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Research Article

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Development and Performance Characterization of Double Antigen Sandwich ELISA for *Mycoplasma bovis* Antibody Detection

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Abstract

Background: *Mycoplasma (M.) bovis* is an important pathogen of cattle, which is associated with the occurrence of many. Currently there is no effective vaccine to prevent *M. bovis* infections, and no commercial vaccines currently available, so the detection approach shows the importance for the control the disease and surveille the prevalence of *M. bovis*.

Objective: To develop and optimize an ELISA, and evaluate the analytical, diagnostic and epidemiological performance.

Animals: Field serum samples (n=368) from 9 cattle farms in different counties in Ningxia Province, China.

Methods: Different principles of antibody detection ELISA were compared, and the parameters of double antigen sandwich (DAS) ELISA with P48 recombinant antigen were optimized. All serum samples were tested with DAS ELISA and reference kit. Accuracy, sensitivity and specificity were evaluated using ROC curve, and Box plot was used for the prevalence and epidemiological performance analysis.

Results: ROC curve analysis for DAS ELISA showed the area under curve was 0.807 ($p<0.001$). With cutoff of OD 1.5, the diagnostic sensitivity was 69.16% (95% Confidence interval [CI], 59.5% to 77.7%) with a diagnostic specificity of 72.8% (95% CI, 67.0% to 78.1%). 6 of 9 farms infected with *M. bovis* were detected, and the relevance rate were 81.43%, 75.00%, 65.79%, 33.33%, 38.64% and 72.84% with DAS ELISA respectively, higher than 50.00%, 50.00%, 28.95%, 3.33%, 29.55, 40.74% with reference kit.

Conclusion: The optimized DAS ELISA provided unambiguous improved serodiagnostic performance and sufficient sensitivity, specificity, and it will be an improved tool for *M. bovis* surveillance and prevention.

Keywords

Mycoplasma bovis; Double antigen sandwich ELISA; Serodiagnostic; Analytic performance; Diagnostic performance; Epidemiological performance; Prevalence

Introduction

Mycoplasma (M.) bovis is an important pathogen of cattle, which belongs to the genus Mycoplasma within class of Mollicutes, and was first detected and isolated in 1961 in the United States [1]. This wall less bacterium is associated with the occurrence of many diseases including mastitis, pneumonia, arthritis, genital disorders and keratoconjunctivitis, and otitis media only occurs in younger animals [2-4]. After isolated, *M. bovis* has been detected in many countries, especially in these years it has speared worldwide, and now currently recognized as main occurring mycoplasma pathogen threatening livestock production, even in some apparent healthy herd the *M. bovis* was isolated [2,5,6]. Associations between mycoplasmas and other pathogens were often observed, from some research *M. bovis* was detected with different microbes, such as Pasteurella, Mannheimia species [6,7], and different Mycoplasma species [8]. The resulting clinical signs and diseases by *M. bovis* are increasingly recognized as having a significant adverse impact on animal welfare and the economy of cattle farming around the world [9].

Prevention of *M. bovis* infections in cattle is inherently difficult. To date, there is no effective vaccine to prevent *M. bovis* infections and no commercial vaccines currently available [10,11]. Although we now know more about the biology of this pathogen, information is lacking about appropriate protective antigens, the type of immune response that confers protection and adjuvants selection [12]. Recent researches about vaccine showed some improvements, but this was not enough to confer protection against *M. bovis* [13-16], and in some research the calf even could be infected after inoculation with M *bovis* [17]. If an infection occurs, then early antimicrobial treatment with drugs effective at treating mycoplasma infections offers the best opportunity to control disease, but therapy with antibiotics is scarcely efficient and increasing antimicrobial resistance is reported [18]. In vitro studies show that many of the current *M. bovis* isolates have high minimum inhibitory concentrations (MIC) for many of the commercially available antimicrobials [19-21]. Mutations responsible for high macrolide, lincomycin, florfenicol, and pleuromutilin antibiotic MICs were identified in genes encoding 23S rRNA [22].

To control the disease and surveille the prevalence of *M. bovis*, the detection approach shows the importance. Isolation of *M. bovis* by culture is the definitive method for diagnosis, but several factors may influence the outcome, such antimicrobial treatment of animals prior to sampling, presence of a polymicrobial infection with faster growing organisms and inadequate handling, storage or processing of the samples [23-25]. Some studies about molecular diagnosis and serological tests were developed for *M. bovis*, which are convenient to use. In general, molecular methods can be used for identify the animals infected or carrying *M. bovis* [26-28], but the factors influencing culture also exist. The serological tests Detection of antibodies to *M. bovis* is usually used for herd screening and these tests always are useful as a herd test and also for demonstrating lack of infection [29,30].

Materials and Methods

M. bovis strains and recombinant antigen

M. bovis isolates Ningxia-1, Ningxia-2 and Ningxia-3 were originally isolated from the lesioned lung of beef calf in different

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herd, which were confirmed using molecular technology [31], and the whole-genome sequence of isolate Ningxia-1 has been reported [32]. The strains were grown at 37°C for 24 hours in Mycoplasma culture medium. The recombinant antigen of P48 used in vitro study were produced by our lab [33], using expression in E.coli bacterial with the synthetic P48 gene of *M. bovis* isolate Roma, whose GenBank reference number is DQ020482.1.

