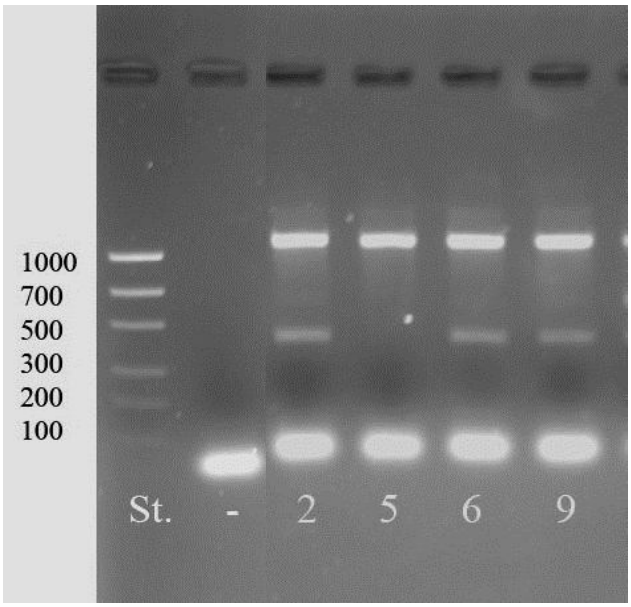

Figure. 6. PCR (16S RNA, MPB70) with (-) representing negative controls, (2) representing the DNA from *M. bovis* Vallee L-J, (5) representing the DNA of isolates from PFE, infected with tuberculin M .avium, (6) representing DNA of isolates from PFE, infected with tuberculin (UFACF) *M.bovis 8* and (9) the DNA from cultures from tuberculin PPD *M.bovis*.

and inactivated BCG tuberculosis immunization. But unfortunately, there are also possible severe side effects associated with taking repeated tuberculin. Hospitalizations have resulted from severe side effects from the administration of a single intradermal PPD. In addition, the mycobacteriophages inside of the tubercular forms found in tuberculin can, through genetic transfer, make an existing tubercular infection worse. Today tuberculin is of course not indicated for immunization. It is only approved as a diagnostic test. According to our findings, it is an indication not without risk.

Funding: This work was supported by a financial grant from The National Academy of Sciences of Belarus. (grant No. 20065951).

Conclusions and future perspectives

1. According to the results of research at 48 hours after inoculation on MycoCel DW medium other CWD mycobacterial forms are in evidence in each sample, such as non-acid-fast (NAF) honeycomb-like and ring forms. Within 3-5 days all tuberculin smears show the familiar coccoid and rod-shaped forms of CWD tuberculosis.

2. With FAM probe for the *M. tuberculosis-M. bovis* complex DNA all isolates got amplification from a 7-27 cycle. With JOE for *M .tuberculosis* alone, synthesis of specific sites coincided only with isolates from PPD *M. tuberculosis T-3840*.

3. After the subcutaneous injection of isolate from UFACF *M. bovis* on autopsy no changes, no AF forms happen, but after planting tissue homogenates of lungs, liver or kidneys on MycoCel DW medium mostly CWD NAF tubercular cocci may grow within 3 to 4 days.

4. Both CWD and classic AF forms of the tubercle bacilli appear when plating the fluid from each tuberculinized chicken embryo's allantoic sac on growth-enhancing MycoCel DW medium.

The results received in the study can be used for optimization of further development of diagnosis, prophylaxis and treatment of tuberculosis.

References

A family of autocrine gtowth factors in Mycobacterium tuberculosis / Mukamolova G., Turapov O.A., Young D.I., Kaprelyants A.S. [et al.] // Molecular Microbiology. - 2002. - Vol. 46, № 3. - P. 623-35.

Atherosclerosis and tuberculosis: are they both chronic infectious diseases? / [Anestad G., Hoel T., Scheel O., Vainio K.] // Scand J Infect Dis. - 2001. - Vol. 33, № 10. - p. 797.

Broxmeyer L. Is mad cow disease caused by a bacteria? Med Hypotheses / L. Broxmeyer. - 2004. - Vol. 63, № 4. - P. 731-739.

Cantwell A. The Cancer Microbe / A. Cantwell. - Los Angeles: Aries Rising Press. - 1990. - 283 p.

Cantwell A.R. Jr. Variably acid-fast bacteria in a fatal case of Hodgkin's disease / A.R. Jr Cantwell, D.W. Kelso // Arch. Dermatol. - 1984. - Vol. 120, № 3. - P. 401-402.

Cantwell A.R. Variably acid-fast cell wall-deficient bacteria as a possible cause of dermatologic disease / A.R. Cantwell. - In, Domingue GJ (Ed.) Cell Wall Deficient Bacteria. Reading: Addison-Wesley Publishing Co. - 1982. - P. 321-360.

Chandrasekhar S. Ratnam S. Studies on cell-wall deficient non-acid fast variants of M.tuberculosis. Tuber Lung Dis. - 1992. - Vol. 73, № 5. - P. 273-279.

