Clinical and Experimental Medical Sciences, Vol. 2, 2014, no. 2, 55 - 73
HIKARI Ltd, www.m-hikari.com
http://dx.doi.org/10.12988/cems.2014.445

The Tuberculin Skin Test: How Safe is Safe? - the Tuberculins Contain Unknown Forms Capable of Reverting to Cell-Wall-Deficient Mycobacteria

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Abstract

Tuberculin is made from proteins derived from tubercle bacilli that have been "killed" by heating. Yet in both Zwadyk's 1994 study and Bemer-Melchior's 1999 investigations 'heat-killed' tuberculosis and its related mycobacteria, whether in tuberculin, vaccination or otherwise, have dormant, practically indestructible cell-wall-deficient forms which can revert back to virulent TB bacilli "killed" — by neither heat nor sterilization. The ability and actual preference of mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium bovis* to form filterable, multi-shaped cell-wall-deficient (CWD) forms and spores in order to survive unfavorable conditions has in fact been known for some time. But the possibility of PPD tuberculins for human use containing such potentially virulent CWD forms, even after autoclaving, sterilizing and ultrafiltration, has not. Autoclaved ultra-filtrates of the various mycobacteria used to produce tuberculin skin tests, consisting of *M. Tuberculosis*, *M. bovis*, and *M. avium* were investigated. All samples were mixed with growth stimulant, incubated, and placed on a special nutrient medium with a 1% agar base. Within 2-10 days after incubation colonies of a variety of non-acid-fast forms were noted, yet all of these proved, through PCR real time with FAM probe to still have antigens in common with their classic tubercular parent-form, from which they originated. Moreover, in true cell-wall-deficient fashion, the isolates, upon guinea pig inoculation, did not immediately produce visible lesions, but nevertheless persisted. However, tissue homogenates of the infected animals, once placed on a growth-enhancing medium showed cell-wall-deficient mycobacterial forms interspersed with classical acid-fast rods. And a repeated passage of such tissue homogenates back into non-infected guinea pigs, not only induced small mycobacterial granulomas in their livers, but a distinct increase in acid-fast rods. Moreover, similar cell-wall-deficient mycobacterial forms with acid-fast rods occurred when embryonated chicken eggs were inoculated with PPD tuberculins as well.

The autoclaved and supposedly "sterilized" purified protein derivative [PPD] used in tuberculin skin tests contain cell-wall-deficient forms capable of eventually reverting back to virulent acid-fast tuberculosis, both typical and atypical.

Keywords: Tuberculin; Mycobacterium bovis; thermo stability; Cell-Wall-Deficient mycobacteria

INTRODUCTION

Mycobacterium tuberculosis (MTB) and other species of mycobacteria remain the greatest infectious killers of the 21st century.[1] Besides classic "tuberculosis", mycobacterial disease has been repeatedly implicated in other diseases such as cancer, cardiovascular disease, and other illness.[2-13] Mattman relates that the preferred configuration of MTB are its cell-wall-deficient (CWD) forms.[14] In fact the success of MTB as the continuing

scourge of mankind is tied to its unique ability to assume such resistant CWD forms as an adaptation and survival strategy when MBT is faced with adverse conditions.[15] CWD tuberculosis is stubbornly resistant to the harshest of physical insults including extreme heat, a fact which must always be kept in mind when manufacturing products like tuberculin. It has already been established that tuberculosis's spore-like, dormant forms have a much greater survival rate at 60º C than does its vegetative form.[16,17] But using special growth stimulants and nutritional media has shown that the thermostability of tuberculosis's cell-wall-deficient forms is even greater.[18,19]

Certainly, in view of its extensive diagnostic use, there are already in place strict rules for the manufacturing and standardization of tuberculin, controlling its sterility and trying to achieve the absence of mycobacterial cells. 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 survive the most rigorous sterilization systems.

The purpose of this study was to therefore probe the possibility of the existence of such viable CWD forms of *M. bovis*, *M. tuberculosis*, *and M. avium* in 'sterile' tuberculins, using a mycobacterial growth-stimulating medium to facilitate this.

MATERIALS AND METHODS

Tuberculins used in commercial packaging:

1. PPD BOV AN5 - 1st International standard purified protein derivative (PPD) *M.bovis* AN5 (Central Veterinary Laboratory, Lion), 1.8 mg PPD (in ampoules) dissolved in 1.8 ml of sterile water for injection with 0.32 % phenol.
2. PPD *M. bovis* (Russia), a standard solution (0.86 mg / ml with 0.4 % phenol).
3. PPD M. *avium* (Russia), content of a vial (10 mg) dissolved in 10 ml sterile solvent for mycobacterial allergens with 0.4 % phenol.
4. PPD tuberculina mamifera AN5 (Rosenbusch), a standard solution (1 mg / ml with 0.4% phenol).
5. PPD bovituberculin AN 5 (Biovet, Polska, P-481/98, 01/2008), a standard solution (0.5 mg / ml with 0.4 % phenol).
6. Tuberculine bovine HCSM AN 5 (Intravrac Poly Miffa Merieux, lot 4112) with 0.4 % phenol.

