

The bovine tuberculosis burden in cattle herds in zones with low dose radiation pollution in Ukraine

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Summary

The authors describe a study of the tuberculosis (TB) incidence in cattle exposed to low doses of radiation resulting from the Chernobyl (pronounced 'Chornobyl' in Ukrainian) nuclear plant catastrophe in 1986. The purpose of the study was to determine if ionising radiation influences the number of outbreaks of bovine TB and their severity on farms in the Kyiv, Cherkasy and Chernigiv regions of Ukraine. These farms are all located within a 200 km radius of Chernobyl and have had low-dose radiation pollution. Pathological and blood samples were taken from cattle in those regions that had positive TB skin tests. *Mycobacterium* spp. were isolated, differentiated by PCR, analysed and tested in guinea-pigs and rabbits. Species differentiation showed a significant percentage of atypical mycobacteria, which resulted in the allergic reactions to tuberculin antigen in the skin test. Mixed infection of *M. bovis* and *M. avium* subsp. *hominissuis* was found in three cases. The results concluded that low-dose radiation plays a major role in the occurrence of bovine TB in regions affected by the Chernobyl nuclear disaster.

Keywords

Bovine tuberculosis, Cattle, Chernobyl, Chornobyl, Low dose, Mycobacteria, *Mycobacterium bovis*, Radiation, Tuberculosis, Ukraine.

Il peso della tubercolosi bovina nelle mandrie site nelle zone a basso inquinamento radioattivo in Ucraina

Riassunto

Gli autori descrivono uno studio sull'incidenza della tubercolosi (TB) condotto sul bestiame esposto a bassi livelli di radiazioni provocate dal disastro nucleare di Chernobyl ('Chornobyl' in ucraino) nel 1986. Scopo dello studio era stabilire se le radiazioni ionizzanti influenzano il numero di focolai di TB bovina e la loro gravità nelle fattorie delle regioni di Kiev, Cherkasy e Chernigiv in Ucraina. Tutte queste fattorie si trovano entro un raggio di 200 km da Chernobyl e sono state esposte a un basso livello di inquinamento radioattivo. I campioni patologici ed ematici sono stati raccolti dai capi di bestiame di queste regioni che avevano risultati positivi ai test cutanei della TB. È stata accertata la presenza di Mycobacterium spp., che è stato isolato, differenziato mediante PCR, analizzato e testato su cavie e conigli. La differenziazione delle specie ha evidenziato una percentuale significativa di micobatteri atipici, con reazioni allergiche all'antigene della tubercolina al test cutaneo. In tre casi è stata riscontrata un'infezione mista da M. bovis e M. avium subsp. hominissuis. Sulla base dei risultati è stato concluso che le radiazioni a basso livello giocano un ruolo importante nell'insorgenza della TB bovina nelle regioni colpite dal disastro nucleare di Chernobyl.

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Parole chiave

Basso livello, Bestiame, Chernobyl, Chornobyl, Micobatteri, *Mycobacterium bovis*, Radiazione, Tubercolosi, Tubercolosi bovina, Ucraina.

Introduction

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by *Mycobacterium bovis*. In many countries, bovine tuberculosis is a major infectious disease of cattle, other domesticated animals and certain wildlife populations (35).

Bovine tuberculosis constitutes a public health problem as *M. bovis* is a zoonotic pathogenic agent, i.e. it can be transmitted from animals to humans; it has been identified in humans in many countries. *M. bovis* causes a disease clinically indistinguishable from tuberculosis (TB) infection caused by *M. tuberculosis* (4, 35).

M. bovis infection has a significant economic impact with worldwide annual losses to agriculture of US\$3 billion (8). This fact supports the worldwide TB eradication programmes which prescribe the slaughter of cattle showing positive reactions to the intradermal tuberculin skin test. This test remains the standard method for ante-mortem diagnosis of bovine TB and forms the basis of measures aimed at TB eradication in Ukraine.

In Ukraine, TB diagnosis is conducted using a combination of epidemiological, allergic (e.g. skin test), pathological and bacteriological methods. Ante-mortem TB diagnosis is based solely on the skin test. All cattle in Ukraine must be skin tested with the mammalian tuberculin antigen (made from *M. bovis*) twice a year and all reactors must be removed from the herd immediately and slaughtered. In addition, the entire complex of veterinary, sanitary and organisational measures, specified in the current instruction, must be performed (16, 17, 18, 19, 36).