Detection of Mycobacterium tuberculosis in lung cancer tissue by indirect in situ nested PCR / Song L.Y., Yan W.S., Zhao T. [et al.]. - 2002. - Vol. 22, № 11. - P. 992-993.

Foddai A. Rapid assessment of the viability of *M.avium* subsp. paratuberculosis cells after heart treatment, using an optimized phage amplification assay / A. Foddai, C.T. Elliot, I.R. // Grant. Appl Environ Microbiol. - 2010. - Vol. 76. - P. 1777-1782.

Fontes A. Bemerkungen ueber die tuberculoese infection und ihr virus. Mem. Inst.Oswaldo Cruz. - 1910, № 2. - P. 141-146.

Growth, cell division and sporulation in mycobacteria / [Singh B., Ghosh J., Islam N.M., et al.]. - Antonie van Leewenhoek. - 2010. - Vol. 98, № 2. - P. 165-177.

Guliang H., Tefu L. Mycobacterium tuberculosis L-forms / H. Guliang, L. Tefu // Microbial Ecology in Health and Disease. - 1999. - Vol. 10. - P. 129-133.

Hababou-Sala I. A propos de la filtrabilite du bacilli tuberculeux pour le tissue ganglionnaire / I. Hababou-Sala // C rend. Soc. Biol. - 1928. - Vol. 99. - P. 1215-1217.

Hodgkin's lymphoma and tuberculosis coexistence in cervical lymph nodes. Leuk Lymphoma / [Centkowski P., Sawczuk-Chabin J., Prochorec M., Warzocha K.]. - 2005. - Vol. 46, № 3. - P. 471-475.

Investigation of the thermal resistance of Mycobacteria tuberculosis. Probl. Tuberk. Bolezn. Legk / Lysenko A.P., Lemish A.P., Krasnikova E.L. [et al.]. - 2007. - Vol. 2. - P. 42-46.

Livingston V. Presence of consistently recurring invasive mycobacterial forms in tumor cells / V. Livingston, R.M. Allen. - Microscop Soc Bul I- 1948. - Vol. 2. - P. 5-18.

Livingston V.W. Mycobacterial forms in myocardial vascular disease / V.W.

Livingston, E. Alexander-Jackson // J. Amer Med Wom Assoc. - 1965. - Vol. 20. - P. 449-452.

Lucksh F. Die Filtierbarkeit des Tuberkelbazillus und anderer Bakterien, C-bl. f. Bakt. I Or. / F. Lucksh 1930. - Vol. 117. - P. 1-17.

Lung carcinogenesis induced by chronic tuberculosis infection: the experimental model and genetic control / Nalbandian A., Yan B.S., Pichugin A. [et al.] // Oncogen. - 2009. - Vol. 28, № 17. - P. 1928-1938.

Ma X.L. Experimental studies on pathogenicity of Mycobacterium tuberculosis L-form / X.L. Ma // Chinese Journal of Microekology. - 1995. - № 01. - P. 110-115.

Mattman L.H. Cell Wall Deficient Forms - Stealth Pathogens / L.H. Mattman. - CRC Press Boca Raton 3rd ed. - 2001. - 416 p.

Meirowsky E. On the biological position of the Spirochaeta pallida and its development / E. Meirowsky // Brit. J. of Dermat. - 1914. - Vol. 26. - 185 p.

Mellon R. New studies on filterability of pure culture of the tubercle group of microorganisms / R. Mellon, L. Fisher // J. Infect. Dis. - 1932. - Vol. 51, № 1. - P. 117-128.

Morozova E. Zur Frage der filtierbaren Formen des Tuberculoseerrger. C-bl. f. Bakt. I, Or; / E. Morozova. - 1929. - Vol. 113. - 200 p.

Morphological, biological, and immunological studies on isolates from tumors and leukemic bloods [Seibert F.B. Feldmann F.M. Davis R.L. Richmond I.S.] - Ann. N.Y. Acad. Sci. - 1970. - Vol. 174. - P. 690-728.

Ramsine S. Sur les formes filtrables des Straptocoques et sur la nature de la toxine de Dick. C. rend. Soc. Biol / S. Ramsine. - 1926. - Vol. 94. - 1010 p.

Robinson D.H. Pleomorphic mammalian tumor-derived bacteria self-organize as multicellular mammalian eucaryotic-like organisms: morphogenetic properties in vitro, possible origin, and possible roles in mammalian "tumor ecologies" / D.H. Robinson // Med. Hypothesis. - 2005. - Vol. 64, № 1. - P. 177-185.

Rosenzweig D.Y. Atypical mycobacteriosis / D.Y. Rosenzweig // Clin. Chest. Med. - 1980. - Vol. 1. - P. 273 - 284.

Rosenzweig D.Y. Pulmonary mycobacteria infections due to Mycobacterium avium complex. Clinical features and course in 100 consecutive patients / D.Y. Rosenzweig. - Chest., 1979. - Vol. 75. 115 p.