Indirect ELISA preparation with *M. bovis* and P48 recombinant antigen

Indirect ELISA tests include plates coated with the Mycoplasma and recombinant antigen, and HRP conjugated Rabbit anti Bovine IgG (Beijing Berseebio Science & Technology Co., Ltd., Beijing, China). The Mycoplasma medium containing *M. bovis* were centrifuged 15 min at 12000 rpm and washed once in 0.02 M PBS pH 7.4. The *M. bovis* were centrifuged again, and then suspended in 0.02 M PBS pH 7.4 with the same volume of medium. Then *M. bovis* lysis was got after 10 freeze-thaw cycles, and the suspension of lysis was collected after 15 min centrifuge at 8000 rpm. Then the protein concentration of lysis suspend was measured, then the Mycoplasma coating solution was ready after lysis suspend was diluted with 0.02 M PBS pH 7.4 to the concentration of 0.5 mg/mL. The blank plates (Xiamen Yijiamei Lab Instruments Co., Ltd, Xiamen, China) were coated with the Mycoplasma coating solution 100 uL per well in 37°C for 2 hours, then we washed the plates and blocked with blocking buffer (5% BSA, 2% Gelatin, 0.5% Casein, in 0.02 M PBS pH 7.4) in 37°C for 2 hours, at last washed and dried the plates to get the *M. bovis* plates. In order to get the P48 plates, the recombinant antigen of P48 was dialyzed to 0.02 M PBS pH 7.4, and then diluted to 1.0 mg/mL with 0.02 M PBS pH 7.4 for the P48 recombinant antigen coating solution. Then the P48 plates were coated with the same protocol described for Mycoplasma plate's preparation.

DAS ELISA preparation with P48 recombinant antigen

Double antigen sandwich ELISA tests include plates coated with P48 recombinant antigen and the HRP conjugated P48 recombinant antigen. With the protocol described above, the P48 plates of 100 ng/well P48 recombinant antigen were prepared. To prepare the HRP conjugated P48, 250 μL of 20 mg/mL $NaIO_4$ solution was added to 250 μL HRP solution within 5 mg HRP drop by drop with stirring gently, then the mixture was left stand in 4°C for 30 min. 5 mL ethylene glycol was resolved in 25 μL H_2O, then added to the mixture drop by drop, then the mixture, activated HRP solution, was collected after 30 min standing in room temperature. Put the P48 recombinant antigen solution, which was dialyzed in 50 mM CB pH 9.5 and diluted to 5 mg/mL, into the activated HRP solution with the ratio of 1:4, then all the mixture was dialyzed in 50 mM CB pH 9.5 for 6 hours in 4°C. 100 μL of freshly prepared 0.1 M $NaBH_4$ was added to per mL of the previous mixture of HRP and P48, then the mixture was stirred at 4°C overnight. At last, the conjugate mixture was dialyzed against 0.1 M PBS overnight at 4°C, then 50% Glycerol, 1% BSA and 0.02% Thimerosal was added to get the concentrated HRP conjugated P48.

ELISA testing procedure

All washing steps for ELISA were performed using a microplate washer (Wellwash™ Microplate Washer, Thermofisher Scientific, USA) in 0.02 M PBS pH 7.4 containing 0.05% Tween-20. 10 μL samples were added into the ELISA plate well, and 100 μL diluted HRP conjugation, which was diluted in HRP diluent of 0.02 M PBS pH 7.4 within 0.1% Casein and 0.2% Tween-20, were added to the well also. Then the plate was incubated for 1 hour in 37°C. The plates were washed 5 times, and developed for 15 min in 37°C after the 100 μL TMB-ELISA substrate solution (Beijing Hotgen Biotechnology Co., Ltd., Beijing, China) were added to well. OD450 was read soon using a plate reader (Multiskan™ FC Microplate Photometer, Thermofisher Scientific, USA), and the OD value was used for analysis and measurement.

Serum samples

Field samples of serum from cattle (n=368) were collected from 9 farms in different counties in Ningxia, which is located in Northwest China, and 9 counties are Helan, Litong, Zhongning, Zhongwei, Jingyuan, Longde, Pengyang, Xiji, Yuanzhou. The location of Ningxia and 9 counties was showed in Figure 1. The serum samples were collected with standard sampling. Gibco Fetal Bovine Serum (Thermofisher Scientific, USA) was used as control serum sample.

