

Establishment of an Efficient Enzyme-Linked Immunosorbent Assay for the Detection of *Eperythrozoon suis* Antibody in Swine

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ABSTRACT. The *Eperythrozoon suis* (*E. suis*) antigen was purified using a Sephadex G-200 chromatograph, and thereby, a high-affinity, specific *E. suis* antigen was collected and confirmed with Western blotting. Using this antigen, an enzyme-linked immunosorbent assay (ELISA) system to detect the antibody against *E. suis* in swine was established. There was no cross-reaction with swine sera, which were affected with *Mycoplasmal pneumonia*, swine fever, swine colibacillosis, or toxoplasmosis. A comparison of this ELISA system with an indirect hemagglutination (IHA) test using 78 swine samples revealed that the ELISA system significantly improved the sensitivity, specificity, and stability for the serodiagnosis of swine *E. suis*.

KEY WORDS: ELISA, *Eperythrozoon suis*, purified antigen.

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Although the disease caused by *E. suis* infection in swine was first observed in 1934 [6, 9, 10], there has been limited knowledge about this organism and disease. In addition, it remains uncertain whether the organism is transmitted from swine to humans and causes disease. One of the reasons for such poor understanding about this organism is the lack of appropriate methods for diagnosis. To establish a suitable and precise method to diagnose *E. suis* infection, we purified the *E. suis* antigen and developed an ELISA method. An indirect hemagglutination test (IHA) was also performed to evaluate the potential of this ELISA.

The crude *E. suis* antigen was purified using a Sephadex G-200 chromatograph as indicated in the literature [2, 5] and then applied to Western blotting as previously described [8]. *E. suis*-infected and healthy piglets were provided by the Laboratory of Veterinary Microbiology, Yanbian University, for use as donors of positive and negative sera, respectively.

The test was carried out following the conventional procedures of the ELISA method. In brief, the purified *E. suis* antigen diluted in a 0.05 M carbonate buffer (pH 9.6) was used as a capture antibody to coat microtiter plates at 4°C overnight. Positive sera, negative sera, and horseradish peroxidase (HRP)-labeled goat anti-swine IgG (IgG-HRP) (KPL Co., Gaithersburg, Maryland, U.S.A.) were used as primary and secondary antibodies. The optical density (OD) was measured with a micro-plate reader (Multiskan MK3 Type, Thermo Company, Waltham Finland) at a wavelength of 405 nm. The average OD value of a positive serum/the average OD of a negative serum (P/N ratio) was

used to determine each ELISA parameter. The detection limit was defined as a mean value of OD₄₀₅ of the 20 negative sera which had been confirmed to be *E. suis*-negative by IHA [1]. The critical value was expressed as $x \pm 3$ standard deviations (SD).

Sera of swine infected with *Mycoplasma pneumonia*, swine fever, swine colibacillosis, and *T. gondii* were tested using the ELISA to assess any cross-reaction. The sera from swine infected with *E. suis* were used as a positive control. Twelve sera from an animal farm of Yanbian University were tested with the newly established ELISA using the same or different batches of antigen in triplicate, and the mean of the coefficient of variation (C. V.) was determined to judge the precision of each antigen. To evaluate the precision of the newly established ELISA, 78 swine sera from the animal farm were analyzed by ELISA, and the results were compared with those by IHA [7, 11].

As shown in Fig. 1, the Sephadex G-200 chromatograph gave four protein peaks of the *E. suis* antigen. Each combined elution from the peaks was used for Western blotting (Fig. 2). The specific bands were at about 58 kDa and 31 kDa, and an antigen with a molecular weight of 31kDa was used as the *E. suis* antigen for ELISA in the following tests.

The specific value of P/N is at its maximum when the concentration of the antigen is 3.5 µg/ml with 2-hr incubation, the serum dilution is 1:160 with 1-hr incubation, IgG-HRP dilution is 1:4,000 with 1-hr incubation (Tables 1 and 2), and 2, 2'-amino-di(2-ethyl-benzothiazolinesulphonacacid-6) ammonium salt is the substrate solution.

E. suis-negative sera taken from 20 swine were analyzed with ELISA under the optimal conditions. The average OD₄₀₅ value was 0.098, the standard deviation was 0.019, and the 99% confidence interval was OD₄₀₅+3S=0.155. For convenience, the cutoff point of 0.16 was chosen as the critical value. To reduce false-positive and false-negative

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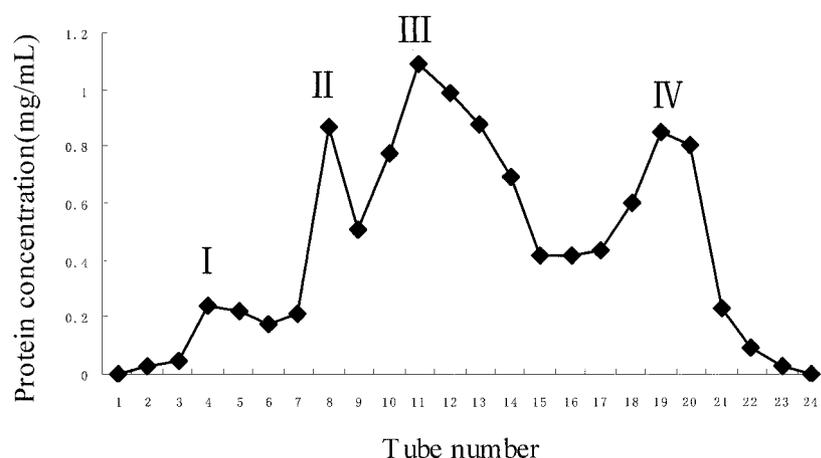


Fig. 1. The elution curve of *E. suis* soluble antigens separated using a Sephadex G-200.

