Scientific Works. Series C. Veterinary Medicine. Vol. LIX (1)
ISSN 2065-1295, ISSN CD-ROM 2343-9394, ISSN Online 2067-3663, ISSN-L 2065-1295

COMPARATIVE ANTIGEN TESTING FOR AN ELISA DIAGNOSTIC METHOD IN OVINE PARATUBERCULOSIS

Alexandrina (TURCU) ALECU

Faculty of Veterinary Medicine of Bucharest, 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania

Corresponding author email: lxandralecu@yahoo.com

Abstract

In this paper it was conducted a study on four types of antigens prepared from Mycobacterium avium subspecies paratuberculosis cultures in order to build an indirect ELISA method with diagnostic sensitivity as high as possible. The optimization of the method's parameters method was developed together with the interpretation's identifications of the cut-off limit. It was found that the antigen Ag 2, is most appropriate for detecting the level of antibodies in sheep serums, is followed that performant by the antigen Ag 3. In testing the serums of 60 sheeps the antigen Ag 2, obtained by molecular filtration, resulted in a diagnostic accuracy of 78.83% compared with the method of commercial kits.

Key words: *antigens, ELISA, paratuberculosis, serums, sheep.*

INTRODUCTION

Paratuberculosis (John's disease) is caused by *Mycobacterium avium subspecies paratuberculosis*, and it remains an important issue for animal and human health. Paratuberculosis diagnosis is performed in two stages: clinical diagnosis and detection of subclinical infections (Samarineanu M. et al., 1996). Determination of subclinical infections are the most important in the prevention and control of paratuberculosis in livestock, both nationally and internationally.

Currently, ELISA (enzyme-linked immunosorbent assay) is considered the most sensitive and specific method for detection of serum antibodies anti-*Mycobacterium avium subspecies paratuberculosis* in ruminants. Sensitivity is higher than the CFR test (complement fixation reaction) allowing identification of infected subclinical carriers (when there are small amounts of antibodies) and their removal from the livestock. The speed of performing this method allows also, a testing of a large number of serums in a short period of time.

In this paper is presented the testing of several antigens prepared in order to choose a suitable antigen for the ELISA technique that can be applied to the testing of ruminant serum.

MATERIALS AND METHODS

It was prepared four types of antigens in view of their application in ELISA.

Antigen Ag 1 – the antigen was obtained by preparing a fraction rich in protein and carbohydrates from the bacterial corpus. From a culture of *Mycobacterium paratuberculosis*, TEPS strain, on the Reid medium, 13 weeks aged, inactivated by autoclaving for 1 hour at a temperature of 100^{o}C and ultrasonicata, it was retained the supernatant which was precipitated with ammonium sulfate up to 25% saturation. The precipitate was slowly dissolved in a small volume of PBS pH 7.2 and was dialysed against the buffer, the obtained antigen is noted Ag 1.

Antigen Ag 2-This type of antigen was prepared from a culture of *Mycobacterium paratuberculosis*, the 8578 strain, 10 weeks aged. The obtaining technique was adapted from the described method by Jark et al., 1997, as follows: it was sampled the bacterial mass, it was inactivated for 15 minutes at 100^{o}C, it was ultrasonicated for 30 minutes, and the supernatant was treated with proteinase K in a Tris-EDTA buffer and subsequently passed through a filter with an exclusion limit of 30,000 daltons.

Antigen Ag 3 – The antigen was prepared according to the method of Milner et al., 1990, as follows: the culture of *Mycobacterium paratuberculosis*, the 8578 strain, on the Reid medium, 12 weeks aged, washed with PBS, was

inactivated by autoclaving. After ultrasonication and centrifugation, the bacterial extract was ultracentrifugated at 10000xg, for 30 minutes. The supernatant was retained as antigen Ag 3.
Antigen Ag 4-This antigen was prepared according to the method described by Molina et al., 1991, with modifications: the culture of *Mycobacterium paratuberculosis*, the 8578 strain, on the Reid medium, 12 weeks aged, washed with PBS, was inactivated by tyndallization 7 hours at 56^{o}C. After centrifugation the supernatant was retained as antigen Ag 4.
The immunoenzymatic assay (ELISA) applied was an indirect type, composed by the following steps:
- The antigen microplate lining: the obtained Mycobacterium paratuberculosis antigens is fixed on the wells of the microplates in carbonate buffer pH 9.6.
- The reaction with sheep serums: the serums to be (tested/examined) were adequately diluted in 0.01 M PBS with 1% bovine serum albumin and 0.05% Tween-20. For optimization were used control serums: the positive serum (P) is a cumulative serum from vaccinated sheeps with a commercial vaccine and the negative serum (N) a serum from an animal unvaccinated and from a herd free of paratuberculosis.
- The reaction with anti-IgG sheep conjugate: the conjugate used was a commercial one, coupled with peroxidase.
- The reaction with chromogen and substrate mixture: the chromogen was 2.2 Azino-di (3-ethyl benztiazolin-6-sulfonic acid) (ABTS) in 2.3 g% citrate buffer, pH 4.
- The stopping of the reaction: it was performed with 1.5% sodium fluoride.