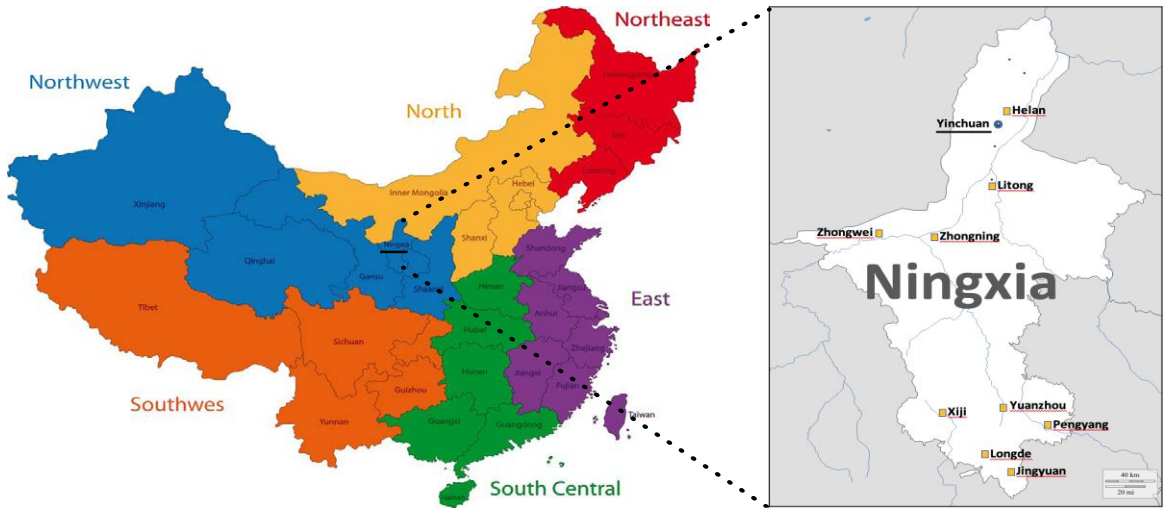

Figure 1: Location of Ningxia, and 9 counties. Ningxia is located in Northwest China, and 9 counties, where field samples were collected, were labeled on the map of Ningxia. Helan was located in the north of Ningxia, Litong, Zhongwei, Zhonging are located in the middle of Ningxia, and Yuanzhou, Xiji, Pengyang, Longde and Jingyuan are located in the south of Ningxia.

Reference kit and the testing procedure

The reference kit for *M. bovis* is ELISA Kit for serodiagnosis of *M. bovis*, catalog BioK 260 (Bio-X Diagnostics S.A., Rochefort, Belgium), and the testing procedure is based on the instruction for use. The testing value of BioK 260 was calculated based on Delta OD of samples, which was OD of samples minus OD of negative control, and Delta OD positive, which was OD of positive control minus OD of negative control. Then the percentage ratio between Delta OD samples and Delta OD positive was calculated as testing value of reference kit. The samples with value above 37% were judged as positive. Described on instruction for use of BioK 260, the positive results could be defined as "+", "++", "+++", "++++" and "+++++" with the value from 37% to 60%, from 60% to 83%, from 83% to 106%, from 106% to 129% and above 129% respectively.

Statistical analysis

Statistical analysis was performed using MedCalc (MedCalc Software, Ostend, Belgium), and the accuracy performance of the tests were evaluated with ROC curve, in which the area under the curve can be used to select the threshold value and evaluate the performance. Then based on the criterion, the testing results could be judged, then the diagnostic performance, including sensitivity, specificity, positive predictive value, negative predictive value, etc. was analyzed.

Results

ELISA tests principle evaluation

The strains of *M. bovis*, Ningxia-1, Ningxia-2 and Ningxia-3, were gathered after being cultured, and then the lysate of the Mycoplasma was employed for coating the ELISA plate. At the same time the P48 recombinant antigen was coated on the ELISA plate. Then the HRP conjugated secondary antibody-diluted 1000 times was used for the testing as the indirect ELISA, and the HRP conjugated P48 recombinant antigen-diluted 1000 times was used also for the double antigen sandwich (DAS) ELISA with P48 plate. 4 field samples, 2 positive and 2 negative, control FBS and 0.02 M PBS pH 7.4 were tested by these 5 kinds of ELISA tests. The BioK 260, ELISA Kit for Serodiagnosis of *M. bovis* from Bio-X Diagnostic S.A. was used as reference kit. All the results were showed in Table 1.

In the 4 kinds of indirect ELISA tests, only the P48 plate showed the remarkable difference between 2 positive samples and 2 negative samples. The 3 kinds of indirect ELISA tests with Mycoplasma *bovis* coated plates could not tell the difference between positive samples and negative samples. Meanwhile, we got high OD value with the control samples on 3 kinds of indirect ELISA tests, and the P48 plate gave low OD. The DAS ELISA with P48 plate showed similar results as indirect ELISA test with P48 plate, and the positive samples gave high OD, while negative samples and control samples gave low OD.

Comparing the results of indirect ELISA and DAS ELISA with P48 plate, we found that both of them showed obviously difference between positive samples and negative samples. Although the OD value of positive samples is on similar level, the OD value of negative and control samples with indirect ELISA was remarkable higher than those with DAS ELISA. So the DAS ELISA showed best diagnostic performance in the comparing experiments, and it was selected for the further development.

Optimization of P48 DAS ELISA tests

To optimize the P48 DAS ELISA, the different parameters of the ELISA tests were adjusted, including the dilution ratio of P48 HRP conjugate and the antigen volume for ELISA plate coated. The different volume of antigen was coated on plates, and the HRP conjugate was diluted with different ratio, then the 2 positive samples, 2 negative samples and 2 control samples were tested, the OD value was generated. Then the ratio of average OD value of positive samples to average OD value of negative samples, positive samples to control samples, negative samples to control samples were calculated. The final results were showed in Figure 2. No matter which condition of parameter was applied, the ratio of negative results to control results was at the same level, but the ratio of positive results to negative results and positive results to control results varied, due to the OD value generated by field samples varied. The P48 DAS ELISA with 100 μL/well coating and 1:1000 dilute ratio of HRP conjugate was optimized.

