Part 2. Biosafety

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MICROBIOLOGICAL STUDY OF FIELD CULTURES OF *MYCOBACTERIUM* SPP. AS CONTAMINANTS OF MILK AND THE ENVIRONMENT

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Summary. Atypical nonpathogenic nontuberculous mycobacteria are common in the environment and can contaminate livestock facilities, feed, animals, animal products, and manure. These prokaryotes are saprophytes, but they share antigenic similarities with emerging mycobacterial pathogens. Upon contact with animals, they trigger an immune response through complexes of mycobacterial antigens in the host. This can cause errors in immunodiagnostic tests and microbiological assessments of outbreak situations. Microbiological monitoring of livestock products and milk has shown widespread environmental contamination with nonpathogenic, saprophytic, atypical mycobacteria of various species, mainly group IV by Runyon. These are fast-growing, hardy prokaryotes tolerant to a wide range of cultivation conditions and temperatures, with broad adaptability for extracellular enzymatic breakdown of organic macromolecules. The epidemiological concern is that atypical mycobacteria belong to the genus *Mycobacterium* and share antigenic complexes with pathogenic mycobacteria, leading to false-positive allergic reactions and diagnostic confusion in microbiological tests. Biological testing on guinea pigs demonstrated that ubiquitous atypical mycobacteria, common contaminants of milk and manure, do not cause pathological changes in internal organs during necropsy. However, during simultaneous allergy testing, they had significantly stronger reactions to sensitin from atypical mycobacteria. In contrast, responses to tuberculin from pathogenic mycobacteria were weak or absent. Saprophytic mycobacteria are part of the normal, transient microbiota of animals and serve as a barrier in integumentary tissues

Keywords: atypical mycobacteria, ubiquity, nonpathogenicity, manure and milk contamination, false-positive allergic reactions, bioassay, guinea pigs

Introduction. The microbial world of the environment is characterized by endless biodiversity of representatives from all four domains of life, based on the dominance of carbon in metabolic processes that support life and the functioning of genetic information. The most widespread organisms in natural environments, in terms of species diversity and total biomass, are prokaryotic cellular organisms from the domains Bacteria and Archaea. They are phylogenetically the oldest forms of cellular life, tracing back to LUCA (Hotsulia et al., 2021; Kassich et al., 2019; Zazharskyi et al., 2023).

For about four billion years, microbial forms of organic life have carried out the functions of global biogeochemical cycling through enzymatic extracellular dissimilation of complex organic biomolecules. Nonpathogenic mycobacteria are among the natural members of microbial communities that break down organic matter. They actively participate in various biodegradation processes and play an important role in soil formation, working closely with actinomycetes and other organisms of different taxonomic groups. Nonpathogenic mycobacteria make up a large group of prokaryotic species that have adapted to the metabolic conditions of the inanimate environment. During the first two billion years of Earth's formation, they existed solely as saprophytes (Runyon, 1965; Tkachenko et al., 2010; Zazharskyi et al., 2023).

However, as multicellular life forms evolved, interspecies relationships with parasitic potential emerged. This resulted in the development of infectopathology, which is the macroorganism's protective and adaptive response to the invasion of genetically foreign substances into its internal environment. Of the many mycobacterial prokaryotes, only a few species have become pathogenic toward poikilothermic and homeothermic multicellular animals. In the last seven million years, they have also become pathogenic toward the genus *Homo* and, more recently, *Homo sapiens sapiens* (O'Brien et al., 2004; Tkachenko et al., 2016).

These pathogenic mycobacteria induce tuberculosis and leprosy, and they are classified as emergent prokaryotic agents of severe infectopathologies. They can maintain an active epidemic process in susceptible macroorganisms. In addition to epidemic variants of pathogenic mycobacteria, numerous nontuberculous mycobacteria with potentially pathogenic capabilities circulate among humans and animals. These bacteria cause sporadic cases of nontuberculous infections with low epidemic potential, also known as 'diseases of the future'. These infectopathologies are called mycobacterioses, and their agents belong to the category of potentially or conditionally pathogenic mycobacteria. These mycobacteria are also referred to as atypical, unclassified, or anonymous. The term 'atypical mycobacteria' is

the most widely used (Kassich et al., 2019; O'Brien et al., 2004).

Atypical mycobacteria belong to the phylum Actinobacteria, class Actinobacteria, order Corynebacteriales, family Mycobacteriaceae, genus *Mycobacterium*, and are characterized by significant species biodiversity.

According to Runyon's classification, atypical mycobacteria are divided into four groups. The most widespread in the environment of farm animals — and therefore the most frequent contaminants of livestock products — are group IV rapidly growing saprophytes, including *M. phlei*, *M. smegmatis*, *M. fortuitum*, *M. flavescens*, *M. vaccae*, *M. chelonae*, *M. peregrinum*, *M. diernhoferi*, and others (Hotsulia et al., 2021; Bihdan et al., 2018; Gomez-Buendia et al., 2024; Magee and Ward, 2015).