7. PPD *M.bovis Vallee* dry *and* PPD *M. tuberculosis* T-3840 dry (LenNIIVS, Russia), dissolved in a solvent for mycobacterial allergens with 0.4 % phenol.
8. UFACF *M. bovis* (lots 37, 66, 74, 83, 85) - ultrafiltrate of autoclaved culture of the filtrate from *M. bovis.* The strain *M. bovis 8* was cultured on Soton medium for 8 weeks, autoclaved at 121º for 30 min and filtered. 0.25% phenol and 0.02% of bacteriostatic sodium azid was added to the culture filtrate before filtering through a .22 $_{MK}$ Durapore® sterilizing filter. This was then separated with a polyethersulfone membrane (Biomax©® 300K), collecting an ultrafiltrate (UFACF) of 0.5-0.6mg/ml. Phenol 0.4% was added.

Nutrient Medium and Growth stimulant.

Growth stimulant (MycCel DW experimental composition on water base) a sterile, transparent liquid with 0.1% chorhexidine bigluconate. Nutrient medium (MycCel DW experimental composition on 1% agar base). The components were suspended in deionized water, dissolved at 100ºC, and then placed in sterile glass test tube slants and autoclaved for 15 minutes at 121º C. The medium was used after a 48-hour control period for sterility.

Preparation and inoculation of tuberculins

All tuberculins, under aseptic precautions, were mixed with growth stimulant (1:2) and then incubated for 48 h at 37°C. After that 0.3ml of each mixture was placed in sterile test tube slants with nutritive media and incubated for 10-15 days at 37°C. As a control, growth stimulant was inoculated by itself on nutritive media.

Investigation of isolates from tuberculins

Upon the appearance of colonies, smears were made and stained with Ziehl-Neelsen (Z-N) and by modified immunoenzyme technique (MIET): heated for 1.5 h at 65^0C, and inactivating endogenous peroxidase by 3% hydrogen peroxide (30 min) and 95% methanol (5 min). Incubation was for 1.5 h with a conjugate (1:80 in PBS with 0.002% Tween 20) of affinity-purified antibody for *M. tuberculosis* $H_{37}Rv$ or *M. bovis 8* with peroxidase. Incubation for 20 min with substrate solution DAB followed (10 mg 3,3 diaminobenzidine «Fluka» in 2 ml DMSO, 10 ml of distilled water with

10 мl 33 % H_20_2 – which was then counterstained for 5 minutes with Kinyoun carbolfuchsin (through a filter paper), decolorized by acid alcohol for 70-90 sec, and finally subject to 3 minutes of 0.25% methylene blue. After every step the smears were washed with deionized water.

Antibody (Ig) from the rabbit antisera to *M. tuberculosis $H_{37}Rv$* and *M. bovis 8* was purified on Affigel (Bio-Rad) with antigens of sonicates. *M. tuberculosis $H_{37}Rv$, M. bovis 8* and conjugated (Nakane, 1977) with a peroxidase (Sigma Type VI). With MIET stain AF forms were stained a crimson red color, surrounded by brown colored non-acid-fast (NAF) forms with a red or lilac shade. Non-tubercular microflora were colored in a dark blue or greenish-blue color, providing a distinct contrast from cell-wall-deficient MTB. Results of microscopy were recorded using the digital microscope «Olympus 56BX».

ELISA research of isolates from tuberculins

Isolates grew in 5-6 days, after which the bacterial mass was washed twice by 0.3 % phenol solution and centrifuged at 3000 g. The sediment was then suspended in 0.15% phenol solution (pH 7.4) and disrupted on Bandelin Sonopuls 2400. Sonicates (100 μg /ml in 0.1M carbonate-bicarbonate buffer) were placed for 1 hour at 37 °C on microtiter plates of Biofil®. Then these plates were washed 4 times with a washing buffer.

Sheep and bovine antisera to sonicates of *M. tuberculosis $H_{37}Rv$, M.bovis Vallee, M.bovis 8 and* also affinity purified antibodies from sheep antiserum to CWD *M. tuberculosis* were used. As a control, the blood from tuberculin negative cows and sheep were employed.