RESULTS AND DISCUSSIONS

The total protein composition determination of the prepared antigens showed that Ag 2 has the highest concentration, the obtained values were: 1.66 mg/ml for antigen Ag 1, 3.80 mg/ml for antigen Ag 2, 1.11 mg/ml for antigen Ag 3 and 0.30 mg/ml for Ag 4.
The immunochemical testing, performed by ELISA, aimed the behavior of the reacting antigens with the control serums and afterwards the diagnosis of some serums from livestocks.
In Ag 1 titration it was observed that at the concentration of 10 mg/ml the concurrent conditions of the biggest ratio and the biggest difference between the optical density values (O.D.) of the control serums. The results are presented in table 1.

Table 1. Values OD x 1000 in Ag 1 antigen titration with sheep serum

	Antigen concentration (µg/ml)									
	160	**80**	**40**	**20**	**10**	**5**	**2.5**	**1.25**	**0.63**	**0.32**
Serum positive (P)	2098	1817	1646	1602	1919	1661	1141	1006	916	583
Serum negative (N)	200	210	253	206	181	161	135	99	62	41
Serum difference P-N	1898	1607	1393	1396	1738	1500	1006	907	854	542
Serum ratio P:N	10.49	8.65	6.50	7.77	10.60	10.31	8.45	10.16	14.77	14.21

Using this concentration of antigen it was performed the testing of 132 sheep serums with a 1/200 dilution. The serums came from 4 farms and were previously tested by the CFR technique. The diagnosis was achieved by imposing an interpretation limit value (cut-off limit) equal to 2.1 x OD negative control serum. In the table 2 are presented the comparative diagnoses by the two techniques and the diagnostic accuracy.
It is found a highly variable diagnostic accuracy between 12.90% and 100% the higher consistents are for negative serums. This aspect may improve if it is chosen a cut-off value which takes into account the possible weaker recognitions between serum antibodies and the antigen.

Table 2. Comparative table (ELISA with Ag 1 and CFR) of the diagnosis for 132 sheep serums

	Number samples	ELISA		CFR		diagnostic accuracy
		Positive	Negative	Positive	Negative	
Ferm 1	28	8	20	13	15	60.71%
Ferm 2	31	4	27	31	0	12.90%
Ferm 3	40	0	40	20	20	52.50%
Ferm 4	33	0	33	0	33	100%

For a greater precision is indicated also to be introduced a category of so-called dubious serums. At the same time, also the using of a purified antigen would help to reduce the nonspecific reactions or to distinguish the small quantities of antibodies in some serums, all this causing a safer diagnosis.

The antigen Ag 2 was titrated using citrate buffer saline pH 6 for fixation, the results are presented in table 3. It is noted that at the concentration of 2.5 μg/ml it is registered the optimal ratio and difference between the control serums.

With this antigen, applied at the above concentration, were tested 12 sheep serums previously diagnosed also by ELISA with the IDEXX kit for ruminants. It was obtained a diagnostic accuracy of 83.33% applying a cut-off limit = 0.2 x OD positive control serum. The more elaborate purification of the antigen and changing the lining conditions led to a variant of the method with high sensitivity and specificity.

The antigens Ag 3 and Ag 4 were simultaneously tested at a concentration of 10 μg/ml, using the sheep control serums with 8 dilutions. The results are presented in table 4.

It was observed that antigen Ag 3, at the 1/200 serum dilution, has the ratio and the difference between the most representative OD values. The antigen Ag 4 showed no appropriate reaction to any serum dilution. The antigen Ag 3 was titrated in 8 decreasing concentrations (from 160 to 1.25 μg/ml) in order to find the optimal concentration for microplate fixation, and the appropriate serum dilution. The data presented in table 5 have revealed the optimal concentration of 10 μg/ml antigen and the optimal dilution of 1/200 for serum.

