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Determination of Proteins in Bovine and Avium PPD Tuberculin

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Abstract

Tuberculin skin test is one of the oldest and widely used *in vivo* methods for detection of *Mycobacterium bovis* infections in cattle and *M. avium* infections in birds and pigs. Tuberculins used for the test are purified protein derivatives (PPDs) containing a mixture of antigens - proteins, polypeptides, nucleic acids and polysaccharides. The specificity and activity of PPD depends mainly on the protein content.The aim of the development is to determine the amount of protein in Bulgaria produced dry and liquid PPDs from *M. bovis* and *M. avium* by determining the nitrogen content of the micro-Keldahl method. The process occurs in three stages - incineration, distillation and titration. Average losses of PPD tuberculins at various stages of production reached the highest values after sterile filtration, about 25-30%. At a dry substance concentration of 1 mg/ml, we obtained 0.98 mg/ml of protein, while in the non-filtered product the mean amount was 0.80 mg/ml. In a study of dry tuberculins, part of the samples were diluted in order to avoid foaming and to achieve complete burning. The protein losses were about 20%, which may be in the production process, tuberculin drying or in the analysis itself (pipetting, dilution of samples, etc.).

Regardless of protein losses during the filtration of liquid unfiltered bovine and avian PPD tuberculins as well as the filtered end product, this process is necessary and of great importance for increasing the content of specific proteins, hence the antigenic activity of produced lots of PPD.

Keywords: bovine and avian PPD, proteins, micro-Kjeldahl method

Резюме

Туберкулиновият тест е един от най-старите и широко използвани *in vivo* диагностични тестове за откриване на *Mycobacterium bovis* инфекции при говедата и *M. avium* инфекции при птици и свине. Използваните за теста туберкулини представляват пречистени протеинови деривати (PPD), съдържащи смес от антигени - протеини, полипептиди, нуклеинови киселини и полизахариди. Специфичността и активността на PPD зависят главно от съдържанието на протеини. Цел на разработката е да се установи количеството протеини в произведени в България сухи и течни PPD от *M. bovis* и *M. avium,* чрез определяне съдържанието на азот в тях по микро-Келдал метода, протичащ в три етапа - изгаряне, дестилация и титруване. Средните загуби на PPD туберкулини в отделните етапи от производството достигнаха най-високи стойности при стерилното филтруване, около 25-30%. При вложена концентрация на сухо вещество 1 mg/ml получихме 0,98 mg/ml протеин, докато при нефилтрувания продукт средното количество беше 0,80 mg/ml. При изследване на сухи туберкулини, част от пробите бяха разредени, за да се избегне пенообразуването и да се достигне пълното им изгаряне. Загубите на протеини бяха около 20%, които може да са в производствения процес, при сушенето на туберкулина или при самия анализ (отпипетирване, разреждане на пробите и пр.).

Независимо от загубите на протеини по време на филтрирането на течни нефилтрирани говежди и птичи PPD туберкулини, както и филтрирания краен продукт, този процес е необходим и от голямо значение за увеличаване на съдържанието на специфични протеини, следователно и на антигенната активност на произведените партиди PPD.

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Inroduction

The increasing pressure of modern transport and commerce requires rapid and simple diagnostic tests to detect *Mycobacterium bovis* infections, in addition to mass-scale surveillance testing procedures for the inspection of domestic and wildlife populations infections. In the past century, the tuberculin skin test has been the most commonly used *in vivo* diagnostic test worldwide, as a primary screening test in most countries to diagnose *M. bovis* infections in cattle and *M. avium* infections in birds and pigs (Adams, 2001).