Field samples testing

Total 368 field samples were collected, and at first they were tested with reference kit, BioK 260 to get the serodiagnostic background, or called reference result. The results are calculated and judged with the instruction for use strictly, and summarized testing results were showed in Table 2. Then the field samples were tested with P48 DAS ELISA, and the OD value were recorded and showed in Figure 3, in which the OD value of P48 DAS ELISA was organized based on the serodiagnostic background of the samples. The distribution of the samples varied in a huge range, which meant that there were some results were not accord with reference with reference kit, but the mean value of the samples with positive reference was higher than those with negative reference, so P48 DAS ELISA could distinguished the positive samples and negative samples basically.

Criterion selection of P48 DAS ELISA based on diagnostic performance

ROC curve was employed to analyze the accuracy performance of P48 DAS ELISA, so the testing OD value was analyzed with reference results as the classification, which included positive group of 107 samples (29.08%), and negative group of 261 results (70.92%). The ROC curve was got as Figure 4, and the area under curve (AUC) was 0.807, P<0.001, with 95% Confidence interval (CI), the AUC was between 0.763 and 0.846. So the total diagnostic performance of P48

Table 1: Evaluation results of different test principle.

ELISA Plates	HRP conjugate	Samples					
		6-24	10-27	6-43	10-25	FBS	PBS
P48 plate	Rabbit Anti Cattle HRP conjugate	2.476	2.034	1.097	0.864	0.546	0.219
Mycoplasma plate - Ningxia1		2.726	3.072	2.560	2.595	2.960	2.412
Mycoplasma plate - Ningxia2		2.548	2.740	1.890	2.451	2.326	2.035
Mycoplasma plate - Ningxia3		2.254	2.197	1.490	2.376	1.868	1.971
P48 plate	P48-HRP	3.019	2.245	0.366	0.216	0.124	0.167
BioK 260		+++++	+	-	-	-	-

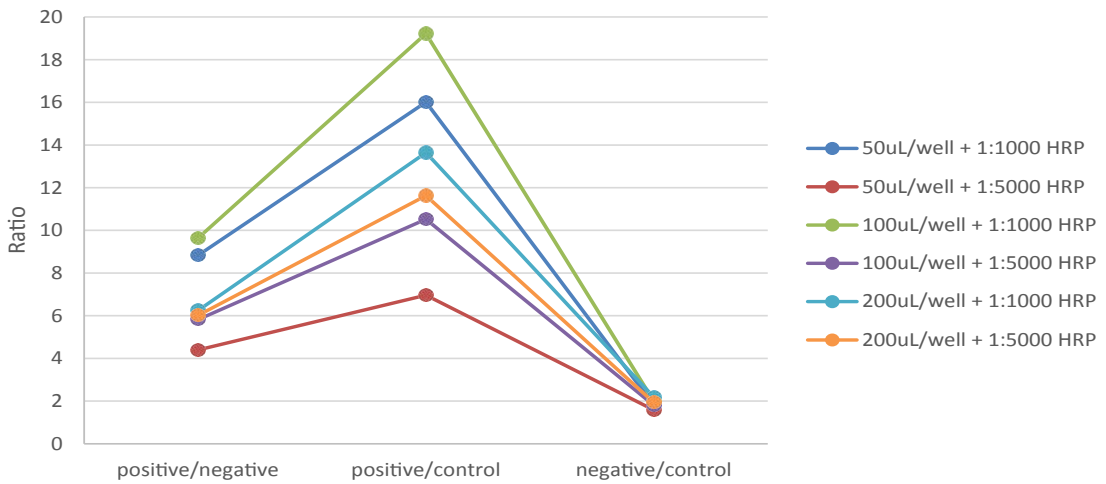

Figure 2: Evaluation of two ELISA parameters. The volume of P48 recombinant antigen per well and the diluent ratio of P48 HRP conjugate were evaluated, and the ratio of OD value between positive samples and negative samples, positive samples and control samples, negative samples and control samples were demonstrated respectively to optimize P48 DAS ELISA.

Table 2: Distribution of filed samples and the serodiagnostic background with reference kit.