On artificial nutrient media, including MPA and MPB, atypical mycobacteria form macroscopically visible colonies within 7–10 days after inoculation from biomaterial, and in subcultures within 3–5 days at a temperature of 37–38°C in stationary aerobic conditions. Their colony biomass is abundant and forms in S- or R-types of colonial growth. Both pigmented and nonpigmented variants exist. Atypical mycobacteria are not demanding in cultivation conditions; for example, *M. phlei* can grow even at a temperature of 42°C. Colonies of some atypical species continue to grow at room temperature (20–22°C), though more slowly (Reber et al., 2016; Runyon, 1965; Tkachenko et al., 2010).

When animals are exposed to water or feed contaminated with atypical mycobacteria, they can develop short-term sensitization in the form of delayed-type hypersensitivity to tuberculin protein antigens. Bioassays on guinea pigs and farm animals repeatedly confirmed that oral or parenteral infection with atypical mycobacteria cultures results in typical sensitization to tuberculin sensitins. This is due to the antigenic similarity between tuberculous and nontuberculous atypical mycobacteria within the genus *Mycobacterium* (Abdulla et al., 2024; Atlas, 2010; Chen et al., 2025; Van Ingen et al., 2013; Solaghani et al., 2023).

Aim of the work was bacteriological study of milk and manure from clinically healthy dairy cows to detect genus *Mycobacterium* spp. and to characterize the basic properties of atypical mycobacteria circulating in the cows' environment, milk, and manure.

Materials and methods. Microbiological studies were conducted at the educational and research laboratory and vivarium of the Department of Infectious Diseases of the Faculty of Veterinary Medicine in Dnipro State Agrarian and Economic University.

Pre-seeding processing of cow feces was carried out according to the method of Alikaeva, using a 20% sulfuric acid solution with an exposure of at least 20 min, followed by thorough washing of the biomaterial with sterile saline. Milk samples were prepared following the official instructions for laboratory diagnosis of tuberculosis.

For the isolated mycobacterial cultures, species identification recorded the timing of initial growth, features of colony development, and morphology, pigmentation (or lack thereof). As differential diagnostic criteria, growth potential and accumulation of bacterial mass at a temperature of 25°C, 37°C, and 45°C were assessed, as well as haloresistance to 5% sodium chloride, growth in the presence of sodium salicylate, ability to reduce potassium tellurite, hydrolysis of Tween-80, catalase activity, and amidase activity.

Smear preparations from isolated mycobacterial cultures were stained by Ziehl–Neelsen and examined under immersion in a light microscope.

Simultaneous allergic testing of laboratory animals and dairy cows with PPD-tuberculin and atypical mycobacterial allergen (AAM) was carried out to determine the possibility of infection with pathogenic and nonpathogenic mycobacteria — that is, latent mixed mycobacteriosis — as well as the sensitizing potentials of field mycobacterial variants. Preparations were injected intradermally at a dose of 0.1 cm^3, with the injection site clipped and disinfected with 70% ethanol beforehand. Reactions were assessed after 24 h in guinea pigs, and after 48 h and 72 h in cows, by palpation and measuring skinfold thickness. In cows, an increase of ≥ 3 mm was considered positive; in guinea pigs, local signs of inflammation, edema, and necrosis were recorded.

Biological experiments were conducted on randomized short-haired guinea pigs weighing 300–400 g. They were infected with isolated field cultures of atypical mycobacteria to determine pathogenic potential and sensitizing properties. Two-week-old cultures suspended in saline were injected intradermally (inguinal region) at a dose of 1.0 mg. Clinical observation lasted for about two months, with simultaneous allergic tests using PPD-tuberculin and AAM performed 30 days and 15 days according to the instructions.

Results and discussion. At the beginning of the epizootic survey of farm animal health, simultaneous intradermal allergic testing of dairy cows was conducted using PPD-tuberculin for mammals and AAM produced by the Sumy Biofactory. After 72 h, examination of the reactions showed that all cows responded to both PPD-tuberculin and AAM. However, the intensity of skin reactions was significantly different: reactions to AAM were reliably stronger than those to PPD-tuberculin. The mean skinfold thickness after PPD-tuberculin injection was 6.3 ± 0.9 mm, while after AAM injection it was 22.6 ± 1.6 mm ($p \leq 0.05$).

During microbiological studies of general manure and milk samples from dairy cows, mixed cultures of atypical (nontuberculous) mycobacteria were isolated, including ten monocultures of a single species. The atypical mycobacteria were Gram-positive, acid-fast, polymorphic rods with pronounced saprophytic activity. They were not classified as pathogenic for humans or animals.

