

SENSITINS FOR DIFFERENTIATING NONSPECIFIC REACTIONS TO PPD TUBERCULIN MAMMALIAN IN CATTLE

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ABSTRACT

The paper presents the study of the habitat of mycobacteria in various natural and climatic zones of the Karaganda region, which showed that out of all the isolated cultures, 307 (94.5%) were atypical mycobacteria (*M. scrofulaceum*, *M. avium*). At that, 64.5% of atypical mycobacteria cultures (*M. scrofulaceum*, *M. avium*) were isolated from the diagnostic material and objects of the external environment of the steppe zone. The sensitins from epizootic strains of mycobacteria were tested in terms of such criteria as sterility, safety, reactogenicity, lack of sensitizing properties, activity, and specificity. The sensitins were administered to guinea pigs and cattle using an intradermal needleless syringe. Preliminary experiments on guinea pigs found that sensitins had biological and specific activity, identical to avian purified protein derivative tuberculin. The tests of developed sensitins (*M. scrofulaceum* and *M. avium*) on 59 cattle showed that the sensitins were highly effective in differentiating nonspecific reactions and demonstrated the expedience of their application. Using sensitins for lifetime differentiation of nonspecific reactions to tuberculin mammalian in healthy animals and cattle recovering from tuberculosis prevents the premature slaughtering of commercially valuable animals of a reproductive age.

Keywords: atypical mycobacteria, cattle, mycobacteriosis, sensitins, tuberculosis.

INTRODUCTION

Recent years witnessed an increase in cases when it was impossible to confirm the tuberculosis (TB) diagnosis by anatomopathological and bacteriological methods, hence these reactions were considered nonspecific. Numerous studies show that the main reason for nonspecific reactions to tuberculin in cattle at satisfactory farmsteads are atypical mycobacteria (Shtukis and Kublitskas, 1968; Katale *et al.*, 2013; Biet and Boschiroli, 2014).

Researchers studied various diagnostic tests to differentiate nonspecific reactions. However, such tests failed to become widely used for different reasons (García-García *et al.*, 2006; Nelson, 2007). The members of the *Mycobacterium* genus are controversial and mostly based on host preference; some permanent characteristics mostly seem to reflect a potential source of the infection (Rodríguez-Campos *et al.*, 2014). *M. bovis* is more viable in nature, living in and transmitting among cattle, goats, East-Asian water buffaloes, deer, bison, and brush-tailed possums (Behr and Gordon, 2015). *M. bovis*-specific antigens that showed good T cell stimulation, such as CFP-10, ESAT-6, Rv3615c, etc., have been used in skin

tests, but there have been no large-scale clinical studies on these antigens (Xin *et al.*, 2013). The main goal of recent studies is to develop and implement new methods and processes that have the potential to provide more cost effective, efficient and measurable results (Rhodes, 2015).

In the Karaganda region of the Republic of Kazakhstan, nonspecific tuberculin reactions are commonly registered in farm cattle, despite the low incidence of TB among cattle (2 to 3 unsatisfactory cases were registered on average during the last 10 years). With two examinations per annum, 0.2 to 0.8% of animals reacting to tuberculin are discovered, i.e. about 0.3 thousand animal units. However, postmortem anatomopathological examination not always confirms the presence of TB. Atypical mycobacteria (AM) are often isolated during laboratory studies of the diagnostic material (Sadikova, 2007; Basybekov, 2011).

Instructions for diagnosing TB in animals (1999) suggest using a simultaneous test with PPD tuberculin from AM for differential diagnosis of nonspecific reactions. However, researchers have different opinions regarding the information value of AM. They believe that using the AM allergen in the set of diagnostic studies when differentiating tuberculin reactions in animals at

arbitrarily satisfactory farmsteads allows understanding, to a certain extent, the epizootic situation in terms of TB among cattle (Michel, 2008).

However, there is no tuberculin with full specificity of reactions found only in animals infected with TB caused by bovine mycobacteria. Cattle, infected with avian mycobacteria, were found to react to PPD tuberculin mammalian the same way as cattle, infected bovine mycobacteria did (Amadori *et al.*, 2002; Donchenko *et al.*, 2004).