Sporulation in mycobacteria. Proc Natl Acad Sci USA / Ghosh J., Larsson P., Singh B. [et al]. - 2009. - Vol. 106, № 26. - P. 10781-10786.

The effect of repeated tuberculin skin testing of cattle on immune responses and disease following experimental infection with Mycobacterium bovis / Thom M., Morgan J.H., Hope J.C. [et al.] // Vet. Immunol Immunopathol, 2004. - Vol. 102. - P. 399-412.

Vallat B. Bovine Tuberculosis, The Tuberculin Test in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Terrestrial Manual) / B. Vallat. - World Organization, 2010. - http://www.cabi.org/ahpc/default.aspx?page 3323.

Vlasenko V.V. Tuberculosis in focus of problem contemporarity / V.V. Vlasenko. - (in Russian) Vinnica: Nauka, 1998. - 350 p.

WHO Report 2010. Global Tuberculosis Control 2010. World Health Organization; WHO Library Cataloguing-in-Publication Data 2010. - P. 216-218.

Лисенко О.П., Власенко В.В., Броксмайер Л., Леміш А.П., Новик Т.П., Палій Г.К., Палій І.Г., Сорокоумов В.П.
ТУБЕРКУЛІН МІСТИТЬ БІОЛОГІЧНІ ФОРМИ, ЗДАТНІ ДО ВІДНОВЛЕННЯ ЖИТТЄЗДАТНОСТІ У КИСЛОТОСТІЙКИХ БАКТЕРІЙ І МІКОБАКТЕРІЙ З ЧАСТКОВОВТРАЧЕНОЮ КЛІТИННОЮ СТІНКОЮ

Резюме. *У дослідженні вивчено існування життєздатних форм з дефектною клітинною стінкою у M. tuberculosis чи M. bovis у комерційних туберкулінах. За допомогою використання поживних середовищ, що стимулюють ріст мікобактерій, доведено, що туберкулін не є абсолютно безпечним.*

Ключові слова: *туберкулін, очищений протеїновий дериват, Mycobacterium tuberculosis, Mycobacterium bovis; клітини з дефектною клітинною стінкою, стерилізація туберкуліну.*

Лысенко А.П., Власенко В.В., Броксмайер Л., Лемиш А.П., Новик Т.П., Палий Г.К., Палий И.Г., Сорокоумов В.П.
ТУБЕРКУЛИН СОДЕРЖИТ БИОЛОГИЧЕСКИЕ ФОРМЫ, СПОСОБНЫЕ К ВОССТАНОВЛЕНИЮ ЖИЗНЕСПОСОБНОСТИ В КИСЛОТОУСТОЙЧИВЫХ БАКТЕРИЙ И МИКОБАКТЕРИЙ С ЧАСТИЧНОПОПОТЕРЯННОЙ КЛЕТОЧНОЙ СТЕНКОЙ

Резюме. *В исследовании изучено существование жизнеспособных форм с дефектной клеточной стенкой в M. tuberculosis или M. bovis в коммерческих туберкулинах. Посредством использования питательных сред, стимулирующие рост микобактерий, доказано, что туберкулин не является абсолютно безопасным.*

Ключевые слова: *туберкулин, очищенный протеиновый дериват, Mycobacterium tuberculosis, Mycobacterium bovis, клетки с дефектной клеточной стенкой, стерилизация туберкулина.*

Стаття надійшла до редакції 13.02.2013 р.

Lysenko A.P. - doctor of Veterinary, professor, the head of the department of tuberculosis and leucosis of farming animals Institute of the experimental Veterinary Science n. a. S. N. Wyshelesski (Belarus, Minsk);
Vlasenko V.V. - doctor of biology, professor, the head of the department of Microbiology and Technology of Processing, Podolsk Center of Tuberculosis (Ukraine, Vinnitsa);
Broxmeyer L. - doctor of medicine, professor of N. Y. Institute of Medical Research in Bayside (New York, USA);
Lemish A.P. - the head of the department of vaccines and serum, philosophy doctor of of veterinary, senior researcher of Institute of the experimental Veterinary Science n. a. S. N. Wyshelesski (Belarus, Minsk);
Novik T.P. - philosophy doctor Candidate of biology Assistant Professor of the department of microbiology in Minsk State Medical University;
Paliy G. K. - Honoured Worker of science and techniques of Ukraine, Doctor of medical science, Professor, the Head of the department of Microbiology, Virology and Immunology in Vinnitsa National N. I. Pirogov Memorial Medical University (Ukraine, Vinnitsa);
Paliy I. G. - doctor of medical science, Professor the department of Internal and family medicine in Vinnitsa National Pirogov Memorial Medical University (Ukraine, Vinnitsa);
Sorokoumov V. P. - philosophy doctor Candidate of medicine, Assistant Professor of the department of Pathologic Anatomy in Vinnitsa National Pirogov Memorial Medical University (Ukraine, Vinnitsa).