However, skin test results are influenced by a number of variables such as the stage and severity of disease, prevalence of cross-reacting organisms and a host of other factors (27, 32). On many farms, the comparative skin test, utilising both tuberculin for mammals and

allergen prepared from *M. scrofulaceum* and *M. intracellulare* (allergen from atypical mycobacteria: AAM), is used to discriminate between animals infected with *M. bovis* and those sensitised to tuberculin due to exposure to non-pathogenic mycobacterial organisms of environmental origin (20).

The Chernobyl (pronounced 'Chornobyl' in Ukrainian) nuclear plant catastrophe in 1986 changed the ecological situation in Ukraine dramatically. It caused radioactive nuclide pollution of over 35 million hectares. The human population in this area exceeded 5 million. In the 30-km zone affected by the disaster, as well as in the adjacent territories, there was a broad spectrum of radionuclides of different forms, half-life periods and energies of radiation. In the polluted territory, small doses of long-term radiation by isotopes ^{134}Cs , ^{137}Cs and ^{90}Sr were observed (29).

After the Chernobyl disaster, TB-infected cattle were held for extended periods because the pollution of their meat by radioactive substances had resulted in them exceeding maximum permissible indices for slaughter. As a result, in the Narovlyansk district of the Gomel region, tuberculosis lesions were detected in 516 carcasses for four months in 1987 and in 570 carcasses in 1988 (14, 25).

The lack of veterinary specialists and technical workers on the TB-affected farms caused the delayed and poor-quality implementation of organisational, economic, veterinary and hygiene measures. The high radioactive pollution resulted in the spread of the TB infectious agent and increased the number of animals that reacted to the tuberculin skin test (13).

At present, a large cattle population is reared on a territory that was polluted by a low-dose of radiation, i.e. the Kyiv, Cherkasy, Zhytomyr, Rivne and Chernigiv regions. Previously, we reported the results of a study on the influence of the Chernobyl catastrophe on the intensity of the bovine TB epizootic in radioactively polluted and 'clean' territories of Ukraine. More intensive epizootics of bovine TB were observed in areas polluted by radioactivity. However, it is remarkable that

during the past 24 years, in both 'clean' and polluted areas, there are regions (Crimea, Rivne) where no outbreaks of TB occurred (31). Ionising radiation can influence both the infectious disease agent and the host. The level of such influence depends on the characteristics of radiation, the dose, dose rate and many other factors (29).

The state of health of the host plays an important role in susceptibility to TB. In humans who lived in areas polluted by radioactivity; the form of TB observed was usually acute and it developed in a short period, with massive bacterial excretion of the fibro-cavernous forms of the agent. Furthermore, there was a large increase in the primary tuberculosis reactivation frequency (1, 33). This was conditioned by over-exertion of compensatory-adaptive mechanisms in the immune system which led to atrophy of the thymus and immune deficiency (33).

It was reported, that radiation, above the background level, leads to a decrease of the pool of circulating lymphocytes in the blood. This results in the development of a secondary immunodeficiency and the initiation of autoimmune processes in the animal (3).

On the other hand, mycobacteria isolated from humans exposed to a low radiation dose (0.03-0.09 Sv) were more virulent in the mice model and the agent had a virulence index of 1.01 ± 0.034 while the control group (mycobacteria isolated from humans who were not exposed to the radiation) had a virulence index of 0.85 ± 0.041 . At the same time, other authors noted that the ability of 'radioactive' mycobacteria to form the cell-wall deficient variants had increased (12).

Low doses of gamma radiation (0.00645-0.0654 C/kg) contribute to the acceleration of growth on nutrient media of both reference and field strains of the tuberculosis agents, as well as atypical mycobacteria. The mechanism of the small dose gamma radiation stimulating effect is due to the more rapid lipid metabolism in the chain of lipid peroxidation in the membranes of mycobacteria. However, under natural conditions in a polluted environment, the radiation doses do not reach such a 'stimulating' level (15).