AM are a group of mycobacteria, whose morphological and biological properties differ from those of true pathogenic mycobacteria. AM may cause mycobacteriosis in human. Animal infection often manifests as temporary carriage of AM. Some types produce morphological alterations, typical of TB (Pavlik *et al.*, 2005).

AM are common in nature. They are found in feed, water, soil, and other environmental objects, from which they enter the organism and cause hypersensitivity to tuberculin. In favorable conditions, certain types of AM are capable of reproducing in the external environment (Waters *et al.*, 2006). Among 20 *M. bovis* immune dominant antigens for cattle, at least 16 to 17 are common with AM antigens (Lysenko, 1991; Wu *et al.*, 2008).

The main cause of nonspecific reactions in animals is the organism's sensitization to AM or avian mycobacteria (Shtu kis and Kublitskas, 1968; Brown *et al.*, 1981; Katala *et al.*, 2013).

The current simultaneous allergic diagnosis of TB is based on simultaneously administering different allergens to the examined animals and comparing results with regard to reactions.

The problem of timely discovering nonspecific reactions to tuberculin requires researchers to investigate the said factor for mammals, which is often caused by atypical (arbitrarily pathogenic) mycobacteria that include more than 300 species commonly found in the environment (Daugaliyeva and Piontkovsky, 2003).

The analysis of literary data shows that etiological factors were studied the most among issues related to various aspects of nonspecific sensitization of cattle to tuberculin. At the same time, the differentiation between nonspecific tuberculin reactions and specific ones is understudied.

Additional optimization approaches to improve test performance were examined and showed that the application of "a priori exclusions" of test results on the basis of reactivity to fortuitum PPD (sensitin produced from *Mycobacterium fortuitum*) and to a lesser degree, avian PPD, increased specificity without losing sensitivity (Michel *et al.*, 2011). Homologous sensitins caused significantly greater reactions than heterologous sensitins did (Michel, 2008).

Bacteriological studies with conventional methods that are suited to distinguish only the bacterial forms of the agent cannot serve as the basis for ruling out TB in animals. With the existing epizootic situation in terms of TB, it is necessary to conduct allergy tests for animals with sensitins produced from AM. This will allow understanding the epizootic situation in terms of cattle TB and developing measures for preventing premature slaughtering of animals at farmsteads recovering from TB.

Therefore, designing allergen sensitins capable of promptly differentiating such reactions in animals becomes especially relevant for veterinary science and practice and determines the novelty of this study.

The purpose of the study is to determine the area of mycobacteria spread in different natural and climatic zones of the Karaganda region and to develop sensitins from local epizootic strains of AM to differentiate nonspecific reactions to tuberculin in cattle, which can be later used in world veterinary science.

MATERIALS AND METHODS

The study was conducted to the World Medical Association Declaration of Helsinki (WMA, 2008), European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CE, 1998) and Declaration of Principles on Tolerance (Popovic, 1995). Ethics Commission of Kazakh Medical University of Continuing Education for 15.12.2015 decided to consider this study as non-controversial bioethical standards implemented in compliance with relevant international provisions on experimental work and clinical studies. It may be used in publishing materials.

The experiment that determined the specificity of the drug used 18 healthy guinea pigs with a live weight of 300 to 350 g, divided into six groups, three animals per group. At first, guinea pigs were tested for TB by a SST. Then, group 1 (No. 1, 2, 3) were administered *M. avium*; group 2 (No. 4, 5, 6) were administered *M. bovis*-8; group 3 (No. 7, 8, 9) were administered *M. kansasii*; group 4 (No. 10, 11, 12) were administered *M. TB*; group 5 (No. 13, 14, 15) were administered *M. scrofulaceum*; group 6 (No. 16, 17, 18) were administered *M. phlei*. Animals in six groups were administered respective cultures subcutaneously at a dose of one billion of mycobacterial culture per 1 cm³ of suspension, prepared in sterile physiological saline in accordance with the BCG-vaccine turbidity standard. The infection was done subcutaneously, into the inner hip area.

