Mycobacterial Diseases

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Evaluation of Fibronectin-Binding Protein Ag85-B as Target for Serodiagnosis of Swine Mycobacteriosis in Living Animals

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Abstract

The aim of the current study was to evaluate the fibronectin-binding protein Ag85-B as a potential antigen for ELISA-based serodiagnosis of swine mycobacteriosis in living pigs. The validity of Ag85-B, in comparison with tuberculin purified protein derivatives (PPD), for the serodiagnosis of swine mycobacteriosis was investigated in the current study. A total of 89 serum samples from previously confirmed *Mycobacterium* and non-*Mycobacterium*-infected slaughtered pigs were used for the evaluation study. These included 66 serum samples from confirmed cases with swine mycobacteriosis and 23 samples from non-*Mycobacterium* infected cases. Obtained ELISA results revealed an overall accuracy index, sensitivity, specificity, positive predictive value, and negative predictive value of 87.6%, 87.9%, 86.9%, 95.1%, and 71.4%, respectively for Ag85-B as compared to 80.9%, 86.4%, 65.2%, 87.7%, and 62.5%, respectively for tuberculin PPD. In conclusion, the study revealed the reliability of Ag85-B as a potential candidate for ELISA-based serological assay that would provide for early and rapid diagnosis of swine mycobacterial infections in living animals.

Keywords: Antigen 85-B; Serodiagnosis; Swine mycobacteriosis

Introduction

During the last two decades, infection with tuberculous, as well as non-tuberculous mycobacteria has become increasingly important among human beings and farm animals [1,2]. The pandemic of HIV infection in many part of the world, as well as the emergence of multi-drug resistant (MDR) tuberculosis have complicated the disease control process [3]. *Mycobacterium tuberculosis* complex and *Mycobacterium avium-intracellulare* complex are the most frequently isolated mycobacteria from AIDS patients [4,5]. Origin of mycobacterial infections in humans is still a matter of speculation. Previous studies have shown that pigs are among important sources of mycobacterial infections to man [6,7].

Diagnosis of swine mycobacteriosis in living pigs has been a major hindrance in efforts to study the disease. Clinical signs of diagnostic value are usually absent and reliability of the tuberculin skin test has been questioned [8]. Majority of cases are diagnosed only during regular meat inspection in slaughterhouses by detection of suspected mycobacterial lesions in carcasses [9,10]. However, many cases can escape post-mortem examination especially during early stage of infection [11,12]. In some other cases, it is difficult to differentiate lesions caused by mycobacterial infections from other lesions [13,14]. Unreliability of the tuberculin skin test for diagnosis of swine tuberculosis has drawn attention towards serodiagnosis as an alternative for diagnosis of swine mycobacteriosis in living animals [8,15,16].

Culture filtrates prepared from tuberculous *Mycobacterium spp.* are complex mixture of several antigenic proteins [17]. A major portion of the secreted proteins in *M. tuberculosis* and *M. bovis* BCG culture filtrate is formed of the antigen 85 (Ag85) complex, a 30-32 kDa family of three fibronectin-binding proteins (Ag85-A, Ag85-B, and Ag85-C) [18]. All members of Ag85 complex possess a mycoloyltransferase enzyme activity required for the biogenesis of cord factor, a dominant structure necessary for maintaining cell wall integrity [19,20]. The Ag85 complex molecules have been reported to be the dominantly secreted

antigens expressed by nearly all mycobacterial species [18]. Ag85 complex molecules are highly conserved among *Mycobacterium spp.* and thus revealed species-specific properties [15,21].

The aim of the current study was to evaluate the reliability and validity of Ag85-B as a potential ELISA candidate, in comparison with tuberculin PPD, for serological diagnosis of swine mycobacterial infections in living animals.

Materials and Methods

Study samples

A total of 89 serum samples from previously investigated and confirmed infected pigs, based on mycobacteriological and molecular examination, [22] were used for the current evaluation study. Investigated samples included 66 samples from animals whose suspected lesions were previously confirmed positive for *Mycobacterium bovis* [7], *M. tuberculosis* [22] and other non-tuberculous *Mycobacterium spp.* [23] The remaining 23 samples were from animals whose suspected lesions were positive for bacteria other than *Mycobacterium* spp. (non-*Mycobacterium* isolates) and were used to assess the cross-reaction probability of Ag85-B to other non-mycobacterial bacteria. In addition, 28 serum samples were obtained from apparently healthy lesions-free pigs and were used as healthy control subjects for the purpose of calculating the optimal cut-off points for distinguishing between

positive and negative results for both Ag85-B and tuberculin PPD antigens.