In the radioactive polluted zones of the Semipalatinsk region, extensive changes in mycobacterial biological properties (pathogenic, atypical, L-forms) were detected (6, 7, 28).

It was observed that 76.5% of coccoid cultures had high virulence which the authors believe is evidence of an increase in 'virulent characteristics' of the *M. tuberculosis* isolated in the area polluted by radiation (9). Furthermore, the authors concur with the findings of Golyshevskaya *et al.* who believe that the affect of ionising radiation on micro- and macro-organisms is insufficiently known and requires further study.

The aim of our study was to analyse the impact of bovine tuberculosis on farms located in the territories where low dose radiation pollution was recorded, as a consequence of the Chernobyl catastrophe.

Materials and methods

Skin tests were performed using the purified protein derivative (PPD) tuberculin prepared from *M. bovis* in the Sumy Biological Factory. A 0.1 ml dose of the PPD was injected intradermally in the middle of the left neck with a needleless injection device; the hair on the site had been clipped and the site was treated with 70% alcohol. The reaction to the tuberculin was observed and recorded 72 h after the injection.

A total of 81 pathological samples and 28 blood samples were taken from cattle at slaughter that had revealed positive skin tests. Samples were collected from animals in the Kyiv, Cherkasy and Chernigiv regions.

The following tissues, from slaughter cattle, were collected for pathological examination: submaxillary, retropharyngeal, prescapular, upper udder and mesenteric lymph nodes as well as lung, liver and spleen. Before inoculation for bacterial isolation the material was treated with 3-5% H₂SO₄.

All samples were inoculated into beef-extract broth and onto slants with 'medium for cultivation of mycobacteria' (NSC 'IECVM', Kharkiv) with and without 5% NaCl. Three tubes of each medium were inoculated and cultures were incubated at 25°C, 37°C and

45°C to obtain enough bacterial mass for the biochemical tests. The following physiological characteristics of mycobacteria were investigated (5, 19):

- rate and characteristics of culture growth
- morphology and pigmentation of colonies
- catalase, nicotinamidase and pyrazinamidase activities
- ability to hydrolyse Tween-80 and urea
- tolerance to sodium chloride
- reduction of tellurite and the ability to take up iron.

The sensitisation and pathogenic characteristics of *Mycobacterium* cultures isolated were studied by experimental inoculation of guinea-pigs, rabbits and chickens.

Mycobacteria were differentiated to the species level according to the results of biochemical tests, experimental infection in laboratory animals and polymerase chain reaction (PCR). The latter was conducted in the following way: 1-5 µl of isolated DNA was added to a master

mix which contained 5 µl PCR-buffer (10×) with MgCl₂ (1.5 mM), 2 µl dNTP (2 mM), 1 µl forward and 1 µl reverse primers (20 pM), 0.2 µl *Taq*-polymerase (5 U/µl), PCR-grade water and 2 µl betaine (5 M) for reaction optimisation. Amplified loci, annealing temperature, number of cycles and references are presented in Table I.

Results

Several farms were selected for skin test investigation based on their TB history. TB screening was performed on two farms located in the Kyiv region, three in the Cherkasy region and two in the Chernigiv region. In total, 7 134 head of cattle were skin tested and 359 (5.03%) animals gave positive results (Fig. 1).

Gross pathological examinations at slaughter of the animals that had reacted to the skin test revealed 72 animals with TB lesions (20.05% of

Table I
Differentiation of *Mycobacterium* species and species complexes using the polymerase chain reaction