Mycobacteria strains were cultivated in the Löwenstein-Jensen medium, Pavlovsky's potato medium, Sauton's medium, meat infusion agar, meat infusion broth (State Standard 20730), and Sabouraud agar.

Sensitins were produced by the developed method (Innovation patent of the Republic of Kazakhstan 78813 dated 06.01.2012 “Means of obtaining an allergen from the . *scrofulaceum* typical bacteria” and No. 78819 dated 06.01.2012 “Means of obtaining an allergen from . *avium*”) with 12 to 15-day cultures of epizootic strains, cultivated in Sauton’s liquid medium.

Sensitins were tested in comparison with avian PPD tuberculin (manufactured by the Kursk biofactory). It was used following criteria: sterility, harmlessness, reactogenicity, absence of sensitizing properties, activity, and specificity in experiments on 38 guinea pigs, 15 white mice, and 175 cattle units (Assistance Standard 6537 “Tuberculin PPD mammalian and avian”, 1999; State Standard 23881 “Tuberculin PPD avian”, 1989).

Industrial testing of sensitins was conducted at the limited liability partnerships (LLP) of Karaganda region, Kazakhstan (“SHON” family-owned farm, LLP named after Chapayev, LLP named after Asylbekov, LLP “Shanyrak”).

Sensitin tests on cattle were based on instructions for administering tuberculin mammalian and a standard solution (Waters *et al.*, 2006).

The quantity in 1 mg of tested drugs was estimated by the following formula:

$$Q = \frac{B}{A} \times 2500, \quad (3.3)$$

where A is the sum of reaction intensiveness to avian PPD tuberculin in guinea pigs;

B is the sum of reaction intensiveness to tested sensitins in guinea pigs;

2500 is the concentration of IU in 1 cm³ of standard tuberculin lot.

The study used strains of mycobacteria, produced from biomaterial and environmental objects by the “Karaganda Scientific and Research Veterinary Station”. *M. avium* and *M. scrofulaceum* cultures were used to produce sensitins, autoclaved at 120°C for 60 minutes, after a 3-time adaptation in nutrient media (Löwenstein–Jensen medium, Pavlovsky’s potato-glycerol medium, Sauton’s medium). The culture fluid was concentrated with a rotary evaporator until its initial volume reduced by 3 to 5 times.

The bacterial mass was homogenized in sterile distilled water in a 1:3 proportion and re-autoclaved at 120°C for 30 minutes. Extracted active substances were deposited by centrifuge at 3000 r/min⁻¹ for 15 minutes; excess liquid was drained; the sediment was mixed with the concentrated culture liquid and stirred thoroughly. The mixture was mixed with ethanol in proportion from 1:2 to 1:4 and held for 18 hours at 4°C to deposit the protein at pH from four to five. The mixture was then centrifuged at 6000 r/min⁻¹ for 20 minutes; the sediment was washed several times and diluted with physiological

saline until the protein concentration of 1 mg per 1 cm³ was achieved.

Studying the obtained drugs for sterility, harmlessness, reactogenicity, and sensitizing properties showed that sensitins were sterile, harmless, and had no sensitizing properties. Prior to studying the activity, standard and experimental sensitins were diluted. The studied sensitins were administered subcutaneously into depilated parts of the skin at 0.0005 mg, 0.001 mg, and 0.002 mg in 0.1 cm³ of physiological saline to determine the tuberculin units of the sensitins. Avian tuberculin PPD in dilution of 25, 5, and one international units (IU) was injected at least 30 mm away from the spot where the tested sensitin was injected.

The bioactivity of drugs was studied by comparing experimental tuberculin lots with the standard avian PPD tuberculin (Kursk Biofactory, Russia). The sensitins were injected using a BI-7M needless injector (Labsnab, Russia).

In total, 20 albino guinea pigs with a weight of 300 to 350 g were selected to determine the bioactivity and specificity of produced sensitins. The animals were divided into four experimental groups, five animals per group.