Farm	Total	BioK 260	
		Negative	Positive
Litong	38	27	11
Zhongning	44	31	13
Helan	70	35	35
Zhongwei	81	48	33
Yuanzhou	19	19	0
Pengyang	30	29	1
Jingyuan	28	14	14
Xiji	30	30	0
Longde	28	28	0

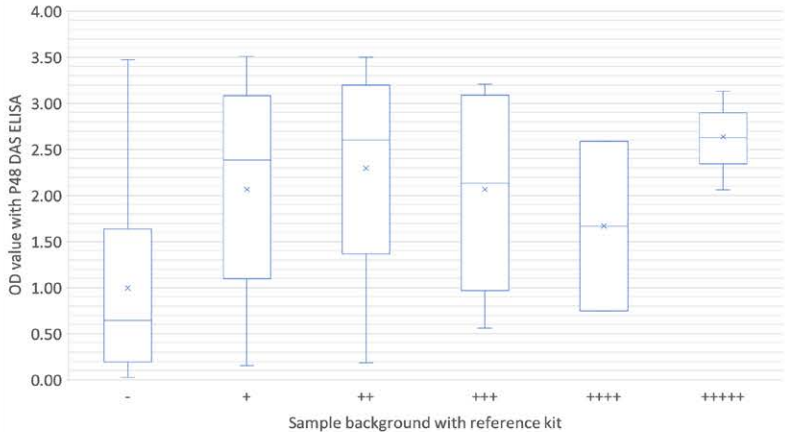

Figure 3: P48 DAS ELISA testing results distribution with the sample background. All samples were classified with background tested with reference kit, the median OD and mean OD of negative samples, "+" positive samples, "++" positive samples, "+++" positive samples, "++++" positive samples and "+++++" positive samples were 0.996, 0.647; 2.064, 2.383; 2.295, 2.599; 2.066, 2.133; 1.669, 1.669; 2.637. 2.627 respectively.

DAS ELISA was good with the ROC curve analysis, comparing with reference kit BioK 260.

In order to select the appreciate criterion for the P48 DAS ELISA test, we select some testing results as initial criterions, which are selected from the OD values tested with P48 DAS ELISA from 368 results. With each criterion, the diagnostic performance was showed in Table 3. With the OD 1.0 as P48 DAS ELISA test criterion, the diagnostic sensitivity was 83.18% and diagnostic specificity was 61.30%, meanwhile with OD 1.5 as criterion, the sensitivity and specificity were 68.22% and 72.80% respectively, and with OD 2.0 59.81% and 80.84% respectively. The accuracy was not 100% at all criterion with any criterion and the analysis with $p<0.01$. Due to

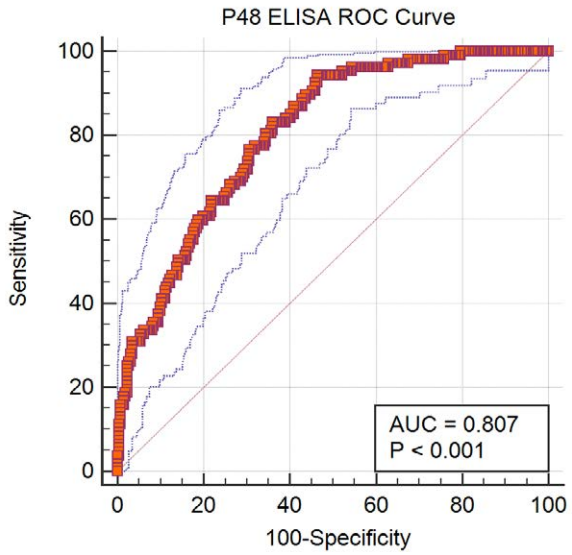

Figure 4: ROC curve of P48 DAS ELISA. Comparing with reference, the ROC curve was analyzed with MedCalc software based on the testing results of 369 field samples.

Table 3: The diagnostic performance based on different criterion.

Criterion	Sensitivity, %	Sensitivity, 95% CI	Specificity, %	Specificity, 95% CI	Coincidence rate, %	PPV, %	NPV, %	p value
1.0	83.18	74.7 - 89.7	61.3	55.1 - 67.2	67.66	46.84	89.89	p<0.001
1.5	69.16	59.5 - 77.7	72.8	67.0 - 78.1	71.74	51.03	85.2	p<0.001
2.0	59.81	49.9 - 69.2	80.84	75.5 - 85.4	74.73	56.14	83.07	p<0.001

the P48 DAS ELISA test will be used as serodiagnostic tools for the epidemiological investigation and prevention of *M. bovis* infection, NPV should be more important than PPV.

Criterion selection of P48 DAS ELISA based on epidemiological performance

The DAS ELISA will be applied as the prevalence surveillance tool and prevention tool for *M. bovis*, so the epidemiological performance should be considered. To show the testing value of BioK 260, Figure 5 showed the results, also Figure 6 showed the results of P48 DAS ELISA OD value for the samples from different farms. The trending in Figure 5 is similar as Figure 6, except samples collected from Pengyang. It was found that the median results were lower in Longde, Pengyang, Xiji, Yuanzhou than those in Helan, Jingyuan, Litong, Zhongning and Zhongwei. And the average results were lower in Longde, Xiji, Yuanzhou than those in Helan, Jingyuan, Litong, Zhongning and Zhongwei. So the epidemiological performance of P48 showed similar to reference kit basically.

To evaluate the criterion better, the number of positive results and negative results and the prevalence data of both BioK 260 and P48 DAS ELISA with different criterions were shown in Table 4. It was found that the prevalence tested was 0% with reference kit in Longde and Xiji, and the prevalence tested also was 0% with all 3 criterions for P48 DAS ELISA. In Yuanzhou samples, if OD 1.5 or 2.0 were selected as criterion, then the prevalence tested was 0% with P48 DAS ELISA, which was tested 0% also with reference kit. But with OD 1.0 there would be one positive result in Yuanzhou, and the results would made Yuanzhou as a farm with *M. bovis* circulation. With considering the prevalence tested between BioK 260 and P48 DAS ELISA, OD 1.5 should be the best criterion for the test.