Following bacteriological identification, three cultures from the isolated pool of atypical mycobacteria were classified as *M. phlei*. When inoculated on Lowenstein–Jensen medium, primary growth of *M. phlei* was observed

as early as 3–5 days of incubation at a temperature of 37°C. *M. phlei* subcultures also produced colonial growth at a temperature of 25°C and 45°C, which is an important species marker. The colonies were dry, rough, wrinkled, and ranged in color from yellowish to orange or cream. Moderate, diffuse growth was observed on liquid media. Ziehl–Neelsen staining preserved the acid-fast properties, resulting in the characteristic ruby-red color of mycobacteria, along with a non-granular cytoplasm. Gram staining revealed large, weakly stained, light-violet, polymorphic rods with rounded edges, sometimes slightly curved, and non-granular cytoplasm (Fig. 1).

Biochemical analysis revealed catalase activity and the ability to reduce nitrates to nitrites, with the formation of niacin and nitrate reductase. Culture growth was observed on glycerol agar and simple nutrient media without the addition of inhibitors. The cultures demonstrated resistance to the main anti-tuberculosis drugs (isoniazid, ethambutol, rifampin), which is typical of non-pathogenic and rapidly growing atypical mycobacteria of Runyon group IV, and particularly *M. phlei.*

To assess pathogenic potency, a bioassay was conducted on guinea pigs. The animals were infected intradermally with a suspension of mixed *M. phlei* cultures and observed for 45 days. During this period, no clinical signs of the disease, such as weight loss, fever, or behavioral changes, were observed. After one month, some of the animals were sacrificed, and postmortem examination was performed. Autopsy revealed no pathological changes in the internal organs characteristic of tuberculosis infection.

An intradermal allergic test with PPD-tuberculin for mammals on 30th day after infection showed no reaction of the macroorganism to the tuberculous sensitin, which confirms the absence of sensitization of guinea pigs to the antigens of *M. phlei* and their inability to induce an immune response typical of tuberculous mycobacteria.

To assess the immunobiological properties of *M. phlei* in guinea pigs, 45 days after intradermal infection with the culture, simultaneous allergic diagnostics were performed using standard PPD-tuberculin for mammals and allergens from atypical mycobacteria. The following indicators were recorded: upon administration of PPD-tuberculin to mammals, there was no reaction; the papule diameter was between 0.1 mm and 0.9 mm; and there was no hyperemia or necrosis. This can be interpreted as an absence of sensitization to the antigenic complexes of pathogenic mycobacteria. Upon administration of AAM, some animals exhibited a mild local reaction with papules measuring up to 3 mm in diameter. There were no signs of systemic inflammation or necrosis.

This indicates partial immunological rearrangement induced by *M. phlei*, but its antigenic structure differs significantly from that of pathogenic mycobacteria. The simultaneous allergy test showed that infection with *M. phlei* does not lead to cross-sensitization to tuberculous sensitin and does not produce false-positive results in allergic intradermal diagnostics of tuberculosis, which is of great diagnostic importance for both veterinary and human medicine when interpreting allergy test results for tuberculosis and mycobacterioses.

Thus, the biological properties of isolated *M. phlei* cultures confirm their saprophytic nature. Field variants of *M. phlei* pose no epidemiological danger but must be differentiated in laboratory diagnostics of tuberculosis and mycobacterioses.

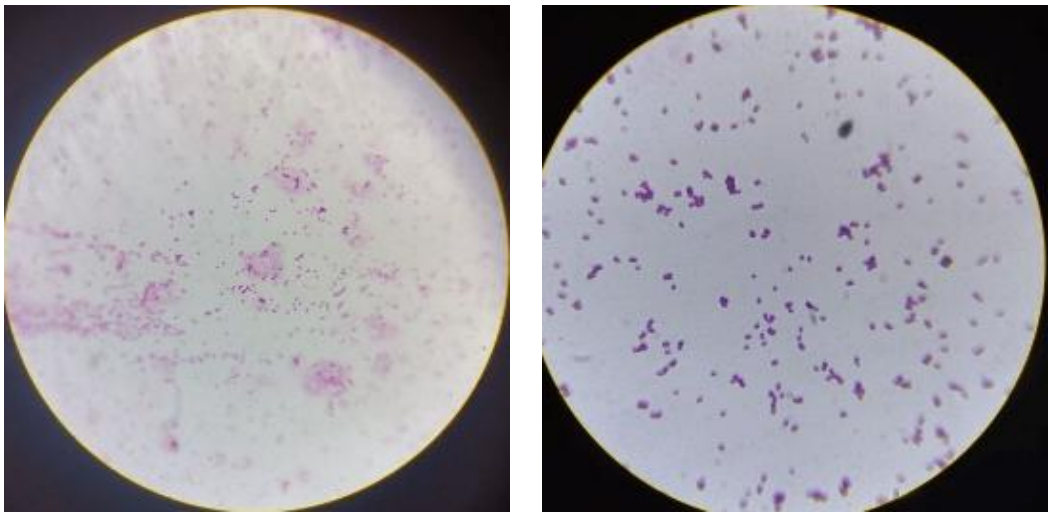
From manure, three pure cultures of *M. vaccae* and four cultures of *M. terrae* were isolated. They had very similar morphotinctorial properties and were obtained from the same manure samples. *M. vaccae* and *M. terrae* are known to be ubiquitous environmental saprophytes with pronounced immunomodulatory potential regarding the nonspecific resistance of macroorganisms. These bacteria are part of the dynamic pool of aerated soil microbiomes and are almost always found on the bodies of clinically healthy cows and in their manure. Thus, they serve as bioindicators of environmental biosafety.