<i>Mycobacterium</i> species	Amplified locus	Primers	Annealing (°C)	No. of cycles	Reference
Genus <i>Mycobacterium</i>	16S rRNA	Mycgen F Mycgen R	60	35	34
<i>M. tuberculosis</i> complex	IS 6110	INS-1 INS-2	65	25	23
<i>M. bovis</i>	RvD1-Rv2031c	JB 21 JB 22	65	25	26
<i>M. tuberculosis</i>	<i>mtp40</i>	PT 1 PT 2	65	25	23
<i>M. avium</i> complex <i>M. kansasii</i>	16S rDNA	Mycgen F MycAv R	65	25	34
<i>M. intracellulare</i>	16S rDNA	Mycint F Mycgen R	60	40	34
<i>M. a. hominissuis</i> <i>M. a. paratuberculosis</i>	FR 300	FR 300 P1 FR 300 P2	65	25	22
<i>M. a. avium</i> <i>M. a. hominissuis</i> <i>M. a. silvaticum</i>	IS 1245	A 1245 B 1245	65	25	11
<i>M. a. avium</i> <i>M. a. silvaticum</i>	IS 901	IS 901 P1 IS 901 P2	65	25	22
<i>M. a. avium</i> <i>M. a. silvaticum</i>	IS 902	P102fµ P103r	65	30	24
<i>M. a. paratuberculosis</i>	IS 900	IS900/P3N IS900/P4N	67	35	2

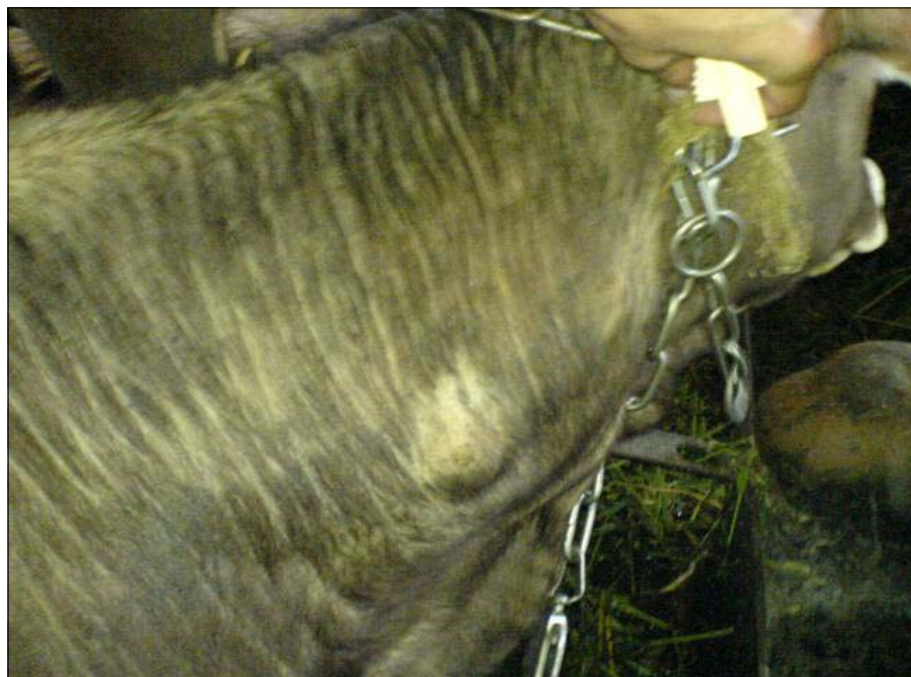


Figure 1
Response to intradermal administration of purified protein derivative-tuberculin
for mammals in cattle infected with *Mycobacterium bovis*

the total number of reactors). Severe lesions were found in submaxillary, retropharyngeal, prescapular and mesenteric lymph nodes and also in the lungs, liver and spleen (Fig. 2).

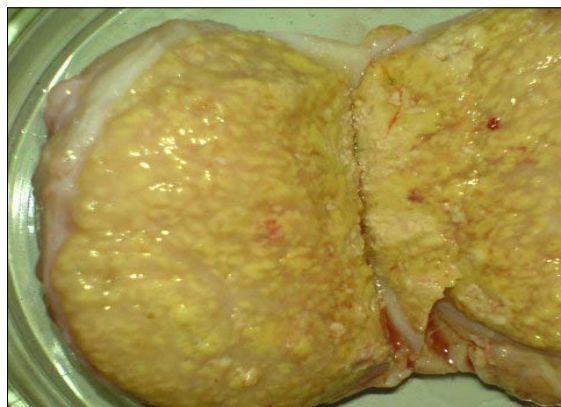


Figure 2
Tuberculosis lesions in lymph node of a cow
infected with *Mycobacterium bovis*

A total of 82 *Mycobacterium* cultures were isolated from 109 tissue and blood samples taken from 72 reactor cattle. The cultures exhibited different morphology, growth rate and pigmentation. Forty-eight cultures were referred to as pathogenic and 34 as atypical mycobacteria. The atypical mycobacteria were

classified as follows using the Runyon classification:

- two belonged to group II
- eight to group III
- twenty-four to group IV.