Table 3. Values DO x 1000 in antigen Ag 2 titration with sheep serums

	Antigen concentration (μg/ml)							
	20	**10**	**5**	**2,5**	**1,25**	**0,62**	**0,31**	**0,15**
Serum positive (P) 1/200	138	417	589	886	835	518	486	522
Serum negative (N) 1/200	63	78	111	140	114	102	133	201
Serum difference P-N	75	339	478	746	721	416	356	321
Serum ratio P:N	2.19	5.34	5.30	6.32	7.32	5.07	3.65	2.59

Table 4. OD values (x1000) in sheep serums titration against antigens Ag 3 and Ag 4

		Dilution sheep serum							
		1/25	**1/50**	**1/100**	**1/200**	**1/400**	**1/800**	**1/1600**	**1/3200**
Antigen Ag 3	Serum positive (P)	3029	2632	2712	2074	1547	899	376	206
	Serum negative (N)	918	591	329	174	109	61	34	18
	Serum difference P-N	2111	2041	2383	1900	1465	838	342	188
	Serum ratio P:N	3.29	4.45	8.24	11.91	14.44	14.73	11.05	11.44
Antigen Ag 4	Serum positive (P)	896	554	418	329	194	101	57	28
	Serum negative (N)	411	233	153	76	43	21	15	3
	Serum difference P-N	485	321	265	253	151	80	42	25
	Serum ratio P:N	2.18	2.37	2.73	4.32	4.51	4.80	3.80	9.33

Table 5. OD values (x 1000) in antigen Ag 3 with sheep serum in 3 dilutions

	Antigen concentration (μg/ml)							
	160	**80**	**40**	**20**	**10**	**5**	**2.5**	**1.25**
Serum positive (P) dilution 1/100	2968	2935	2933	2983	2995	3008	2948	2767
Serum negative (N) dilution 1/100	559	554	627	702	680	524	492	402
Serum difference P-N	2409	2381	2306	2281	2315	2484	2456	2365
Serum ratio P:N	5.30	5.29	4.67	4.24	4.40	5,74	5,99	6.88
Serum positive (P) dilution 1/200	1766	1762	1862	1999	1953	1779	1498	1083
Serum negative (N) dilution 1/200	111	117	125	134	142	275	124	105
Serum difference P-N	1655	1645	1737	1865	1811	1504	1374	978
Serum ratio P:N	15.90	15.05	14.89	14.91	13.75	6.46	12.08	10.31
Serum positive (P) dilution 1/400	568	625	632	794	913	792	575	390
Serum negative (N) dilution 1/400	17	18	24	34	33	38	28	23
Serum difference P-N	551	607	608	760	880	754	547	367
Serum ratio P:N	33.41	34.72	26.33	23.35	27.66	20.84	20.53	16.95

Using Ag 2 and Ag 3 and applying a cut-off limit = 0.2 x OD positive control serum 60 sheep serums were tested previously diagnosed with IDEXX kit. The diagnostic accuracy was 68.33% with Ag 2 and 63.33% with Ag 3.

The 60 sheep serums were compared again regarding the diagnosis applying also a different calculation method for the cut-off limit. Thus, it was calculated a ratio for each sample S (sample) in relation to positive serum, noted S / P% and the interpretation meant the declaration of the serums as: Negative = S / P < 25%, Doubtful = 25% < S / P < 30%, Positives = S / P> 30%. In this way it was found that a better accuracy was obtained for antigen Ag 2, ie 78.83%, where only 13 out of 60 serums had the same diagnosis at IDEXX kit. The accuracy of the method with Ag 3 was 71.66%. Using the MedCalc software the AUC areas were calculated (area under ROC curve) in each testing with the corresponding antigen. ROC curve (receiver operator characteristic curve) is a means of expressing the relationship between sensitivity and specificity of a diagnostic test. Thus, the method with antigen Ag 2 had a value of 0.912, and an estimated cut-off limit of 29.9% (figure 1) and the Ag 3 method had a smaller AUC value of 0.850, and the estimated cut-off limit of 31.5% (figure 2). So the ELISA technique with Ag 2, with a cut-off limit S / P of 30% is more adequate for comparative diagnosis than antigen Ag 3 method.

Milner et al., 1990, who used a type 3 antigen in a ELISA assay on 327 cow serums sampled from a herd infected, obtained a specificity of 98.9%, but the sensitivity varied depending on the stage of the disease in the sense that animals in an early stage could not be detected by the testing. And Molina et al., 1991, who performed an ELISA assay with a type 4 antigen, showed that the clinical signs of the disease appear before the revealing of the serum antibodies.