With OD 1.5 as cut-off for P48 DAS ELISA, we found the positive results were 81.43%, 75.00%, 65.79%, 33.33%, 38.64% and 72.84% in Helan, Jingyuan, Litong, Pengyang, Zhongning, Zhongwei respectively, at the same time the positive results were 50.00%, 50.00%, 28.95%, 3.33%, 29.55, 40.74% in Helan, Jingyuan, Litong, Pengyang, Zhongning, Zhongwei respectively. So in the farms with *M. bovis* infected or circulation, P48 DAS ELISA could detect more animals with antibodies to *M. bovis* than reference. The capability of antibody detection is higher of P48 DAS ELISA than reference.

Performance of P48 DAS ELISA

After the criterion of OD 1.5 was selected, the development of P48 DAS ELISA was done. Comparing with reference kit, BioK 260, the AUC of ROC curve is 0.807 (p<0.001); the diagnostic sensitivity is 69.16% (p<0.001), diagnostic specificity is 72.80%, the coincide rate is 71.74%, PPV and NPV is 51.03%, 85.20% respectively, p<0.001. Considering with the epidemiologic results, there was no false positive result detected in 3 clean farms, and 6 farms with *M. bovis* circulation were detected all. So the accuracy, the diagnostic performance and the epidemiologic performance of P48 DAS ELISA is better than BioK 260, due to the epidemiological results for clean farms are the same with both test, meanwhile the detection of antibodies to *M. bovis* of P48 DAS ELISA is higher than BioK 260.

Discussion

In this study we have developed and optimized the DAS ELISA for *M. bovis*. The different principles for *M. bovis* antibody detection have been compared. Although there we some studies reported about the indirect ELISA with *M. bovis* whole cell antigen [34,35], in this

study the whole cell antigen showed cross reactivity with negative samples. Because the whole cell antigen includes various kinds of antigens, most of which is similar as other Mycoplasma species or other microorganism, the potential performance of indirect ELISA with whole cell antigen may not be used in diagnostic purpose, and some publication reported the performance of ELISA for antibodies against *M. bovis* developed using *M. bovis* whole cell antigen as the coating antigen, which resulted in greater sensitivity but less specificity [36].

To choose the appropriate antigen for ELISA is important, P48 is a good candidate for the antibody ELISA development, because P48 protein was proved to be present in proteomes of all *M. bovis* field isolates and P48 gene was detected in all field isolates also [37,38]. To double confirm the P48 protein is unique and conservative protein in *M. bovis*, we blasted the P48 gene used for our recombinant antigen, and the results showed that all 35 sequences of *M. bovis* in the GenBank database, including Ningxia-1 Whole-Genome Sequence 32, had high coverage with P48 gene used. From the analysis of Blast in Genbank, only 25 sequences of *M. agalactiae* species was found to have similar sequence of P48 Gene. Though *M. agalactiae* is similar to *M. bovis*, and the sequence of *M. agalactiae* is similar to *M. bovis* [39], *M. agalactiae* was detected and isolated from small ruminant animals, like goat and sheep [40]. With the results of ELISA tests principle evaluation, P48 recombinant antigen was proved to be a good antigen for the ELISA development.

In this study, we found many different testing results from reference kit testing. It's normal to have some different testing results, especially when 2 kits are with different antigen used. Because the immune system of animals varies, same pathogen may cause different immunoreaction, and the humoral immunity level was different for each animal, especially recent researches showed that *M. bovis* has capability to modulate the host's immune system [41,42], then the testing results with different serological kits may be different. On another hand the antigen used in the ELISA is key to the performance, due to the P48 was not used in BioK 260, there must be different from 2 test kits, due to the difference of antigen reactivity. But from Figure 6 we found that the median and mean value of P48 DAS ELISA is increased in the samples with positive reference result. The trending showed that the antibodies to *M. bovis* present in animals infected, then the serodiagnostic testing value should be increased, though some of them may not be detected. So the Figure 6 can give us a whole picture of the performance. The testing can distinguish the infected animals and healthy animals, but the individual sample testing result is not a good evaluation method for the new method, like P48 DAS ELISA in this study.

However ROC curve is an effective tool for method evaluation, and AUC is a global measure of diagnostic accuracy; AUC tells us nothing about individual parameters, such as sensitivity and specificity [43,44]. In the ROC curve of P48 DAS ELISA, we found that the AUC was 0.807 ($p<0.001$), so the accuracy of our test is good.

Table 4: Epidemiologic results of reference kit and P48 DAS ELISA with 3 criterions.