Cultures of *M. vaccae* grew on Löwenstein–Jensen medium at a temperature of 25°C and 37 °C, but not at a temperature of 45°C or 4–6°C. Primary growth appeared on days 8–11 as dry, rough, wrinkled colonies with a yellow or orange tint. In liquid media, moderate diffuse turbidity was noted. With Ziehl–Neelsen staining, acid-fastness was confirmed: ruby-red coloration typical of mycobacteria, but with non-granular cytoplasm. With gram staining, *M. vaccae* appeared as weakly stained light-violet polymorphic large rods with rounded edges, sometimes slightly curved, with non-granular cytoplasm.

Biochemical tests revealed catalase activity, the capacity to reduce nitrates to nitrites, and the synthesis of niacin and nitrate reductase. *M. vaccae* grew well on glycerol agar and simple nutrient media at a temperature of 37°C. It tolerated 5% NaCl and grew in media with sodium salicylate. *M. vaccae* also hydrolyzed Tween-80.

Cultures of *M. terrae* exhibited morphotinctorial, cultural, and biochemical properties similar to those of *M. vaccae*. However, their colonies on Löwenstein–Jensen medium were nonpigmented and gray-white (Fig. 2). They were not resistant to 5% NaCl and did not exhibit amidase activity.

The biological properties of *M. vaccae* and *M. terrae* were studied in a traditional bioassay on guinea pigs. After infection with a standard infective dose, the animals were observed for 45 days. No pathological phenomena were recorded; the guinea pigs actively ate, were mobile, and well-nourished. After a month, simultaneous allergic testing was performed with standard PPD-tuberculin for mammals and AAM. The results were as follows: upon PPD-tuberculin administration, the reaction was practically absent. In some animals, slight edema at the injection site and small papules of 0.1–0.6 mm without hyperemia or necrosis were noted. This can be interpreted as an absence of sensitization to the antigenic complexes of pathogenic mycobacteria. With AAM administration, some animals showed a weak local reaction with papules up to 3 mm without systemic inflammation or necrosis.



a b

Figure 1. Microscopy of *M. phlei* culture, Ziehl–Neelsen (a) and Gram (b) staining.

Figure 2. Culture of *M. terrae* on selective nutrient medium.

As with *M. phlei*, the results suggest partial immunological rearrangement caused by antigenic commitment induced by atypical mycobacteria. However, the antigenic structures of *M. vaccae* and *M. terrae* differ significantly from those of pathogenic mycobacteria, preventing cross-sensitization with antigens of both pathogenic and nonpathogenic atypical mycobacteria.

A microbiological examination of milk and cow manure, using standard methods to isolate and identify mycobacterial prokaryotes, revealed their ubiquity, nonpathogenicity, and relative immunological activity when contaminating the internal environment of a macroorganism. It should be noted, however, that their presence in animals is temporary. Saprophytes cannot survive long in the internal environment and are transient components of the normal microbiota of the animal body.

Conclusions. 1. Milk from clinically healthy, tuberculin-negative cows contained no pathogenic mycobacteria, but was contaminated with various nonpathogenic atypical species.

2. Manure from healthy cows was contaminated with saprophytic atypical mycobacteria; no pathogenic strains were isolated.

3. Ubiquitous atypical nonpathogenic mycobacteria are natural co-inhabitants of livestock environments and products. They do not cause disease but can induce transient false allergic reactions to mycobacterial sensitins.

4. These saprophytes are nonpathogenic for mammals, but through transient contact with the immune system, they may cause false-positive skin reactions without pathological changes.

Prospects for further research. Atypical mycobacteria are ubiquitous environmental and animal-associated microbes that are widespread contaminants of livestock and their products. Their phylogenetic similarity to pathogenic species presents challenges in diagnosis. Studying the biological properties of these environmental mycobacteria is essential to improving the laboratory detection and identification of mycobacteria.

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