Group 1 animals were sensitized by a subcutaneous administration of 3 to 4 mg *M. avium* epizootic strain culture in 0.5 cm³. Group 2 animals were sensitized in similar fashion by 3 to 4 mg *M. scrofulaceum* epizootic strain culture in 0.5 cm³. Guinea pigs in groups 3 and 4 were sensitized by *M. bovis*-8 and *M. phlei*, respectively.

30 days after the last injection of respective strains, subcutaneous allergic tests with studied sensitins were done on guinea pigs. At first, guinea pigs were tested for TB by a subcutaneous tuberculin test (STT). The bioactivity of drugs was determined by comparing them with the standard avian PPD tuberculin lot.

Standard and experimental sensitins were diluted before studying the activity of the drugs. Studied drugs were administered subcutaneously on depilated parts of the skin at a dose of 0.0005 mg, 0.001 mg, and 0.002 mg in 0.1 cm³ of physiological saline to determine the tuberculin units of sensitins. Avian PPD tuberculin in dilution 25, 5, and one international unit (IU) was administered not farther than 30 mm from the tested drug.

The mathematical processing of quantitative indexes (papule size) involved the calculation of the arithmetic mean (M) and statistical error (m) (Sadovsky, 1997). Statistical treatment was carried out in Stata14 (StataCorp LP, USA).

RESULTS

The reaction (size of hyperemia and skin swelling) was measured 24 and 48 hours after the drug was administered. The measurement results are presented

in Figure 1. Thus, experimental sensitins have high activity. They are comparable to the industrial avian PPD tuberculin ($\pm 20\%$).

Allergic tests were done on animals in all groups 30 days after the infection. Avian PPD tuberculin was administered on the right side; the tested allergen was administered on the left side at a dose of 125 TU in 0.1 cm³ of sterile physiological saline. Allergic reactions were measured in 24 and 48 hours. The results of the experiment are presented in Table 1. It shows that guinea pigs, infected with a homologous culture of mycobacteria had the most intensive allergic reaction to the administration of the tested tuberculin after 24 hours.

The results of these studies allow concluding that with subcutaneous administration, sensitins have biological and specific activity that is identical to that of avian PPD tuberculin. This allows manufacturing differential tuberculin tests for cattle from newly bred strains of AM in Kazakhstan.

Considering the previous laboratory studies, the diagnostic effectiveness of prepared sensitins was tested during the differentiation of nonspecific reactions to tuberculin in cattle at satisfactory and unsatisfactory farmsteads that faced difficulties in determining the actual epizootic situation in terms of TB.

At "Shanyrak" LLP (Bukhar-Zhyrau District), the sensitins' diagnostic effectiveness was tested on 20 cows, whom planned tests with the standard PPD tuberculin solution (manufactured by "Biovet" Scientific and Practical Center" LLP) found to have TB. The reaction intensiveness of animals was 5.0 ± 0.1 mm. The reacting animals were isolated and re-examined in 40 days with sensitins, produced from atypical strains of *M. avium*, *M. scrofulaceum* and the standard PPD tuberculin solution. The results of skin swelling (in mm) are presented in Figure 2.

Postmortem examination of three carcasses yielded negative results. *M. avium* was isolated from the biological material in the "Karaganda Scientific and Research Veterinary Station" TB laboratory. The remaining 17 units were returned to the herd. They showed negative results in multiple following examinations.

Planned annual diagnoses at LLP named after Chapayev (Osakarov district) always found animals, reacting to PPD tuberculin mammalian. For example, 12 reacting units were found from 2012 to 2013. The animals were slaughtered without verifying the TB diagnosis.

In 2014, the planned diagnostic examination for TB of 124 cattle units at this farmstead found eight (6.45%) reacting units. Animals were reexamined on the 45th day with *M. avium*, *M. scrofulaceum* and PPD tuberculin mammalian to differentiate the animals' reactions to PPD tuberculin. The results of skin swelling are presented in Figure 3.

Two units (one unit with a cross-reaction to tuberculin and one unit with a cross-reaction to *M. avium* + *M. scrofulaceum*) were slaughtered for diagnostic purposes. Anatomopathological examination yielded negative results. AM were isolated from the biomaterial.