Farm	Total	BioK 260			P48 DAS ELISA, OD 1.0			P48 DAS ELISA, OD 1.5			P48 DAS ELISA, OD 2.0		
		Negative	Positive	Prevalence	Negative	Positive	Prevalence	Negative	Positive	Prevalence	Negative	Positive	Prevalence
Helan	70	35	35	50.00%	13	57	81.43%	23	47	67.14%	29	41	58.57%
Jingyuan	28	14	14	50.00%	7	21	75.00%	12	16	57.14%	14	14	50.00%
Litong	38	27	11	28.95%	13	25	65.79%	16	22	57.89%	23	15	39.47%
Longde	28	28	0	0.00%	28	0	0.00%	28	0	0.00%	28	0	0.00%
Pengyang	30	29	1	3.33%	20	10	33.33%	22	8	26.67%	24	6	20.00%
Xiji	30	30	0	0.00%	30	0	0.00%	30	0	0.00%	30	0	0.00%
Yuanzhou	19	19	0	0.00%	18	1	5.26%	19	0	0.00%	19	0	0.00%
Zhongning	44	31	13	29.55%	27	17	38.64%	32	12	27.27%	34	10	22.73%
Zhongwei	81	48	33	40.74%	22	59	72.84%	41	40	49.38%	53	28	34.57%

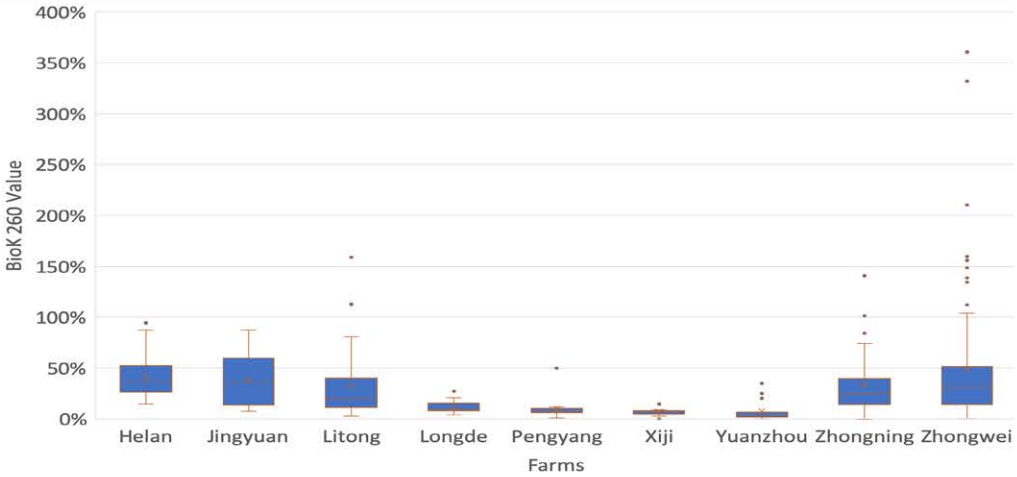

Figure 5: Testing value of reference kit in different farms. The testing value of reference kit was showed in boxplot in different farms, and the median testing value and mean testing value of Helan, Jingyuan, Litong, Longde, Pengyang, Xiji, Yuanzhou, Zhonging, Zhongwei were 41%, 37%; 39%, 36%; 33%, 20%; 11%, 8%; 8%, 6%; 7%, 7%; 8%, 4%; 33%, 25%; 49%, 31% respectively.

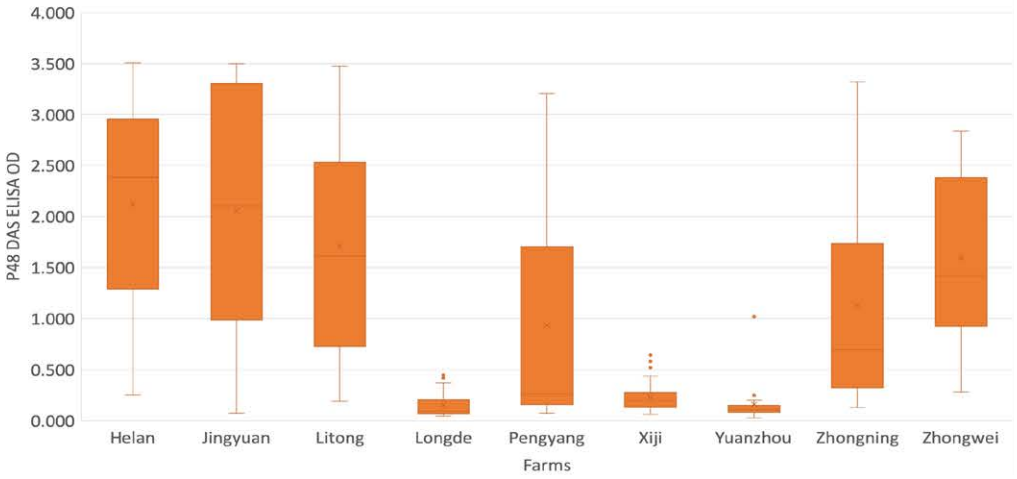

Figure 6: Testing value of P48 DAS ELISA in different farms. The OD value of P48 DAS ELISA was showed in boxplot in different farms, and the median OD value (mean OD value) of Helan, Jingyuan, Litong, Longde, Pengyang, Xiji, Yuanzhou, Zhonging, Zhongwei were 2.118 (2.387), 2.055 (2.105), 1.713 (1.611), 0.151 (0.071), 0.932 (0.259), 0.230 (0.192), 0.160 (0.105), 1.125 (0.695), 1.592 (1.422) respectively.