Plates for the dilution of sera (100 μl of a 1:20-1:1280 solution) were incubated at 37°C for 1hour. Anti-bovine peroxidase conjugate (Sigma A5295 1:2000) in diluting buffer was added (100 μl) and plates then incubated for 1h at 37°C. After washing, TMB substrate solution (100 μl) was added in 7 min-stop reagent (50 μl). The optical density (OD) was measured at 450 nm using an ELISA reader (BioRad). After every step plates were washed four times with washing buffer.

Studying the pathogenicity of tuberculin isolates

Four healthy guinea pigs testing negative for mycobacterial antibodies or DNA in their blood were hypodermically injected with 5 mg. of isolate from UFACF *M. bovis* (one passage). Two healthy guinea pigs, not injected, served as controls. On autopsy

two months later, liver, spleen, and lung tissues were homogenated and decontaminated with 4% sulfuric acid. Then that mixture was hypodermically injected (1 ml) into healthy guinea pigs as a second passage. In addition, a portion of these decontaminated homogenates were mixed (1:2) with growth stimulant, and incubated 24 hours at 37°C. These mixtures were then placed in sterile 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 nutritive media.

Possible persistence of CWD forms of MTB after introduction of tuberculin

Three groups (n=3) of 9-day-old chicken embryos (Lohman, Germany) were injected in their allantoic sac with either 0.2 ml (0.11 mg) UFACF *M. bovis,* 0.2 ml (0.2 mg) PPD *M. avium*, or 0.2 ml of a sterile 0.9 % solution of NaCl as a control. After nine days of incubation, fluid was removed from the allantoic sac of each embryo which was mixed in a 1:2 ratio with growth stimulant and incubated 24 hours before being plated on a medium.

Polymerase Chain Reaction (PCR)

DNA of isolates from tuberculins were investigated with PCR real-time kits (Narvak, Moscow): FAM for the *M. tuberculosis-M. bovis* complex, and JOE for *M. tuberculosis.*

DNA of isolates from the tuberculins and from the chicken embryos were investigated by PCR kits (IBOCH, Minsk) with primers to MPB70 and 16S RNA.

RESULTS

Initially, microscopic examination of sediments after the centrifuging of tuberculins did not seem to contain any microflora of unusual form. But in the course of the incubation of tuberculin UFACF, mixed with a growth stimulant, within 48 hours at 37 degrees Centigrade, unusual broad, shoot-like filament forms were observed, from which separated spiral forms, originating from the sides of each broad filament. (Figure1A) Soon it also became obvious that all of the other tuberculins, when added to growth stimulant MycCel DW and plated grew within a time frame of approximately 2-10 days. Small transparent colonies or "lawn" appeared on culture medium surfaces. Visible colonies grew after "blind" reinoculation. These mycobacterial forms grew from all tuberculins

investigated. As a control, nutrient medium with only growth stimulant added showed no visible changes.

In smears stained with Ziehl–Neelsen (Z-N), non-acid-fast (NAF) honeycomb-like and ring forms were observed initially. (Figure 1B) There were variations within such forms as well, such as "shoot" (filament) formations (Figures 1A, 1C) similar, capillary-like networks (Figure 1D) in which NAF cocci and rods (some of which were also acid-fast (AF) were present. Within 3-10 days all samples showed the familiar AF coccoid and rod-shaped forms, some visibly connected to their non-acid fast counterparts, demonstrating that they originated from and represent the same microorganism — simply evolving into different morphological forms, with different staining capacities. (Figure1E) Importantly, in some isolates, it was the more lengthy cultivation of the tuberculins that showed the classic pathogenic acid-fast rods of tuberculosis. (Figures 2A, 2B, 2C, and 2D)

In isolates of subcultures of the tuberculins grown on Lowenstein–Jensen (L-J) medium and MPA (meat peptone agar), there was growth at temperatures 22^{o}, 37^{o} and 45^{o}C, and in some cases the formation of an orange-brown pigment. (Figure 3) All isolates had common antigens with classic acid-fast tuberculosis, specifically staining brown with MIET. (Figure 4) Also, the sonicates of isolates from the tuberculins reacted in ELISA with the antisera to classical *Mycobacterium bovis* as well as with affinity purified antibody to CWD *M. tuberculosis.* In the range of dilutions of antisera 1:20-1:320 OD indexes essentially did not differ from indexes for the sonicate of classical *M. bovis.* With increased dilution, the intensity towards antisera reactions dropped for CWD tuberculosis as compared to *M. bovis* itself. Nevertheless, the index OD for both organisms, taken in excess of three times, still exceeded indexes of normal sera. (Figures 5 and 6)