Many improvements have been made to Robert Koch‘s original tuberculin test (Pritchard, 1988). Contemporary tuberculins are complex mixtures of soluble antigens - proteins, polypeptides, nucleic acids and polysaccharides, obtained by heat treatment and chemical fractionation of liquid culture from *M. bovis* or *M. avium* (purified protein derivative [PPD]) (Francis *et al.,* 1978). The specificity and activity of PPD tuberculins depend mainly on the amount of proteins in them, which have the ability to induce in the animal organism a specific skin reaction of the delayed-type hypersensitivity (DTH) (Gilot and Cocito, 1993; Adams, 2001).

The aim of this study was to determine the content of proteins in different batches of dry and liquid PPD tuberculins produced from *M. bovis* and *M. avium* in our country and the influence of different stages of production such as heating and chemical treatment on the amount of protein in 1 ml of ready-made tuberculin.

Material and Methods

The Kjeldahl method is one of the most commonly used methods for determining the protein content of different types of samples based on measuring the amount of nitrogen in them. The obtained amount of nitrogen corresponds to the amount of protein in the solution, and represents the antigenicity of tuberculoproteins.

The total nitrogen content was determined in different batches of dry and liquid PPD tuberculin. Bovine tuberculins were produced from *M. bovis* AN-5, and avian tuberculins from *M. avium* D4ER, recommended by OIE (currently, the World Organisation for Animal Health) as suitable for the production of PPD tuberculins (OIE, 2014, 2015). The strains were obtained from the European Reference Laboratory in Lelystad, the Netherlands.

The nitrogen content in bovine and avian PPD tuberculins was determined using micro-Kjeldahl procedure (Benton, 1991). Three different batches of dry and liquid ready-to-use bovine (PPD-24, PPD-25, PPD-28) and avian (PPD-Avium14, PPD-Avium18, PPD-Avium20) tuberculins were tested.

The analysis was conducted according to the protocol of the Center for Veterinary Biology (SAM 513.09) to the USDA (2014). The test samples were in volumes of 5 ml. In parallel, control samples were prepared containing 5 ml of water (in liquid tuberculin) or buffer (Na2HPO4 + K2HPO4), in which the dry matter was dissolved.

Bovine serum albumin was used as a standard at a concentration of 1mg/ml containing ≥ 96% protein and 14.5-16.5% nitrogen.

The sample analysis was carried out in three stages: incineration, distillation and titration. During the burning process, we added 3 ml of H2SO4, catalytic tablets (CuSO4 + Na2SO4) as antifoaming agent and silicone as a softener (BUCHI, 2012). The combustion was carried out until complete discoloration of the samples. Before being tested, tuberculins were filtered and diluted 1:2 to avoid foaming and to ensure complete burning. After cooling, 6 ml of water, 25 ml of 32% NaOH and

Picture 1. Micro-Kjeldahl equipment for testing

Picture 2. Samples of tuberculin after burning process of tuberculins.