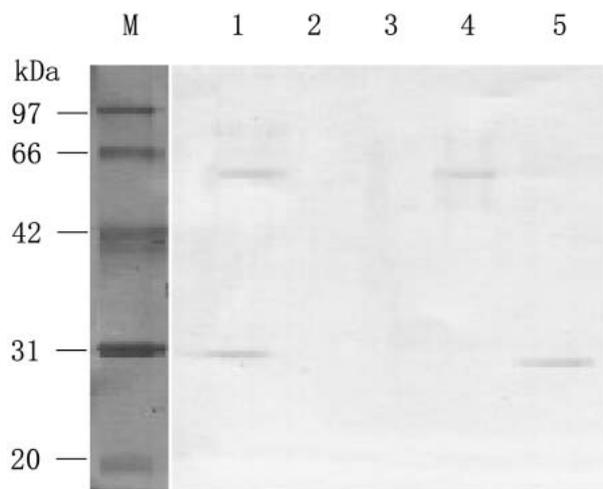


Fig. 2. *E. suis* antigens were analyzed by Western blotting. M: Marker; 1, Crude antigen; 2, 3, 4 and 5, Purified antigen of 1st, 2nd, 3rd and 4th peaks in Fig. 1, respectively. The bands at 31 kDa and 58 kDa are specific for *E. suis*.

results, a questionable range was set by adding or subtracting the standard deviation value from the critical value; in other words, an OD value of test serum ≥ 0.18 was considered as positive, an OD value ≤ 0.14 was considered as negative, and the range from 0.14 to 0.18 was considered as doubtful.

The results of a cross-reaction test indicated that the ODs of the positive sera for *Mycoplasmal pneumonia*, swine fever, swine colibacillosis, or toxoplasmosis were lower than 0.14 (data not shown).

As shown in Table 3, 78 swine sera were assessed with both ELISA and IHA. The positive rate was 82.1% (64/78) in newly established ELISA and 62.8% (49/78) in IHA. The positive rate for ELISA was 19.3 percentage points higher than that of IHA.

For the diagnosis of *E. suis*, various serological methods have been established. However, all of the antigens used for these methods have been crude but not separated and purified, which affects the precision of the diagnosis of *E. suis*. In this study, the *E. suis* antigen was dissociated from plasma by repeated ultrasonic decomposition, freezing, and thawing. Furthermore, the antigen was purified by Sephadex-200 and confirmed by SDS-PAGE and Western blot-

Table 1. Optimal concentration of antigen

Concentration of coating antigen ($\mu\text{g}/\text{ml}$)	112	56	28	14	7	3.5	1.75	0.875
Positive serum OD ₄₀₅ (P)	0.772	0.728	0.687	0.632	0.549	0.472	0.294	0.186
Negative serum OD ₄₀₅ (N)	0.132	0.121	0.112	0.087	0.071	0.056	0.039	0.028
P/N ratio	5.848	6.016	6.314	7.264	7.732	8.428	7.538	6.642

Table 2. Optimal dilution of serum

Dilution of serum	40	80	160	320	640
Positive serum OD ₄₀₅ (P)	0.654	0.547	0.465	0.377	0.231
Negative serum OD ₄₀₅ (N)	0.085	0.068	0.052	0.048	0.037
P/N ratio	7.694	8.044	8.942	7.854	6.243

Table 3. Results of ELISA and IHA for 78 sera from swine

ELISA	IHA		
	Positive	Negative	Total
Positive	62.8% (49/78)	19.3% (15/78)	82.1% (64/78)
Negative	0% (0/78)	17.9% (14/78)	17.9% (14/78)
Total	62.8% (49/78)	37.2% (29/78)	100% (78/78)

ting. The present results indicated that the purified antigen gave high precision of diagnosis for *E. suis* infection. The best concentration of this antigen for the ELISA was 3.5 µg/ml, which was a 0.09-fold concentration of that used by Huiying *et al.* [3] and a 0.12-fold concentration of that used by Hsu *et al.* [4]. In addition, there was no cross-reactivity with the sera from swine infected with *Mycoplasmal pneumonia*, swine fever, swine colibacillosis, or toxoplasmosis.

Seventy-eight serum samples were analyzed by ELISA and IHA. The results indicated that the sensitivity of ELISA was 19.3% higher than that of IHA. Hsu *et al.* set $OD_{492} \geq 0.121$ as a positive reference line [4], but in this study, $OD_{405} \geq 0.18$ was considered as positive and $OD_{405} \leq 0.14$ as negative, and the range of 0.14–0.18 was considered as doubtful.

The present ELISA method using purified antigens significantly improved the sensitivity, specificity, and stability, and therefore, it is a suitable method for detecting *E. suis* antibodies in swine. Furthermore, it will be effective for controlling the epidemics of this disease.

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