The remaining six commercially valuable animals were returned to the herd. They showed negative results in multiple following examinations.

The planned diagnostic examination with PPD tuberculin mammalian of 250 cattle units at LLP named after Asylbekov found 12 (4.8%) units.

Two units were slaughtered to confirm the diagnosis. Postmortem examination showed negative results. The remaining 12 previously reacting units were reexamined in 45 days with the simultaneous test with *M. scrofulaceum*, *M. avium* and PPD tuberculin. All cows had cross-reactions to allergens in different combinations: three (25%) to PPD + *M. scrofula um*, two (16.7%) to *M. scrofula um* + *M. phlei*, one (8.3%) to PPD + *M. avium*, two (16.7%) to *M. scrofula um* + *M. avium*. Only one animal (8.3%) reacted to *M. scrofula um*. The results of skin swelling are presented in Figure 4.

When analyzing the data in Figure 4, it is necessary to note that the animals at LLP named after Asylbekov had more intensive an allergic reaction to *.scrofulac um* (4.8 ± 0.3 mm) and less intensive a reaction to *M. avium* (3.5 ± 0.2 mm) and PPD tuberculin mammalian (3.7 ± 0.1 mm).

TB was ruled out after the postmortem examination of two cow carcasses. *.scrofulac um* was isolated from the biomaterial.

Based on the results of following examinations and postmortem examinations, the farmstead was considered satisfactory in terms of cattle TB. The results also prevented the premature slaughtering of six units of breeding cattle. The LLP manager was recommended taking consolidating measures in accordance with the existing Rules.

Thirteen cattle units that previously reacted to PPD tuberculin were examined at a private farmstead in the Abay district. The results are presented in Figure 5.

Three cows were slaughtered to determine the accuracy of obtained results for allergic reactions to tuberculin. The postmortem veterinary and sanitary examination of the carcasses found hepatic and pulmonary echinococcosis, hyperplasia and hemorrhages in the mandibular lymph nodes. The biological material was studied at the "Karaganda Scientific and Research Veterinary Station" TB laboratory, where *.scrofulaceum* was isolated.

AM sensitins were also tested for specificity at the "SHON" family-owned farm (Kulaygyr village, Abay district), where six units (2.7%) were found during planned examinations.

The animals were examined in 45 days with the simultaneous administration of PPD tuberculin

mammalian (control) and AM sensitins (*M. avium* and *M. scrofulaceum*). The drugs were administered subcutaneously on the left side, into an area in the middle of the neck, sheared and treated with 70° rectified alcohol, at a dose of 0.2 cm³ with 5 to 6 cm intervals between injections. The reaction was checked after 72 hours. The results of drug tests are presented in Figure 6.

Henceforth, TB was ruled out after the slaughtering of two cows. *M. scrofulaceum* was isolated from the biomaterial. Four cows that were left at the farmstead did not react to tuberculin during following planned examinations.

Figure 7(a) shows the subcutaneous administration of sensitin with the BI-7M needle-free syringe. The results of sensitin tests on cattle are presented.

Figures 7(a, b, c) show that animals had more intensive a reaction to *M. scrofulaceum* (10.6 mm). A less intensive reaction was observed for *M. avium* (8.8±0.6 mm) and PPD tuberculin (4.0±0.5 mm).

No alterations, typical for TB, were discovered in the viscera and lymph nodes during the postmortem examinations of two cow carcasses. The remaining four cows that previously reacted to PPD tuberculin mammalian were left at the farmstead under veterinary monitoring.

Table 1. Specificity of sensitins on guinea pigs, sensitized by different types of mycobacteria.