With PCR, the DNA from tuberculin isolates yielded positive results with primers for 16s RNA M. *bovis-M. tuberculosis* (Figure 7), validating that these forms were mycobacterial. In a PCR-real time with FAM probe for the *M. tuberculosis-M. bovis* complex DNA, all tuberculin isolates amplified during a 7-27 cycle. With JOE for *M. tuberculosis* alone, as expected, synthesis of specific sites coincided only with isolates from PPD *M. tuberculosis T-3840*, confirming it as its parent type. (Table 1)

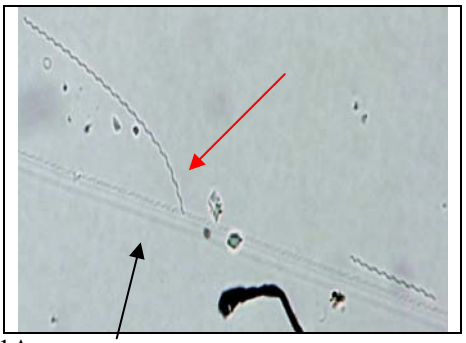
Table 1. The results of PCR real-time with the DNA of tuberculin isolates:

Isolates from	Ct	
	FAM-*M.tuberculosis-M.bovis* compl.	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*	25.98	Not detected
Controls		
M.bovis Vallee L-J medium	5.85	Not detected
M.tuberculosis H37Rv L-J	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

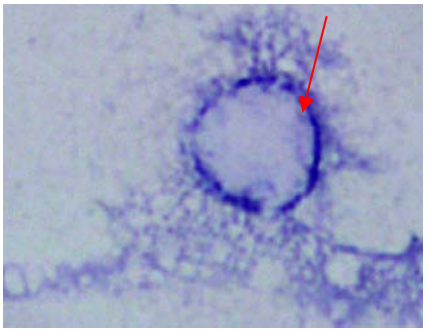
After the subcutaneous injection of 5 mg. from the UFACF *M. bovis* isolate, the guinea pigs remained alive for two months. At autopsy, typical visible lesions for tuberculosis were absent. Z-N staining of lung, liver or kidney tissue revealed no AF forms. But when each of these organ-tissues homogenates were planted on a growth-enhancing medium, although they showed mostly NAF cocci, there were also virulent acid-fast rods. (Figure 8A)

A second passage of decontaminated tissue homogenates from the animals of first passage was injected into a second group of guinea pigs. Although they also remained alive for 2 months - this time autopsy revealed 1-2 mm liver granulomas in half of the animals and plating with growth-enhancing medium yielded many more classical AF rods of tuberculosis on smear then previously achieved in the first passage. (Figure 8B)

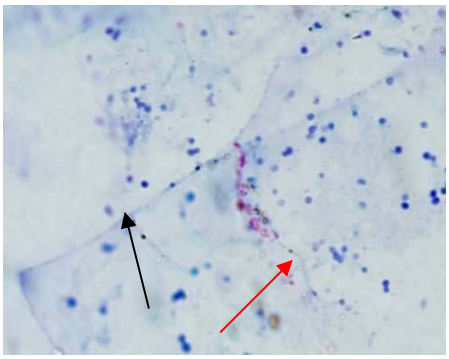
To further show that tuberculins may generate potentially pathogenic cell-wall-deficient forms, pathogen-free chicken embryos (PFE) were injected with either 0.2 ml (0.11 mg of protein) UFACF *M. bovis* or 0.2 mg tuberculin PPD *M. avium*. Nine days after such inoculations, chicken embryos remained alive. Nor, on a day 9 autopsy, were there any essential gross pathological changes showing. But when the fluid from each tuberculinized chicken embryo's allantoic sac was planted on growth-enhancing medium, both CWD and classic AF forms suddenly appeared. Furthermore, the results of PCR with our DNA isolates, using primers 16S RNA and MPB70 were positive. This was true regardless of which one of the two tuberculins in this sub-series was tested, the only difference being that whereas the DNA of culture isolates after injection of UFAC *M. bovis* gave positive reactions with both primers (16S RNA and MPB70), DNA of isolates of culture isolated after injecting PPD *M. avium*, only yielded positive with primer 16S RNA. (Fig.9)



