Full Length Research Paper

A novel approach for very early pregnancy diagnosis in swine by anti-early pregnancy factor (EPF) antiserum blocking enzyme-linked immunosorbent assay (ELISA)

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Early pregnancy factor (EPF) is essential for the initiation and maintenance of pregnancy. Early pregnancy factor activity monitoring has been reported to be the effective method for very early pregnancy diagnosis. In this study, three BALB/c mice were immunized with the synthetic peptide segment corresponding to the amino acid sequence 36 to 55 of EPF (IG_{20}) for anti-EPF antibodies. Mouse anti-EPF antiserum titers were evaluated by an indirect enzyme-linked immunosorbent assay (ELISA), and the titers were 6.4×10^3. Serum samples were taken from 21 Yorkshire × Landrace crossbred sows (12 pregnant and 9 non-pregnant). The presence of EPF in these serum samples was detected by a blocking ELISA using the antigen-antibody (Ag-Abs) reaction between IG_{20}-ovalbumin and mouse anti-EPF antiserum for very early pregnancy diagnosis, blank was used as negative controls. The optical density (OD) values were measured at 450 nm, and the OD ratios of negative control/serum sample (N/S) >2.1 were considered positive, and N/S <2.1 negative. When the test serum samples were in 1/4 dilutions with PBS, twelve samples from pregnant swine were positive, nine non-pregnant serum samples were negative. Very early pregnancy can be determined by using the mouse anti-EPF antiserum blocking ELISA in swine.

Key words: Very early pregnancy diagnosis, early pregnancy factor (EPF), Rosette inhibition test (RIT), blocking enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Early and accurate diagnosis of pregnant sows and gilts has potential economic advantages by reducing non-productive days and increasing reproductive efficiency in pig production. Currently, the porcine pregnancy diagnosis is mainly through the detection of return to estrus (Almond et al., 1986, 1987), as well as the scan of embryo with amplitude-depth (A-mode) (Pyorala, 1989), Doppler and Real-time (B-mode) ultrasonography (RTU) (Zambelli et al., 2006; Williams et al., 2008). However, these methods are only effective after 21 or 24 days of gestation (Krüger et al., 2002), and could not be used for

very early pregnancy diagnosis (in pigs conceptuses 7 to 15 days) in sows (Shaw et al., 1980). It is necessary to establish a fast and efficient method for very early pregnancy diagnosis in sows.

Early pregnancy factor (EPF) is a pregnancy-associated protein, which was firstly detected by rosette inhibition test (RIT) in mice serum as early as 4 h after mating (Morton et al., 1974). Early pregnancy factor has been shown to be essential for the initiation and maintenance of pregnancy, and it was assayed by the RIT through the decreasing ability in the formation of rosettes (Greco et al., 1992; Straube et al., 1989). The activity of EPF has been detected in maternal serum within 24 h of fertilization in all mammalian species tested and persisted for at least the first half of gestation (Morton et al., 1987), and EPF activity monitoring (RIT) has been reported to be the only method suitable for very

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Abbreviation: EPF, Early pregnancy factor.

early pregnancy diagnosis (Straube et al., 1988; Mesrogli et al., 1988). However, RIT was not a quantitative assay and has been proved cumbersome-and time-consuming (Koch et al., 1985).

The amino acid sequence of EPF is fully identical to the extra-cellular form of chaperonin10 (cpn10) (Alice et al., 1996; Morton, 1998), human, bovine and swine share the same sequence of EPF (Englebretsen et al., 2002; Fotini et al., 2003). In the present study, we have undertaken production of anti-EPF antibodies to synthetic peptide segment corresponding to the amino acid 36 to 55 of EPF (IG_{20}). Serum samples were collected from 12 pregnant and 9 non-pregnant sows, the presence of EPF in these sera samples was detected by a blocking enzyme-linked immunosorbent assay (ELISA) using the antigen-antibody (Ag-Abs) reaction between IG_{20} and mouse anti-EPF antiserum, then, the optical density (OD) values were measured at 450 nm, and pregnancy was determined by the OD ratios of control/serum sample (N/S) (Loffeld et al., 1989; Shu et al., 2011; Xie et al., 2007).