Types of mycobacteria	Guinea PigNo.	Allergen name					
		<i>M. avium</i> sensitin		Avian PPD tuberculin		<i>M.scrofulaceum</i> sensitin	
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
<i>M. avium</i> -780	1	11	11	12	10	–	–
	2	13	11.5	11.5	9	–	–
	3	15	11	12.5	9	–	–
±		13.1±1.26	11.1±0.05	12± 0.29	9.3± 0.2	–	–
<i>M. scrofulaceum</i>	13	–	–	–	–	6	–
	14	–	–	–	–	9	7
	15	–	–	–	–	9	8
±		–	–	–	–	8± 1.76	7.5± 0.35

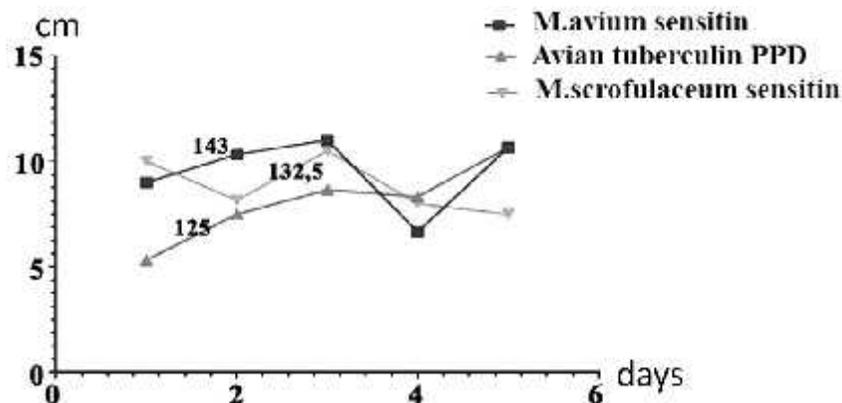


Fig. 1. Reaction of guinea pigs to the tested and standard tuberculin lots after 24 hours

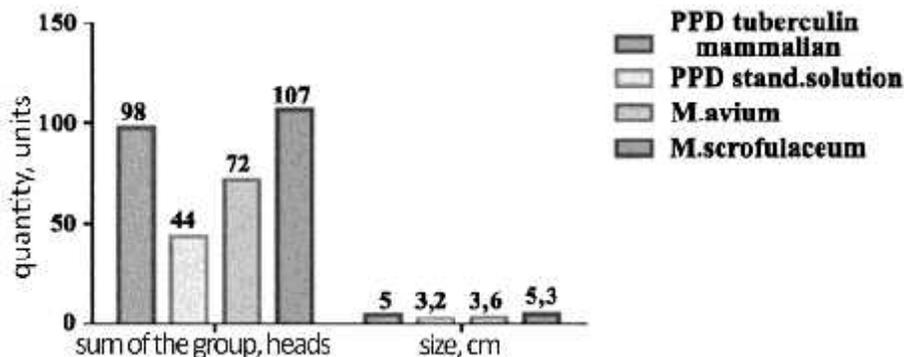


Fig. 2. Results of cattle examination with the simultaneous test at LLP "Shanyrak"

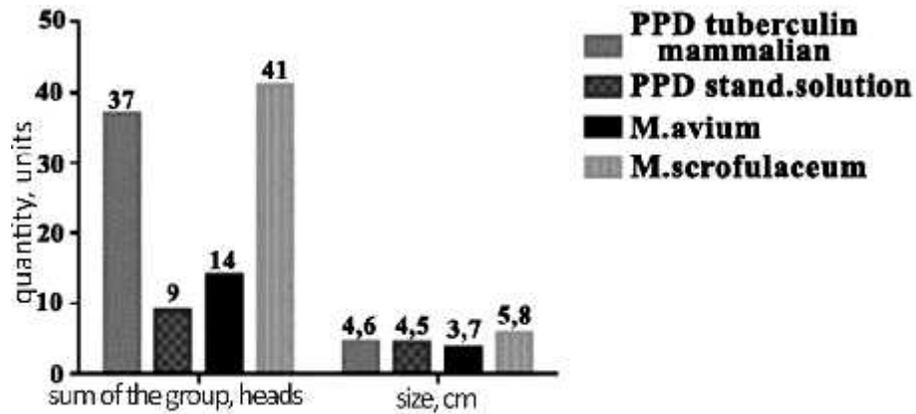


Fig. 3. Results of examining cows that previously reacted to tuberculin with a simultaneous TB test