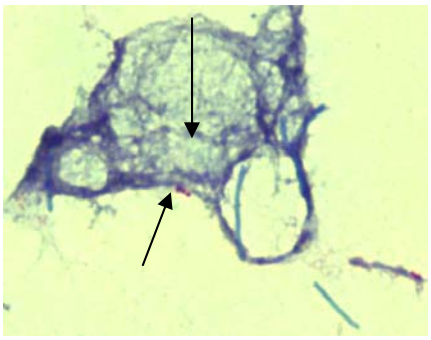
1A



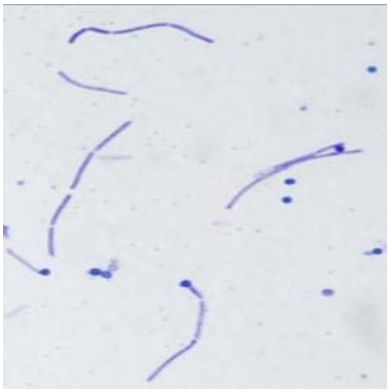
1B



1C



1D



1E

Figure 1:

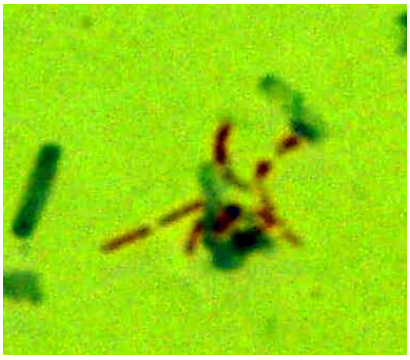
1A.With a mixture of UFACF *M. bovis* (lot 74) and growth stimulant. Light microscopy. Formation of a shoot-like broad filaments (black arrow) and spirochete form. (red arrow).

1B. Growth of PPD BOV AN5. Honeycomb-like structure and ring form (red arrow), Z-N.

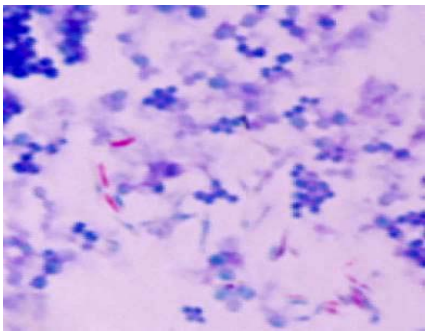
1C. Growth of UFACF *M. bovis* (lot 66). A shoot-like "string" structure (black arrow) in which NAF cocci and AF forms (red arrow) are visible, Z-N.

1D. Growth of PPD BOV AN5 Ring form, capillary-like networks, NAF and AF rods (black arrow) are visible.

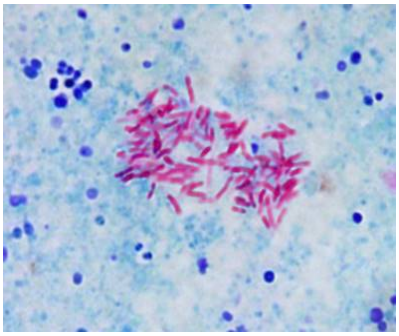
1E. Growth of UFACF *M. bovis* (lot 66). NAF cocci connected to a series of rods, Z-N, 10x100.



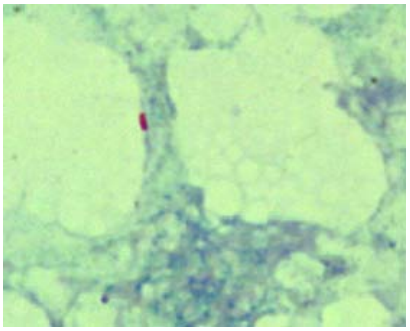
2A



2B



2C



2D

Figure 2 A**.** Growth of BOV PPD AN5 (3 months). **B.** Growth of UFACF *M. bovis* (lot 66, 3 months). **C.** Growth of UFACF *M. bovis* (lot 74, 2 months). D. Growth BOV PPD AN5 (10 days). NAF coccoid forms and rods, capillary-like networks and acid-fast tubercular rods are visible. Z-N, 10x100.

Figure 3. Growth of isolate from PPD BOV AN5 on L-J medium at 37^{0}C (orange-brown pigmentation).