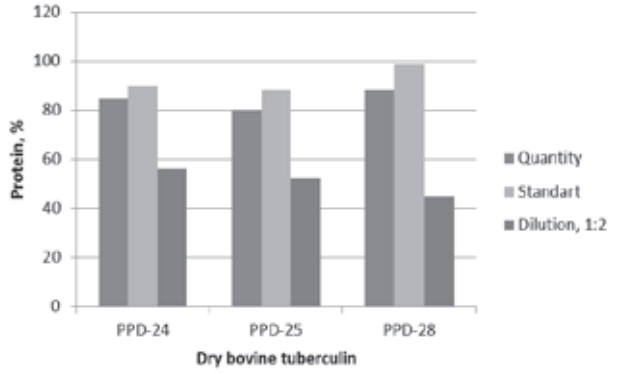

Fig. 1. Protein content in 1 mg/ml dry bovine PPD tuberculin

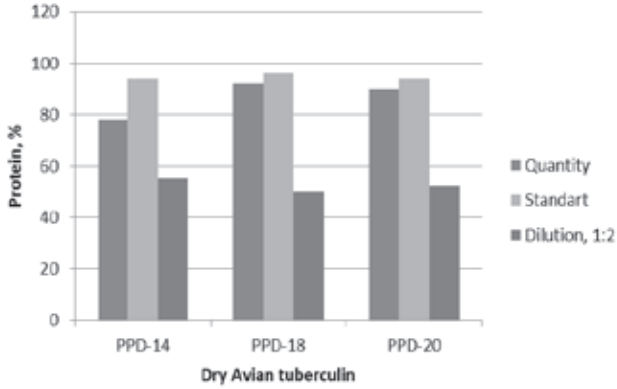

Fig. 2. Protein content in 1 mg/ml dry avian PPD tuberculin

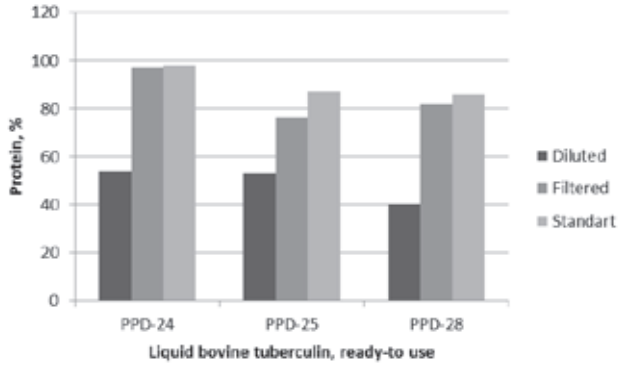

Fig. 3. Protein content in liquid bovine PPD tuberculin after filtration

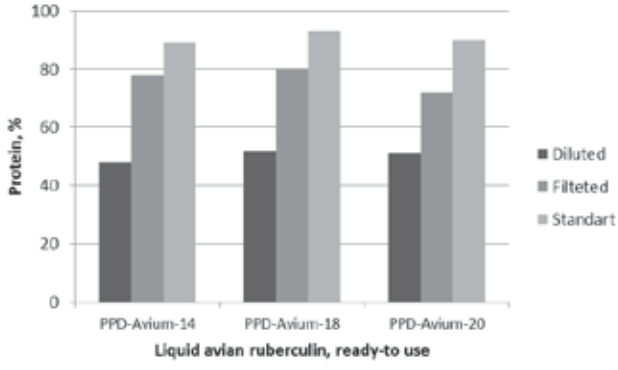

Fig. 4. Protein content in liquid avian PPD tuberculin after filtration

15 ml of 2% H_3BO_3 were added. In the presence of H_2SO_4, organic nitrogen is converted to $(NH_4)_2SO_4$. By distillation, after the addition of NaOH, ammonia was released which is captured from boric acid to form ammonium-borate complex. The residual ammonia was distilled with 0.1% HCl. The amount of the obtained protein was determined by the formula:

Protein mg/ml = ml sample - the amount of HCl necessary for titration of the assayed sample;

ml control - the amount of HCl needed for titration of the control sample;

N HCl - normality of HCl (0.01N); FHCl - HCl factor;

1.4007 – milliequivalent on the weight of nitrogen* 100;

6.25 - conversion factor of %N2 in %protein;

10 - Factor of conversion of %protein into protein mg/ml.

Results and Discussion

The results obtained in the study of bovine tuberculin samples with a starting concentration of dry matter of 1 mg/ml are very close. After the burning process, the protein content of batch PPD-28 was the highest - 88% (0.88 mg/ml) and 14.08% nitrogen/ml, and the lowest was in PPD-25-80% (0.80 mg/ml) protein and 12.8% nitrogen/ml. In batch PPD-24, the protein was 83% (0.83 mg/ml) and 13% nitrogen/ml (Fig. 1, Table 1).

In the dry avian tuberculin PPD-Avium18 the protein content was 92% (0.92 mg/ml) and 14.59% nitrogen/ml, in PPD-Avium 20 the protein was 89% (0.89 mg/ml) and 24% nitrogen/ml, while PPD-Avium14 showed the lowest values - 78% protein (0.78 mg/ml) and 12.36% nitrogen/ml (Fig. 2, Table 1).