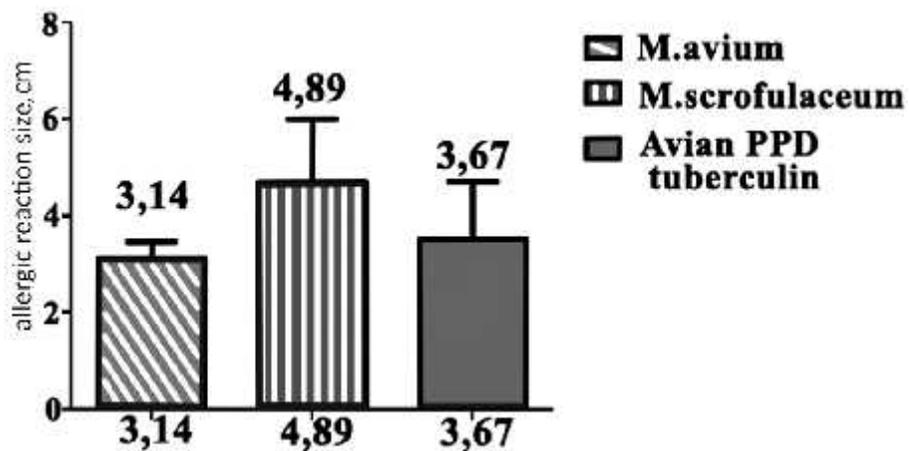


Fig. 4. Results of examining cows with a simultaneous test at LLP named after Asylbekov (Osakarov district).

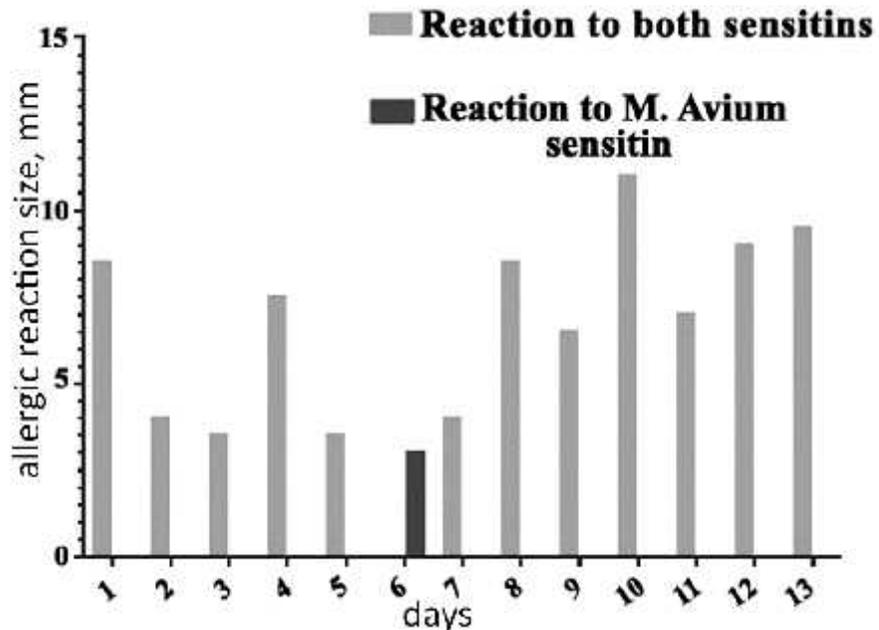


Fig. 5. Allergic reaction of examining cattle for TB with sensitins and PPD tuberculin mammalian at a private farmstead in the Abay district