MATERIALS AND METHODS

Preparation of serum samples

The blood samples (each 2 ml) were taken from the ear vein of 21 Yorkshire × Landrace crossbred sows (12 pregnancies and 9 non-pregnancies) after second parity. Twelve blood samples of pregnant sows were collected on the sixth day after artificial insemination (AI), and pregnancy was confirmed by B-ultrasound after the 40 days of AI. Nine blood samples of non-pregnant sows were collected on the sixth day after estrus (no insemination). These samples were allowed to clot for about 1 h at room temperature, and the sera were separated by centrifugation at 1 000 ×g for 30 min, and deactivated at 56°C for 30 min, then stored at -20°C until testing.

Rosette inhibition test

The human lymphocytes were collected from one of the lymphocyte donors. Lymphocytes were separated through a gradient centrifugation by the Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA) method and processed according to the manufacturer's instructions. The isolated lymphocytes were adjusted to 4×10^6 cells per ml with Hanks balanced salt solution (Hanks', pH7.4, Ca^{2+} and Mg^{2+} free). Sheep red blood cells (sRBC) were obtained from fresh defibrinated sheep blood according to the description of Ghaffari et al. (2008), and sRBC were adjusted to a final concentration (1×10^8 cells per ml) with Hanks'.

Rosette inhibition test was performed as established by Ghaffari et al. (2008) and Athanasas-Platsis et al. (2000) with minor modification. Serum samples of each pig (100 µl) were mixed with human lymphocytes suspensions (500 µl) and incubated at 3°C for 60 min. The reaction solutions were divided into twelve equivalents (50 µl each sample), then 100 µl serially diluted ($1 : 2^1 \times 10^3$, $1 : 2^2 \times 10^3$, to $1 : 2^{12} \times 10^3$) rabbit antihuman-lymphocyte globulin (ALG) were added, and incubated at 37°C for 60 min, washed 3 times with Hanks', respectively. Fifty micolitres sRBC were added and centrifuged immediately at 800 ×g for 5 min. Then one 100 µl 0.8% glutaraldehyde was added to fix the lymphocytes and erythrocytes. Over 200 lymphocytes and rosettes were counted and recorded.

Each sample was repeated three times.

The number of rosettes formed was determined and expressed as percentage of the number of rosettes formed without ALG. Results are recorded as rosette inhibition titers: the highest dilution of ALG [$\log_2$ (reciprocal dilution $\times 10^{-3}$)] to give rosette formation of <75% (samples without ALG = 100% rosette formation).

Preparation of immunogens

A peptide segment (IMLPEKSQGKVLQATVVAVG, IG_{20}) corresponding to the amino acid sequence 36 to 55 of EPF (accession no. NP776771) was synthesized on 0.3 mmol/g Fmoc-wang resin (GL Biochem Ltd., Shanghai, China) using a Symphony® Multiplex Peptide Synthesizer (Protein International Technologies, Inc., St. Louis, MO, USA) (Ahlborg, 1995). A cysteine residue was added at the N-terminal of the peptide segment to facilitate conjugation. The peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) coupled to pneumatically assisted electro spray ionization mass spectrometry (ESI-MS) (Quattro. Micro™ Api, Waters Corp., Milford, MA, USA) with the solid-phase purification over 95% (Sanz-Nebot et al., 2001).

The synthetic peptide was conjugated to a carrier protein of IgG-free bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) using a hetero-bifunctional cross-linker succinimidyl 4-(Nomaleinimido-methyl) cyciohexane-l-car-boxylate (Sulfo-SMCC, Pierce Biotechnology Inc., Rockford, IL, USA) according to the method described by Alexander et al. (1984). Briefly, 4 mg of BSA was dissolved in 0.5 ml phosphate buffered solution (PBS, 0.1 M sodium phosphate, 0.15 M sodium chloride, 0.1 M EDTA, pH 7.2), then mixed with 1 mg sulfo-SMCC dissolved in 50 µl DMSO. The mixture was kept at room temperature for 30 min, and then the buffered solution dialysis was conducted at 4°C overnight. The dialysis fluid was changed three times to remove excess coupling agent. The SMCC-activated BSA (SMCC-BSA) solution was adjusted to a concentration of 5 mg/ml with PBS and stored at -20°C. Two milligrams of IG_{20} were dissolved in 50 µl dimethylformamide (DMF) and added 150 µl conjugation buffer (5 mM EDTA buffered solution 0.01 M PB, pH 7.2). Thereafter, 10 mg/ml of IG_{20} was mixed with SMCC-BSA and kept at room temperature for 30 min, to have the peptide segment conjugated to carrier protein to form the immunogen IG_{20}-BSA. The IG_{20}-BSA was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Ovalbumin (OVA, Sigma Chemical Co., St. Louis, MO, USA) was conjugated to IG_{20} (IG_{20}-OVA, coating antigen) as the similar procedure described earlier.