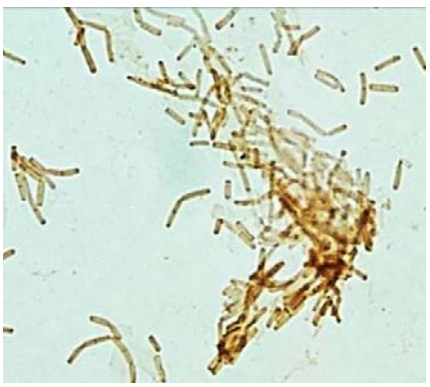
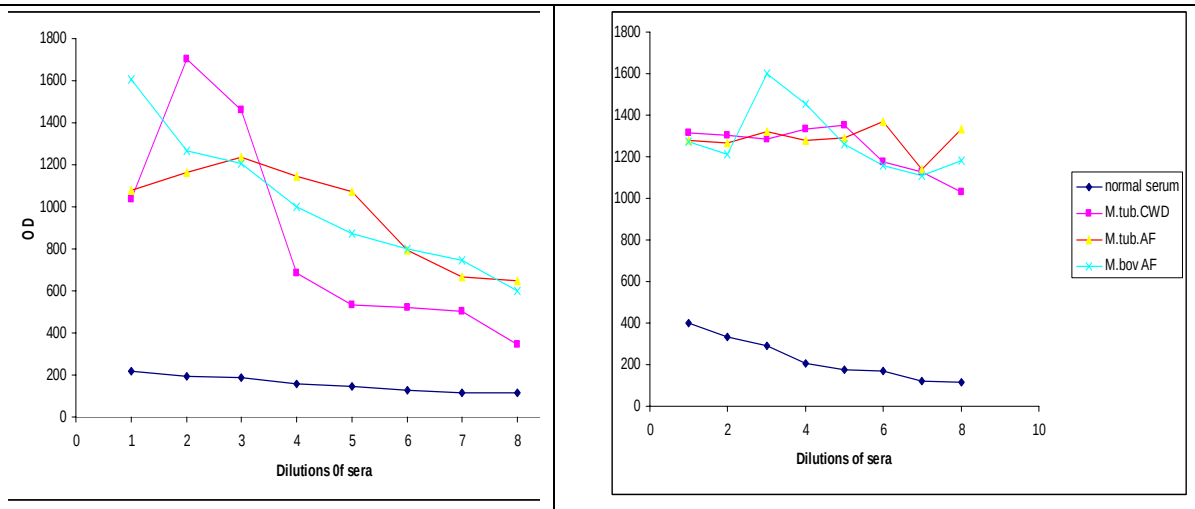


Figure 4. Isolate from PPD BOV AN5, MIET, 10x10



Figures 5, 6. ELISA of sonicates isolates from PPD AN5 (left) and bacillary (AF) form of PPD *M. bovis Vallee* (right)

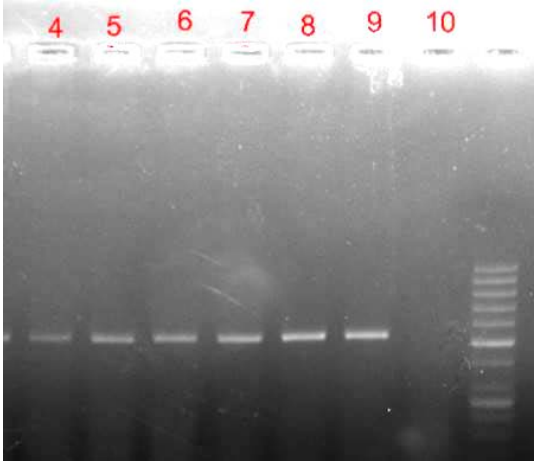
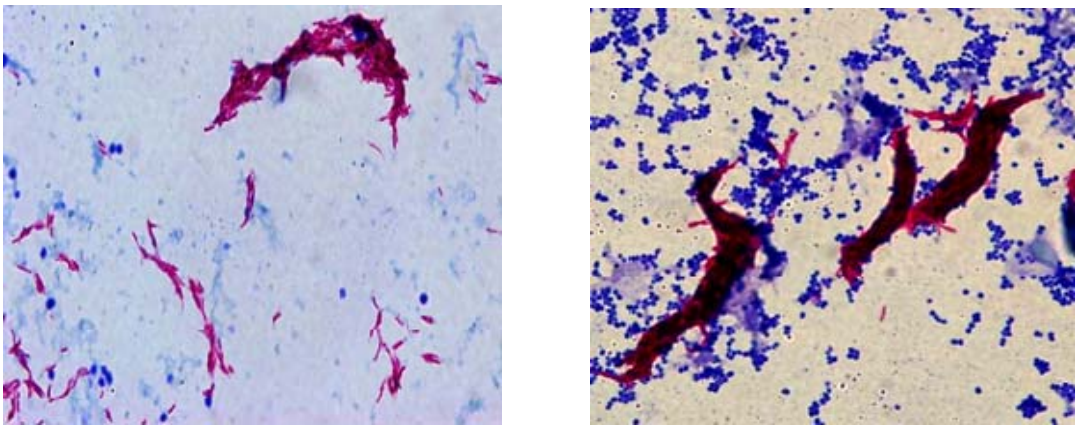


Figure7. Amplification of *M. tuberculosis-M. bovis* 16S RNA from DNA extracted from isolates: 4- UFACF lot 66, 5-UFACF lot 37, 6- PPD BOV AN5, 7- UFACF lot 74, 8 – PPD *M. bovis Vallee*, 9-positive control, 10 –negative control.