Serological examination

Bacterial strains and preparation of antigens: The *M. bovis* AN5 standard strain (supplied by the Central Veterinary Laboratory, Weybridge, England) was used throughout the study. *M. bovis* AN5 cultures were grown to log phase in Middlebrook 7H9 liquid medium supplemented with Middlebrook ADC enrichment[a] then were sub cultured as surface pellicles on the wholly synthetic Sauton medium for 3-8 weeks. The supernatants were collected by centrifugation and then filtered through a 0.22 μm Millipore membrane[b] (Cambridge, MA, USA) to remove remaining mycobacteria. The whole Culture Filtrate (CF) proteins were then precipitated with ammonium sulfate (55%) for 18 h at 4°C, and the resulting precipitate was dissolved and dialyzed against 1 mM sodium phosphate buffer (pH 6.8) for 18 h at 4°C. The PPD antigen was prepared from the heated CF as previously described [24], while the native Ag85-B antigen was purified from the unheated CF by sequential ion-exchange chromatography and reverse-phase high-performance liquid chromatography to yield a single 29 to 30 kd component as previously described [21].

Enzyme-linked immunosorbent assay (elisa)

For serological evaluation, indirect ELISA was carried out using both Ag85-B and tuberculin PPD antigens as previously described [25] with some modifications. Briefly, Immulon II 96 microtiter plates[c] were coated with either Ag85-B (0.5 μg/100 μl carbonate buffer, pH 9.6) or tuberculin PPD (5 μg/100 μl carbonate buffer, pH 9.6, containing 1% carbodiamide) and were incubated for 24 hours. The plates were then decanted and blocked with 2% bovine serum albumin for 2 h. 100 μl of 1/100 diluted serum was added to each well and plates were incubated at 37°C/2h. Horseradish peroxidase-conjugated anti-pig was used as a secondary antibody. The substrate used was OPD[d]. The plates were washed four times between each step with PBS containing 0.1% Tween20. All reaction mixtures were set in duplicate, with the mean value being used for recording and calculations. Results were read on SOFTmax PRO ELISA reader[e] at a wave length of 450 nm.

Evaluation of tests and statistical analysis

Analysis of mean optical density (OD) values of both antigens with different serum samples groups were carried out using Statistical Package for Social Sciences (SPSS) version 10.0. Data were presented as the mean OD ± SD and the OD range. The optimal cut-off points for distinguishing between positive and negative results for both antigens were determined by Receiver Operating Characteristic (ROC) curve analysis using Analyse-it Software[f] [21]. The tested antigens were evaluated in terms of accuracy index, sensitivity, specificity, and positive and negative predictive values as described elsewhere [26].

Results

Results of Ag85-B and tuberculin PPD-based ELISA

The ROC curve was constructed for both antigens and the obtained results revealed cutoff values for Ag 85-B and tuberculin PPD as 0.144 (Figure 1) and 0.212 (Figure 2). Based on the calculated cut-off points, Ag85-B-based ELISA was able to detect a total of 58 (87.9%) out of 66 confirmed *Mycobacterium* species-infected animals and 3 (13.04%) out of the 23 non-*Mycobacterium* species infected animals. On the other hand, tuberculin PPD-based ELISA was able to detect 57 (86.4%) out of 66 confirmed *Mycobacterium* species-infected animals and 8 (34.8%) out of 23 non-*Mycobacterium* species infected animals (Table 1).