Preparation of mice antiserum to EPF

Three BALB/c mice (female, 8 weeks of age) were immunized with IG_{20}-BSA conjugates. The first dose consisted of 50 µg of IG_{20}-BSA conjugates for injection subcutaneously as an emulsion of PBS and Freund's complete adjuvant (FCA, Sigma Chemical Co., St. Louis, MO, USA). The boosters were given at two weeks intervals with the same dosage of IG_{20}-BSA conjugates emulsified in Freund's incomplete adjuvant (FIA, Sigma Chemical Co., St. Louis, MO, USA) (Ebensen et al., 2007).

Blood samples (20 µl) were collected from tail veins 14 days after third immunization. Anti-EPF antibody titer was determined by the indirect ELISA using the following procedure (Kit et al., 1990; Sun et al., 2007). 96 well ELISA plates (Costar) were coated with 1 µg/ml IG_{20}-OVA diluted in carbonate buffer, pH 9.6, in a 100 µl per well and incubated overnight at 4°C. Thereafter, the plates were washed four times with PBST and repeat between each of the following steps, and blocked with 5% chicken serum at 37°C for 1 h, 50 µl 1/100 serial diluted anti-sera were added to the blocked plates and
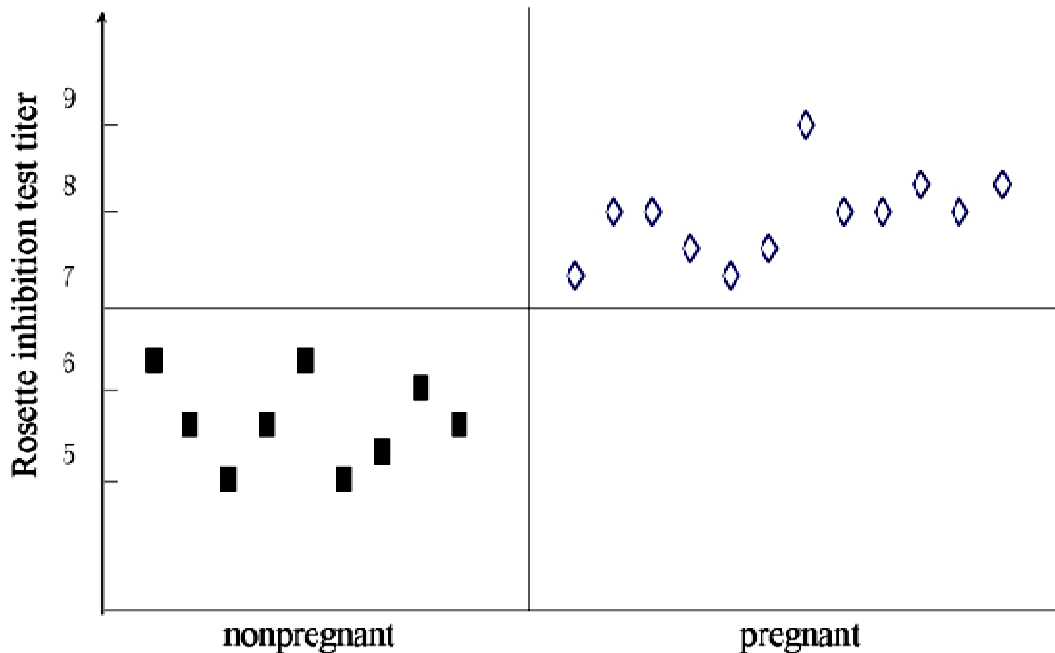
Figure 1. The Rosette inhibition titers (RIT) range of pregnant and non-pregnant porcine serum samples. The Rosette inhibition titers (mean ± SD). Pregnant ($\diamond$): 8.0 ± 0.5**, non-pregnant ($\blacksquare$): 6.0 ± 0.4. ** was P < 0.01 (t-test) between pregnant and non-pregnant serum samples.

incubated at 3 °C for 15 min, then 50 µl 1000-fold diluted horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Abnova Corp., Heidelberg, Germany) was added and again incubated at 37°C for 30 min. The substrate-chromogen solution (TMB-H_2O_2) was added, and the OD (450nm) value was measured using a micro-plate reader. 50 µl 1 µg/ml OVA was used as negative controls and test was repeated three times.