The ready-to-use bovine tuberculin was pre-filtered and diluted 1:2. After filtration the amount of protein in PPD-24 batch was reached from 54% to 98% (0.98 mg/ml). The same was observed in the other two batches of bovine tuberculin - 76% (0.76 mg/ml) in PPD-25, and 82% (0.82 mg/ml) in PPD-28 (Fig. 3, Table 2). In ready-to-use avian tuberculin batches, the amount of protein was also increased after filtration. In PPD-Avium14, the amount increased from 48% (0.48 mg/ml) before filtration to 79% after it, in PPD-Avium 18 from 52% to 80% (0.80 mg/ml), and in PPD-Avium 20 from 51% to 72% (0.72 mg/ml) (Fig. 4, Table 2).

PPD is composed mostly of proteins. Due to the specificity of production, the relative yield of PPD tuberculoproteins varies considerably from one batch to another. Literary data show that losses

Table 1. Amount of protein and nitrogen in tested batches of dry tuberculin

	Batch of tuberculin	Burning time [min]	Protein [mg/ml]	Protein [%]	Nitrogen [%/ml]
Liquid bovine tuberculin diluted 1: 2	PPD 24	45	0.97	97	15.52
	PPD 25	45+10	0.76	76	12.16
	PPD 28	45+45	0.82	82	13.12
Standard average	bovine serum albumin	-	0.90	90	14.45
Liquid avian tuberculin diluted 1: 2	PPD Avium 14	45	0.78	78	12.50
	PPD Avium 18	45+10	0.80	80	12.80
	PPD Avium 20	45+45	0.72	72	11.52
Standard average	bovine serum albumin	-	0.90	90	14.45

Table 2. Amount of protein and nitrogen in tested batches of liquid tuberculin

	Batch of tuberculin	Burning time [min]	Protein [mg/ml]	Protein [%]	Nitrogen [%/ml]
Dry bovine tuberculin	PPD 24	45	0.83	83	13.00
	PPD 25	45+10	0.80	80	12.80
	PPD 28	45+45	0.88	88	14.08
Standard average	bovine serum albumin	-	0.92	92	14.72
Dry avian tuberculin	PPD Avium 14	45	0.78	78	12.36
	PPD Avium 18	45+10	0.92	92	14.59
	PPD Avium 20	45+45	0.89	89	14.24
Standard average	bovine serum albumin	-	0.95	0.95	15.20

can reach 35-40%, the highest being in sterile filtration - about 30% loss of proteins, with concentration, precipitation and dehydration accounting for only 5-10%. The loss during filtration was due partly to adsorption (15%) and partly to the fact that a constant volume of culture filtrate remained in the Seitz and Berkefeld filters after filtration (15%) (Magnusson and Bentzon, 1958; Pritchard, 1988; Benton, 1991; USDA, 2014).

Our analyses show that from 1 mg/ml dry matter input, after filtration, 0.76-0.98 mg/ml protein was obtained in ready-to-use bovine tuberculin, and 0.72-0.80 mg/ml protein in avian tuberculin. In the unfiltered product, the average amount of protein was 0.78 - 0.92 mg/ml (Tables 1 and 2). The protein losses were found to be about 17-20%. They can be both in the tuberculin production and drying, or in the analysis itself (sample preparation, quantity of samples which affects the acid amount, type of catalytic tablets, reagents and equipment, the time of foaming of incineration, the time of burning and distillation of samples). Dry tuberculin is hygroscopic and absorbs moisture, which also influences the final result.

Regardless of protein losses during the filtration of liquid unfiltered bovine and avian PPD tuberculins as well as the filtered end product, this process is necessary and of great importance for increasing the content of specific proteins, hence the antigenic activity of produced lots of PPD.

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