Validity of Ag85-B and tuberculin PPD-based ELISA

Based on the results of both Ag85-B and tuberculin PPD-based ELISA with confirmed *Mycobacterium* species and non-*Mycobacterium* species infected cases, true and false positive and negative values were calculated for each protein (Table 2). Regarding the validity of evaluated antigens for the serodiagnosis of swine mycobacteriosis, obtained results revealed an overall accuracy index, sensitivity, specificity, positive predictive value, and negative predictive value of 87.6%, 87.9%, 86.9%, 95.1%, and 71.4%, respectively for Ag85-B as compared to 80.9%, 86.4%, 65.2%, 87.7%, and 62.5%, respectively for tuberculin PPD (Table 2).

Discussion

Swine mycobacteriosis represents a public health threat in many countries for being a potential reservoir of many mycobacterial infections for other farm animals as well as contact humans [27-29]. Tuberculin skin testing is unreliable for diagnosis of swine tuberculosis in living pigs [16]. Therefore, diagnosis of swine mycobacteriosis and studying of its prevalence in live animals is usually impossible and it still based mainly on post-mortem findings by meat inspectors [10]. In the current study, the fibronectin-binding protein Ag85-B was purified and evaluated serologically, in comparison with the tuberculin PPD, as a potential candidate for rapid screening of swine mycobacteriosis in living pigs. The ROC curve [a plot of the true positive rate (sensitivity) against the false positive rate (1-specificity) that is obtained at each cut-off point] was constructed using the OD readings of both antigens with

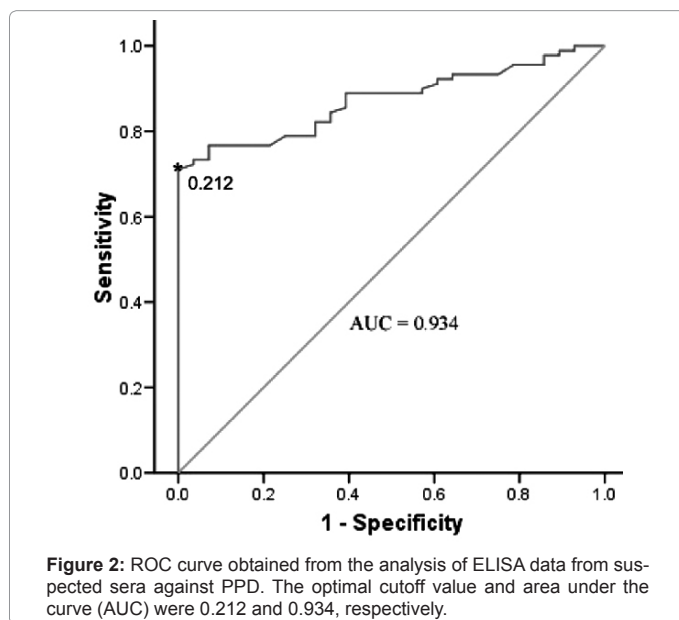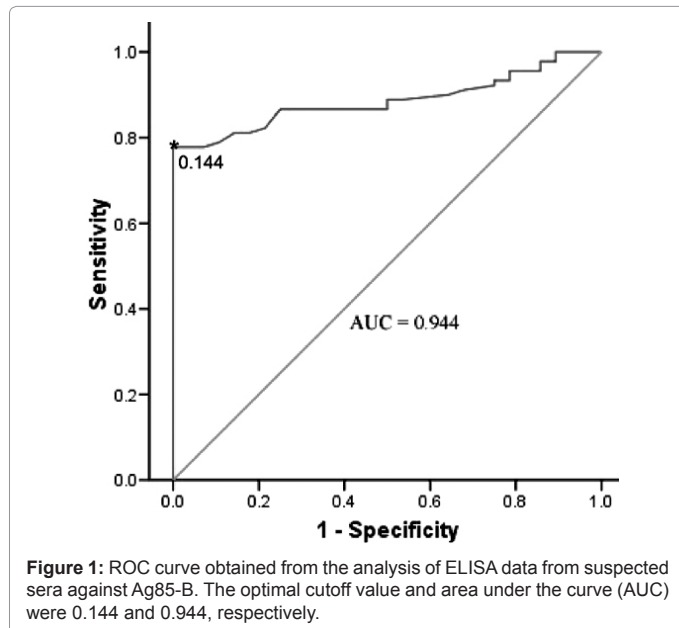| Confirmed bacterial identificati	NO.	Ag58-B			PPD antigen		
		Mean OD ± SD (OD range)	+ve cases *	%	Mean OD ± SD (OD range)	+ve cases †	%
M. bovis	12	0.546 ± 0.156 (0.209– 0.748)	12	100	0.534 ± 0.145 (0.203 – 0.821)	11	91.7
M. tuberculosis	18	0.560 ± 0.203 (0.203 – 0.862)	18	100	0.544 ± 0.131 (0.323 – 0.794)	18	100
Non-tuberculous Mycobacterium spp.	36	0.364 ± 0.173 (0.074 – 0.674)	28	77.8	0.544 ± 0.131 (0.323 – 0.794)	28	77.8
Sub-total	**66**		**58**	**87.9**		**57**	**86.4**
Non-Mycobacterium spp.	23	0.126 ± 0.025 (0.076 - 0.189)	3	13	0.180 ± 0.086 (0.086 – 0.376)	8	34.8