Blocking ELISA detection of EPF presence in serum samples

The procedures of blocking ELISA was conducted as previously described (Kit et al., 1990; Sun et al., 2007). ELISA plates were sealed with 5% chicken serum. Hereafter, 50 µl of each porcine sera sample (9 non-pregnant and 12 pregnant sows, blank group served as negative control) were serially diluted (1/2 to 1/64) with PBS, then mixed with 50 µl 800-fold diluted mice anti-EPF sera in each well, and incubated at room temperature for 30 min. The mixed solutions were transferred to another ninety-six well ELISA plates which were coated with 100 µl 0.5 µg/ml IG_{20}-OVA and sealed with 5% chicken serum, incubated at 37°C for 15 min. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was added, incubated at 37°C for 30 min, TMB-H_2O_2 was added and the OD value at 450 nm was measured by micro-plate reader. The test was repeated three times.

Statistical analysis

Rosette-inhibition titers of 9 non-pregnant and 12 pregnant serum samples were analyzed using the SPSS Statistics 17.0 ANOVA and t-test. The OD values of mouse anti-EPF antiserum blocking ELISA in serials diluted samples (nine non-pregnancies, twelve pregnancies and negative controls) were analyzed using the SPSS Statistics 17.0 ANOVA.

RESULTS

The rosette-inhibition test titers

Early pregnancy factor activities of 9 non-pregnant and 12 pregnant porcine serum samples were tested by RIT using human lymphocytes. There were significant differences between pregnant and non-pregnant sera (P<0.01) (Figure 1).

Evaluation of mice antiserum responses to EPF

The conjugations (IG_{20}-BSA and IG_{20}-OVA) were identified by SDS-PAGE. The calculated molecular combined ratios of IG_{20}/BSA and IG_{20}/OVA were 15:25 and 5:15 (Figure 2), respectively. Three BALB/c mice were immunized with IG_{20}-BSA, and the antibody responses to EPF were measured by indirect ELISA. The serum anti-EPF antibody titers were 1:6.4 × 10^3 (Figure 3).

Evaluation of blocking ELISA

When the test serum samples were diluted 1/4 with PBS, the OD ratios of negative control/serum sample (N/S) were over 2.1 (positive) in 12 pregnant serum samples, and was less than 2.1 (negative) in nine non-pregnant serum samples (Loffeld et al., 1989; Shu et al., 2011; Xie
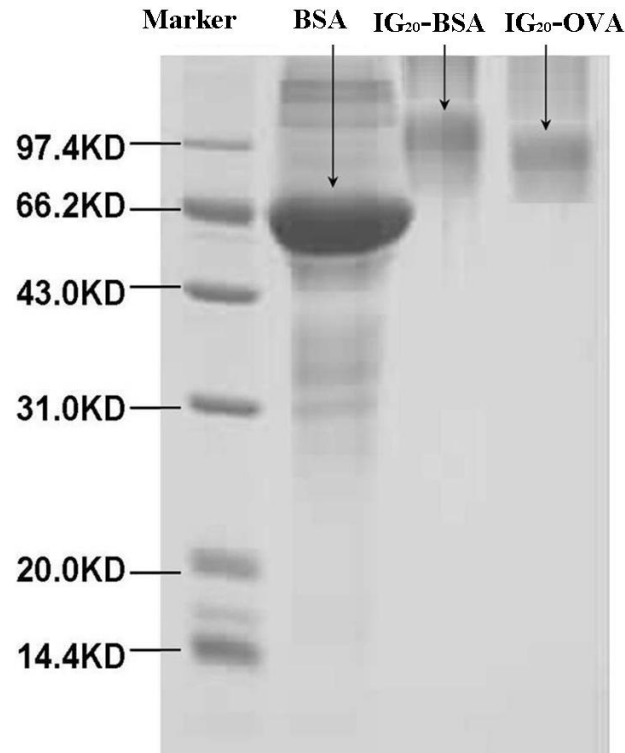
Figure 2. The SDS-PAGE analysis of conjugated products. The molecular weight of IG_{20}-BSA ranged from 97.4 to 110 kDa, and IG_{20}-OVA was from 66 to 97 kDa.
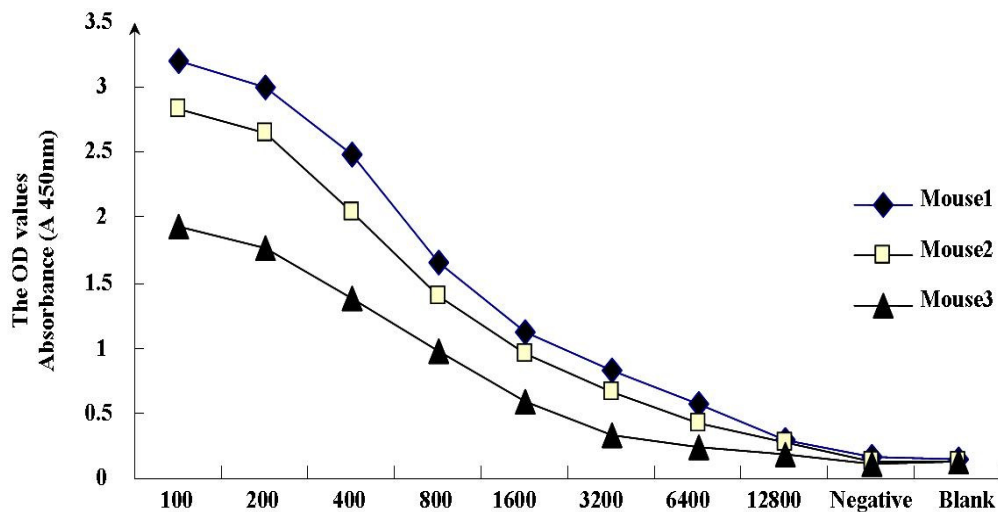