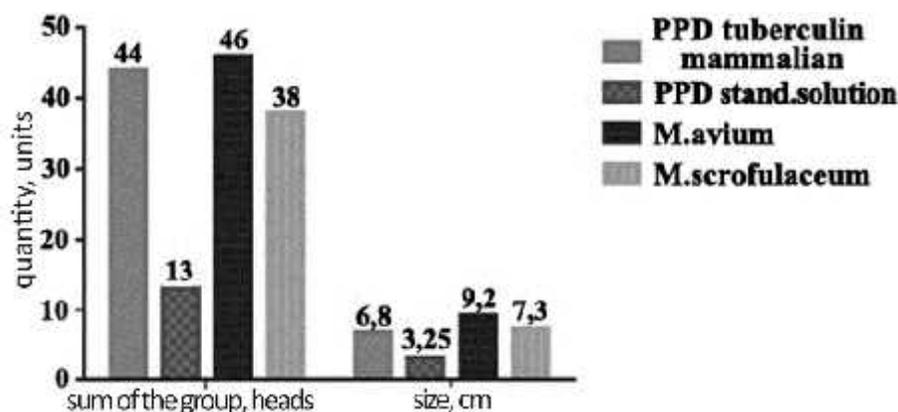


Fig. 6. Comparative analysis results for sensitins at the “SHON” family-owned farm (Abay district) (6 units)

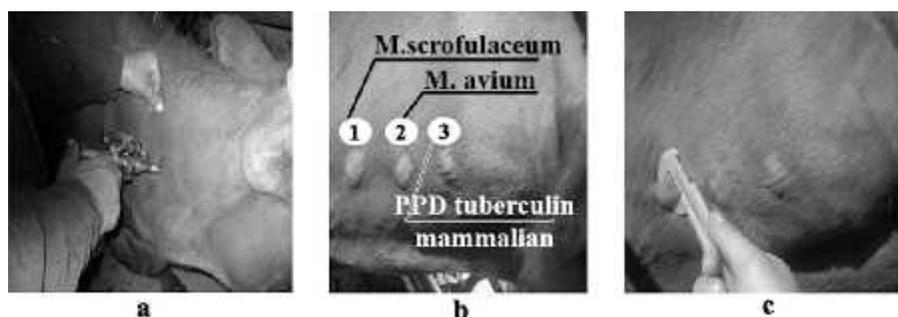


Fig. 7. Cutaneous reaction to sensitins in cattle after 72 hours

DISCUSSION

Data provided in literature and practical observations show the relevance of differentiating nonspecific reactions to tuberculin in cattle. Numerous studies found that the reason for tuberculin reactions in healthy animals, not infected with the TB agent, is their sensitization with AM that are widespread in the environment. In addition to conventional control policies, it is necessary to develop vaccine compatible diagnostic assays to distinguish infected from vaccinated animals (Vordermeier *et al.*, 2015).

Studies of the spread of mycobacteria in different natural and climatic zones of the Karaganda region showed that out of all the isolated cultures, 307 (94.5%) were AM (*.scrofulaceum*, *M. avium*). At that, 64.5% of AM cultures (*.scrofulaceum*, *M. avium*) were isolated from the diagnostic material and objects of the external environment of the steppe zone.

The spread of mycobacteria in the Karaganda region created conditions for the infection of animals and the animals' consequent nonspecific reactions to tuberculin. This requires additional methods for differentiating nonspecific reactions of animals.

Thus, using sensitins in combination with conventional anti-TB measures may prevent the premature slaughtering of cows and save money. For instance, the use of sensitins in the Karaganda region

prevented 47 cows from being slaughtered, which is equivalent to 3.5 million tenge (11 thousand US dollars).

Therefore, the authors developed sensitins from epizootic strains of mycobacteria most frequently encountered in the Karaganda region (*.scrofulaceum*, *M. avium*). Preliminary experiments on guinea pigs found that sensitins had biological and specific activity, identical to avian PPD tuberculin. The tests of developed sensitins *.scrofulaceum* and *M. avium* on 59 cattle at “Mukusheva” LLP, “SHON” family-owned farm, LLP named after Chapayev, LLP named after Asylbekov, and “Shanyrak” LLP for differentiating nonspecific reactions showed their high level of efficiency and the expedience of their application. For example, the combination of sensitins with conventional anti-TB measures in the abovementioned farmsteads prevented the premature slaughtering of 47 cows (more than 3.5 million tenge in monetary form). Sensitin tests can also be used in wildlife monitoring (Livingstone *et al.*, 2015).

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