* cutoff point for Ag85-B = 0.144, † cutoff point for tuberculin PPD = 0.212

Table 1: Serodiagnosis of swine mycobacteriosis by ELISA detection of anti-mycobacterial IgG in swine sera using Ag85-B and tuberculin PPD antigens.

Methods	Test Results				Validity parameters (%)				
	TP*	TN†	FP‡	FN§	SNⁱ	SP#	AI¶	PPV**	NPV††
Ag85-B ELISA	58	20	3	8	87.9	86.9	87.6	95.1	71.4
Tuberculin PPD-ELISA	57	15	8	9	86.4	65.2	80.9	87.7	62.5

*true positive; †true negative; ‡false positive; §false negative; ⁱsensitivity; #specificity; ¶Accuracy index; **positive predictive value; ††negative predictive value

Table 2: Evaluation of Ag85-B and Tuberculin PPD- based ELISA for diagnosis of Swine mycobacteriosis

Figure 1: ROC curve obtained from the analysis of ELISA data from suspected sera against Ag85-B. The optimal cutoff value and area under the curve (AUC) were 0.144 and 0.944, respectively.

Figure 2: ROC curve obtained from the analysis of ELISA data from suspected sera against PPD. The optimal cutoff value and area under the curve (AUC) were 0.212 and 0.934, respectively.

both infected and non-infected sera. The AUC value was calculated as a measure of the accuracy of the test and they were 0.944 for Ag85-B (Figure 1) and 0.934 for PPD tuberculin (Figure 2). This shows that in more than 93% of cases, an animal giving a result above the cutoff value was correctly identified [30].

Serodiagnosis of swine mycobacteriosis using Ag85-B and tuber-culin PPD have showed that both Ag85-B and tuberculin PPD antigens have comparable sensitivity (87.9 and 86.4, respectively), however, the specificity, AI, PPV and NPV for Ag85-B were considerably higher (86.9%, 87.6 %, 95.1 and 71.4%, respectively) than that (65.2%, 80.9%, 87.7% and 62.5%, respectively) of tuberculin PPD (Table 2). The higher specificity and positive predictive value of Ag85-B as compared to that of tuberculin PPD could be attributed to the fact that PPD is a protein complex that contains many shared antigenic epitopes with other non-mycobacterial organism, which greatly affect its specificity in diagnosis of mycobacterial infection [31]. On the other hand, Ag85 complex molecules have been reported to be the dominant secreted antigens that are highly specific and conserved among *Mycobacterium spp.* and thus revealed species specific properties [15,21,32]. In previous studies, the use of multiple overlapping peptides corresponding to the entire sequence of the antigen 85 revealed the presence of at least seven T-cell determinants with high homology between the antigen 85 purified from both *M. bovis* BCG and *M. tuberculosis* [32]. The same epitopes were found in the *M. avium* strains but with much less homology indicating that the antigen could have potential diagnostic capabilities for both *M. tuberculosis* complex as well as *M. avium intracellulare complex* [15,33].

In conclusion, the overall validation parameters of Ag85-B revealed the reliability of the proposed antigen for serological diagnosis of swine mycobacteriosis among living animals. The assay would provide for rapid and early detection of both tuberculous and non-tuberculous mycobacterial infections of living pigs and help to control the disease and prevent its dissemination among other farm animals and contact humans.

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