8A **8B**

Figure 8 A. Growth of liver homogenate of guinea pig infected with isolate from UFACF *M. bovis*. NAF coccoid forms and AF rods are visible. First passage. B. Growth of liver homogenate of guinea pig second passage. Note NAF coccoid and rod forms, with many AF rods visible from this second passage, Z-N, 10x100.

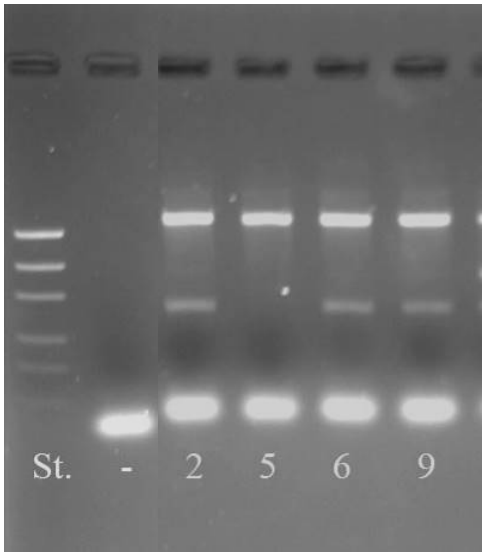


Figure 9. PCR (16S RNA, MPB70) with representing: negative controls (-), (2) DNA from *M. bovis* Vallee L-J, (5) DNA of isolates from PFE infected with PPD *M .avium,* (6) DNA of isolates from PFE, infected with UFACF *M. bovis,* and (9) DNA of isolate from PPD *M. bovis.*

DISCUSSION

In both Zwadyk's 1994 study and Bemer-Melchior's 1999 investigations 'heat-killed' tuberculosis and its related mycobacteria, whether in tuberculin, vaccination or otherwise, have dormant, practically indestructible cell-wall-deficient forms which can revert back to virulent TB bacilli, "killed" by neither heat nor sterilization.[32,33] Yet most manufacturer's documentation regarding the production and control of tuberculin speak highly of their safety and harmlessness. Actually tuberculins are merely checked for the absence of disease causing microbes, the absence of fungal contamination and most importantly, the absence of the classic pathogenic acid-fast red rods of tuberculosis, the disease from which tuberculin originates.

As one example, 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 regard to the TB that might be harbored in tuberculin, such testing in practice only includes investigating for red-staining acid-fast 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".

In light of the safety, harmlessness and sterility proclaimed by tuberculin manufactures therefore, it is surprising that all of the tuberculins investigated, mixed with tuberculin growth stimulant, eventually grew out cell-wall-deficient [CWD] tubercular forms, both non-acid-fast and virulent acid-fast tubercular rods (Figure 3), while controls using this same stimulant in sterile Normal Saline, grew nothing. Moreover, the forms found in this study, resemble the cell-wall-deficient MTB uncovered by many investigators.[2,5,9,11,14,15,18,20,21,22,23]

This suggests that the autoclaved "products of growth" and lysis of mycobacteria in tuberculin manufacturing inadvertently keep thermostable CWD forms in the final product, capable of springing back to life. The existence of such thermostable, filterable tubercular forms, even under the most extreme heat conditions, has been known for some time.[26] Indeed, whether heated or not heated, filterable forms of MTB produce identical lesions in lymph nodes.[23,24] 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 even 0.5% phenol.[25] 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.[14]

The rigors of autoclaving used for the manufacture of tuberculins, with its soaring temperatures, in itself will generate resistant forms. Convincing data about the formation of such resistant cell-wall-deficient (CWD) forms and spores under the duress of extreme heat exist for *M. bovis* as shown by Gosh, Larsson, and Singh's group in 2010.[16,17] And there is no reason to assume that the same does not happen in the generation of such practically indestructible forms under physical duress in the case of *M. tuberculosis* which is structurally similar. Cell-wall-deficient forms of tuberculosis grow more quickly than classical tubercle bacilli.[15,18,25]

The tuberculins all yielded CWD tubercular growth in spite of the fact that they were exposed to sterilizing filtration. Acid-fast cultures of the mycobacteria routinely formed tiny filterable cell-wall-deficient forms[23], fully capable of even penetrating the membrane of a Biomax 300K filter.