Furthermore, the AUC and ROC would not tell us more about the diagnostic performance. With different criterion the performance is different, and the specificity and sensitivity of course varies, so the criterion is the key element for the test development and evaluation. We selected 3 candidates of criterion, then the diagnostic performance was calculated, including sensitivity, specificity, PPV, NPV. The purpose of the test developed should be considered when we precede criterion study, for example the screen test need high sensitivity in order to detect all positive samples and prevent false negative, at the same time the prevalence testing protect should be discussed more about the specificity. In this study, the total sensitivity and specificity was concerned, at the same time the epidemiological performance must be analyzed.

No matter how individual animal's immune system was influenced, the herd with *M. bovis* circulated should be detected by effective test, because there must be some animals infected were detected. From Figure 5 and Figure 6 the trending of testing results of both reference kit and P48 DAS ELISA were similar, that the testing value of some farms was higher. In the Table 4 the detailed information can be analyzed, we found that 8 of 9 farms can be judged as same *M. bovis* circulation status with 2 tests. The results of Yuanzhou can be used for the criterion selection, since the prevalence was different with cut-off of OD 1.0 and 1.5.

If we believed that reference kit is gold standard, then OD 1.5 should be selected. When the criterion was selected as OD 1.5, 3 clean farms were tested with negative results, and 6 farms infected *M. bovis* could be detected by P48 DAS ELISA. After the criterion selection, the performance of P48 DAS ELISA could be generated totally.

The prevalence got by P48 DAS ELISA in the infected farms was higher than those got by reference kit. In 6 infected farms (Helan, Jingyuan, Litong, Pengyang, Zhongning, Zhongwei), it was found that the prevalence is high with P48 DAS ELISA. Since the *M. bovis* must circulate in these farms, so the higher prevalence of *M. bovis* can be treated as reasonable phenomenon. The case of Pengyang can be discussed more. In Pengyang there was only 1 positive animal detected with reference kit, so the Pengyang farm can be judged the infected farm. With P48 DAS ELISA, the prevalence was 26.67%, and 8 animals were detected from herd. In the same farms, more animals detected proved the better epidemiological performance of P48 DAS ELISA.

In our study, there was one sample in Yuanzhou tested with the OD between 1.0 and 1.5, so if the reference kit gave the true situation of *M. bovis* circulation, the OD 1.5 as criterion should be good. Considering there are many animals with chronic diseases or without clinical signals, there may be some carriers of *M. bovis* in Yuanzhou, so the potential risk should be identified, and with OD 1.0 as criterion, the risk can be noticed. For P48 DAS ELISA, OD 1.5 can be used as criterion for result judgement, but OD 1.0 could be some precautious point for the risk identification. With OD 1.0 the NPV will be improved, and the health herd can be identified.

Due to cattle in the farms with *M. bovis* circulation always have the chronic disease caused by *M. bovis* or no clinical signals, apparent cattle in the infectious farms present antibodies to *M. bovis*. So, the capability of antibody detection is important for serodiagnostic tests. P48 DAS ELISA detected more animals with antibodies to *M. bovis* in 6 infectious farms than reference farms, especially in Pengyang, reference kit only detected 1 animal with antibodies to *M. bovis* in 30 animals, and meanwhile P48 DAS ELISA detected 8 animals with antibodies to *M. bovis*. To identify the herd with *M. bovis* circulation, the more sensitivity of detection a testing approach have, the better serodiagnostic tool the testing approach will be.

For *M. bovis*, there is no efficient drugs and vaccine, so the method to control or prevent the infection and circulation is to improve the biosecurity level of farms, and to keep the suspected infected animals or the pathogen carriers away from the healthy heard. Diagnostic approaches will fulfill the requirements of improving biosecurity. The culture isolation and molecular diagnostic approaches rely upon demonstrating the presence of *M. bovis*, and the quality may be influence by many factors, and it's not easy to test all animals in herd, while serodiagnostic approaches, especially ELISA, are available to detect the presence of anti-*M. bovis* antibodies. The purpose of serodiagnostic approaches can identify animals which have been exposed to the pathogen, or carry the pathogen, that means the animals with high risk can be identified with ELISA. In our study, P48 DAS ELISA showed the good performance for epidemiological testing with filed samples, so our study suggests that P48 DAS ELISA

is likely to be useful for screening herds to aid in the development of enhanced biosecurity measures for *M. bovis* and also as a tool for epidemiological survey. Then the test will aid in better estimating the impact of *M. bovis* on animal welfare and productivity and the risk identification for the infection and circulation, as a tool for better understanding and for controlling this pathogen.

Conclusion

In conclusion, DAS ELISA based on P48 recombinant antigen of *M. bovis* showed good accuracy comparing with the commercial serodiagnostic kit. With the same results, the criterion of P48 DAS ELISA was very important for the diagnostic performance. After analyzing the results of 368 samples, the OD 1.5 was selected for P48 DAS ELISA, with the same epidemiological testing performance of reference kit. In the farm, which had been infected with *M. bovis*, P48 DAS ELISA showed higher detection rate than reference kit, and it will be an improved method for surveillance of *M. bovis* and a simplified screening tool for *M. bovis* prevention.

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Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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