Physiological features of isolated mycobacteria and species differentiation based on biochemical test results are presented in Table II.

All samples were positive in PCR when tested with the *Mycobacterium* genus-specific primers. When the *Mycobacterium*-positive samples were tested with IS 6110-specific PCR primers, 48 samples (58.54%) were positive and thus were identified as *M. tuberculosis* complex. All IS 6110-positive samples were also positive in PCR with *M. bovis*-specific primers to the gene *RvD1-Rv2031c*.

To determine if the PCR-positive samples were also mixed with *M. tuberculosis*, all of those that reacted to the IS 6110 and to *RvD1-Rv2031c* primers were also tested using specific PCR primers for *M. tuberculosis*. All samples gave negative results.

The IS6110-negative samples were examined using a PCR with primers complementary to the region of 16S rDNA specific to the

Table II
Species identification of mycobacteria isolated from cattle positive in skin tests
Results are based on the biochemical tests shown

Mycobacterium species	No. of cultures	Growth rate (days)	Growth at 25°C	Growth at 45°C	Pigment	Catalase activity	Nicotin-amidase	Pyrazin- amidase	Tellurite reduction	Tween 80 hydrolysis	Urea hydrolysis	Iron uptake	Growth in 5% NaCl	Results of experimental infection
<i>M. bovis</i>	48	28-45	-	-	-	-	-	-	-	-	+	-	-	Generalised TB 30-60-90 days post infection in guinea-pigs and 30-65 days in rabbits
<i>M. avium</i>	8	12-16	-	-	-	-	+	+	+	-	-	-	-	Sensitising guinea-pigs; septic form of TB in rabbits 16-30 days after infection; TB lesions in chickens
<i>M. gordonae</i>	1	6-8	+	-	+	+	-	-	-	+	+	-	-	Sensitising guinea-pigs*
<i>M. scrofulaceum</i>	1	14	+	-	+	+	+	+	+	+	+	-	-	Sensitising guinea-pigs*
<i>M. fortuitum</i>	7	2-5	+	-	-	+	+	+	+	v	+	+	+	Sensitising guinea-pigs*
<i>M. smegmatis</i>	6	3-4	+	+	-	+	+	+	+	+	+	+	+	Sensitising guinea-pigs*
<i>M. phlei</i>	2	3-6	+	+	+	+	+	+	+	+	+	+	+	Sensitising guinea-pigs*
<i>M. vaccae</i>	2	4-5	+	-	+	+	+	+	+	+	+	+	+	Sensitising guinea-pigs*
<i>M. flavescens</i>	3	3-5	+	-	+	+	+	+	-	+	+	-	+	Sensitising guinea-pigs 1
<i>M. diernhoferi</i>	4	4-5	+	-	+	-	+	+	+	-	+	+	-	Sensitising guinea-pigs*

TB tuberculosis
- negative reaction
+ positive reaction
v variable reaction
* sensitising guinea-pigs to tuberculin for mammals and to allergen from atypical mycobacteria

M. avium complex. Eight samples (9.75%) of the 34 remaining atypical mycobacteria were PCR-positive using these primers. *M. avium* subspecies identification was performed using PCRs with primers specific to insertion elements IS1245, IS901, IS902, genes 16S rDNA and FR300. The results were as follows:

- DNA of *M. avium* subsp. *avium* was identified in three (3.66%) samples
- DNA of *M. avium* subsp. *hominissuis* in 5 (6.1%) samples
- DNA of *M. intracellulare* or *M. avium* subsp. *paratuberculosis* was not found in any sample.

Taking into account growth rates and different colony morphology, three *M. bovis* cultures were suspected of being 'mixed'. These cultures were examined using PCR primers specific to the *M. avium* complex and gave positive results. Further PCR typing revealed that these three cultures were mixed with *M. avium* subsp. *hominissuis*.