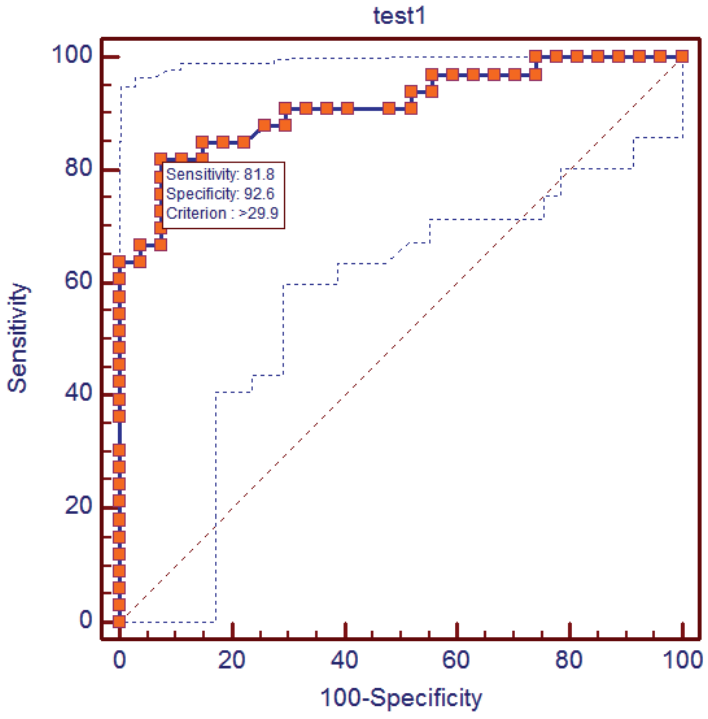

Figure 1. Graph ROC for serum antigen Ag 2 testing (MedCalc software)

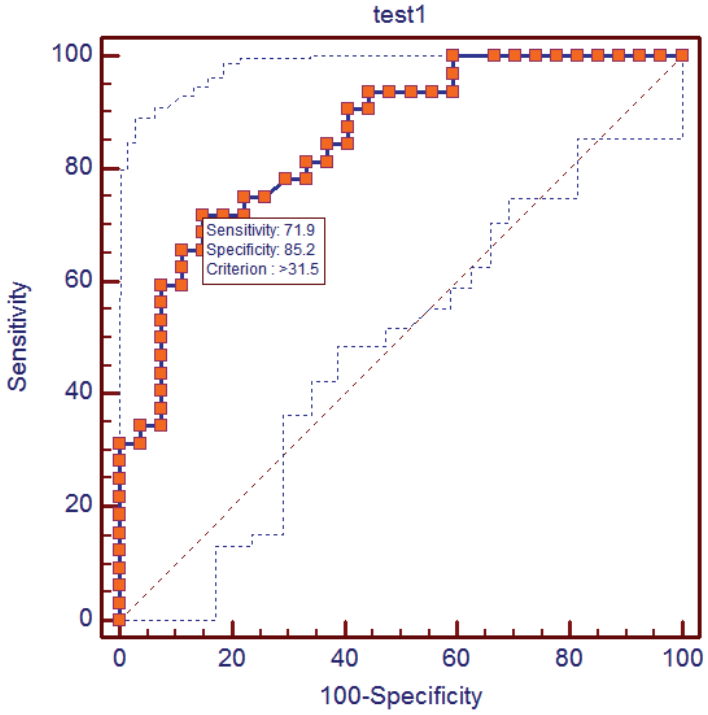

Figure 2. Graph ROC for serum antigen Ag 3 testing (MedCalc software)

CONCLUSIONS

Four types of antigens obtained from cultures of *Mycobacterium paratuberculosis* were prepared and tested by indirect ELISA technique. It was found that the antigen Ag 2, obtained by advanced purification is the most appropriate for detecting the level of antibodies in sheep serums, followed by the Ag 3 antigen performance.

ACKNOWLEDGEMENTS

This work was supported by a strategic financing POSDRU/107/1.5/S/76888 Project 'Doctoral scholarships supporting the research activity in agronomy and veterinary medicine', co-financed by the European Social Fund within the Sectoral Operational Programme of Human Resources Development 2007-2013.

REFERENCES

Jark U., Ringena I., Franz Beate, Gerlach G-F., Beyerbach M., Franz B., 1997, Developement of an ELISA technique for serodiagnosis of bovine paratuberculosis, *Veterinary Microbiology* vol. 51, 189-198.

MedCalc for Windows version 9.5.0.0., MedCalc Sotfware, Mariakerka, Belgium (soft calcul curba ROC si AUC).

Milner A., Mack W., Coates K., Hill J., Gill I. and Paula Sheldrick, 1990, The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trial in cattle, *Veterinary Microbiology vol.* 25, 193-198.

Molina A., Morera L., Llanes D., 1991, Enzyme-linked immunosorbent assay for detection of antibodies against *Mycobacterium paratuberculosis* in goats, *American Journal of Veterinary Research*, vol.52 (6), 863-868.

Samarineanu M., Miciora R., Popovici V., 1996,- Diagnosis Testing in ruminants paratuberculosis by *in vitro* immunostimulation of the lymphocytes (TISL-P). Approved method by the Technical Committee for approving the methods and the methodologies of veterinary medical diagnosis, The Institute of Diagnosis and Animal Health, 25.01.1996.