There can be no doubt that the CWD isolates obtained from all tuberculins investigated possessed antigens in common with their classical tubercular parents, both in reacting with affinity purified antibody to antigens of *M. bovis* and *M. tuberculosis* and staining a specific brown color with MIET. Using ELISA, the intensity of reactions by the tuberculin isolates, even with a dilution of 1:20-1:160 of the antisera to *M. bovis, M. tuberculosis,* and *M. fortuitum* did not essentially differ from those indexes achieved by the bacillary forms of *M. bovis* itself. Indeed, many antigenic properties were shared between classic acid-fast parents and their CWD tubercular offspring.[25]

The fact that isolates from tuberculins introduced to guinea pigs did not produce immediately visible tubercular lesions was expected. Typically, CWD forms of MTB slowly, but persist to eventually invoke tissue granulomas.[14,21,22,25] At the same time tissue homogenates of infected guinea pigs, once planted on a growth-enhancing medium were quick to show an increase in the number of the bright red rods — the traditional hallmark of active tubercular disease. And upon second passage, not only were such acid-fast rods increased in number but small granulomas in portions of the guinea pigs livers became obvious. These results in themselves confirm the growing pathogenicity of CWD forms of tuberculosis with time and passage, as they revert back to the typical forms of virulent tubercular disease[21], as our findings confirmed. Such outcomes have great theoretical value in that under normal non-infectious conditions, with mere uninfected tissue homogenate being introduced into experimental animals, our results would have proved impossible. Similar isolation of CWD non-acid fast and acid-fast forms where obtained through the injection of UFACF tuberculin *M. bovis* and PPD *M. avium*. But whereas tuberculin UFACF proved site specific for both 16S RNA and the bovine

tubercular antigen MPB 70, the cell-wall-deficient forms elicited by M. avium tuberculin were only specific for site 16S RNA.

The phenomenon of having such robust thermostability under extreme temperature elevations is anything but rare for *Mycobacterium tuberculosis*, whose intestinal acid-fast rods recently were found to easily escape with their viability intact, conditions that otherwise would have normally killed them, such as the lethal thermal shock of 134° C..[27]

What effects from the tuberculins then can be assumed, having recognized and documented tuberculin's ability to morph into resistant CWD forms under the influence of spiking temperatures and other conditions of severe stress?

1. First that tuberculins are potentially hazardous. Their mass introduction into a person or an animal probably produces a situation identical to that seen in this study, with long term persistence of cell-wall-deficient *Mycobacterium tuberculosis* which can at some point, revert back to classical TB at any time, especially in the face of a weakened immune system.[21,22]

2. Secondly, the synthetic diffusion of all but immortal, persistent cell-wall-deficient tubercular forms as a result of tuberculin can contribute to the origination of other diseases, even those presently not linked to typical and atypical tuberculosis. For example, some of the forms documented in this investigation, particularly the honeycomb-like pattern and capillary-like network seen in Figure 1, are similar to those formed by microorganisms isolated from tumors.[28] Yet tuberculins have never been tested for their carcinogenic or mutagenic potential.

3. The extremely high thermostability of tuberculosis in general, automatically presents the question as to the efficacy of thermal inactivation not only with the tuberculins, but the sterilization of our foodstuffs subject to mycobacterial disease. Ghosh and others, having proven the existence of the very same tubercular spores that Robert Koch once talked about after sterilization,[16,17] emphasized: "Likewise, mycobacterial spore particles might also be transmitted via our food in spite of sterilization protocols" much in the same manner that Foddai et al.'s data (2010) showed surviving *M. avium* subsp. *paratuberculosis* after the pasteurization of dairy products.[30]

Yet, having said all of this, it is equally important to mention, that on the plus side, that tuberculins can also produce a fixed immunizing effect, as seen in a study where multiple injections of tuberculin and the subsequent infection by CWD tubercular strains in animals to which tuberculin was repeatedly administered was followed by a markedly decreased proliferative response in these animals than with control animals.[31] Such findings are not without historic human precedent. Although tuberculin never fulfilled Koch's

expectation that it would cure tuberculosis in general, let us not forget that at one time, tuberculin was also used, often with success, in treating certain underdiagnosed tubercular conditions such as those of the eyes, resistant to other therapy. Such ocular curative effects were thought "to lie in establishing an active immunity against the disease"[34] Perhaps then, in such cases, the therapeutic action of the tuberculin in our TB skin test was acting more like a milder form of BCG vaccination than a diagnostic test.

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

Conflict of Interest: Prof. Alexander Lysenko is the developer of the mycobacterial growth-enhancer MycCel DW. No other author conflict 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, given at the National University of Environment at Kiev, Ukraine.

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Received: April 29, 2014