Discussion

Data concerning species differentiation of mycobacteria cultures isolated from cattle that had reacted positively to the skin test are summarised in Figure 3. Species differentiation was based on the results of culture, morphological and biochemical methods and the results of the PCR tests.

The high percentage (41.5%) of atypical mycobacteria isolated from the 72 cattle that had reacted to the skin test is significant. The percentage of *M. avium* complex was 9.8%; *M. fortuitum* was 8.5% and *M. smegmatis* was 7.3%. The isolation of other atypical Mycobacterium species from cattle is relatively rare (Fig. 3).

Such a significant percentage of atypical mycobacteria, isolated from cattle, may be the result of a high level of sensitisation to atypical

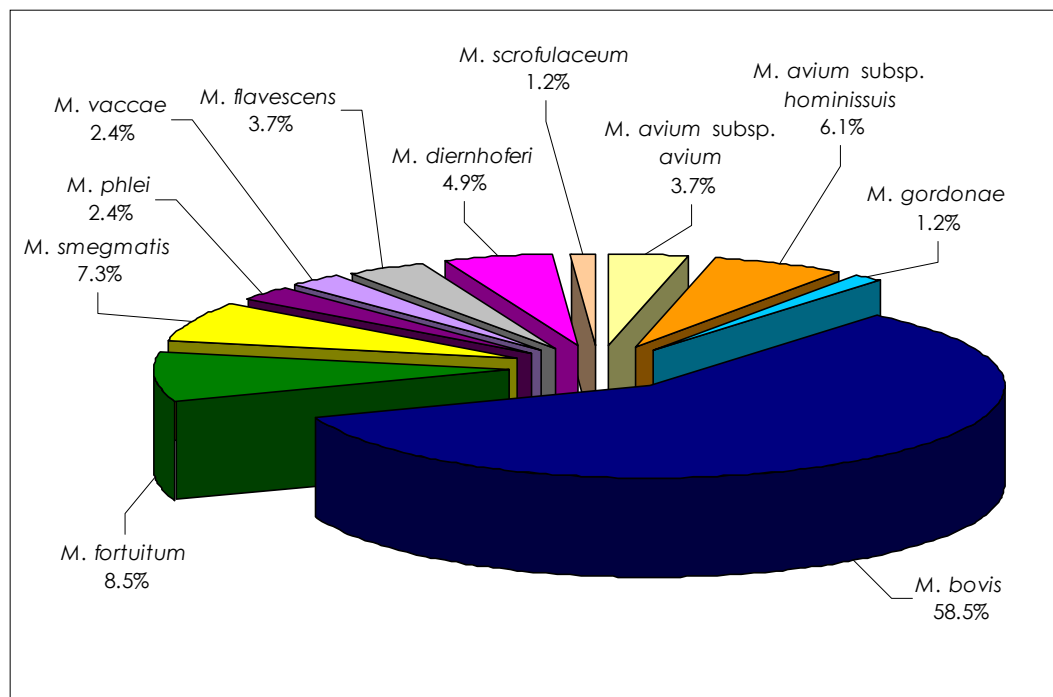


Figure 3
Results of species differentiation of *Mycobacterium* cultures isolated from cattle that gave positive results to the skin test ($n = 72$)

mycobacteria which may have stimulated the skin reactions to tuberculin. These results confirm conclusions of our previous work (10, 30). Inappropriate poor sanitary conditions of animal keeping (10, 21, 36) may have provided the atypical mycobacteria the opportunity to invade and multiply in the extremely weakened animals.

The TB situation in the territories polluted with low doses of radiation remains difficult and the burden of cattle tuberculosis is still heavy. As reported previously, the dynamics of improvement of the TB status of cattle herds in these territories is remarkably lower than in other regions of the country and the impact of TB is appreciably higher (31). This analysis has been confirmed by the isolation of *M. bovis* strains with high virulence from this region;

these strains caused generalised disease in guinea-pigs 38-64 days after experimental injection.

Ante-mortem diagnosis of TB becomes more complicated as a large percentage (41.5%) of tuberculin reactions are caused by atypical mycobacteria.

Results of this study showed the negative influence of low doses of radiation on the TB status of cattle herds. This has increased the impact of cattle TB in regions affected by the Chernobyl catastrophe.

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