Figure 3. The titers of anti-EPF serum antibody. When the OD values of anti-EPF serum antibody were 2.1 times negative, they were positive; and hence negative.

et al., 2007). However, when the sera were 1/2 diluted, six of the nine non-pregnant serum samples were presented as a false-positive. The serum samples were 1/8 dilution or more, the non-pregnant and partly pregnant serum samples showed negative (Figure 4 and Table 1).

DISCUSSION

Accurate and early detection of pregnant and non-pregnant sows has become a key to good breeding management because it is an essential factor for monitoring and controlling fertility in pigs. In commercial
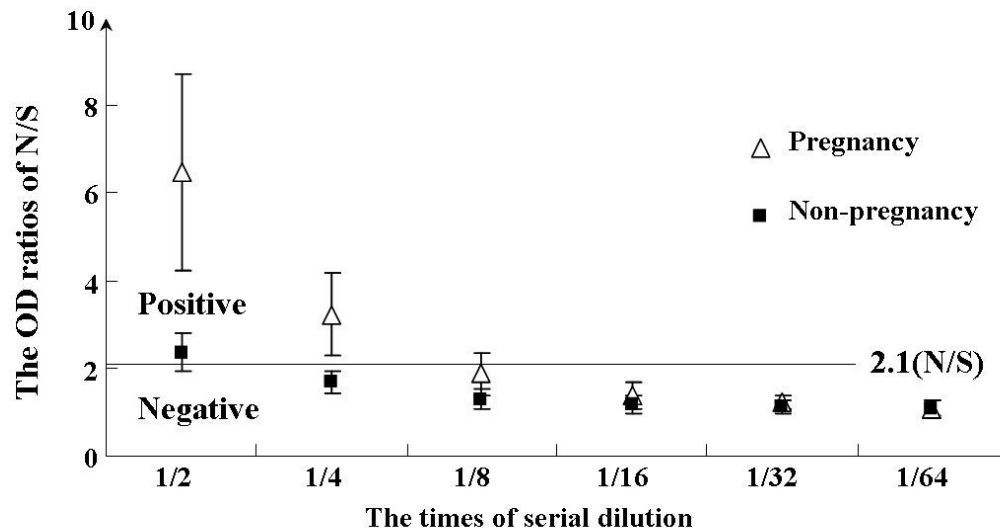
Figure 4. The OD ratios of N/S (negative control/porcine serum sample). N/S > 2.1 were considered positive (+), and N/S value < 2.1 negative (-).

Table 1. The OD values of mouse anti-EPF antiserum blocking ELISA using the serum samples of serial dilution (the mean values of 3 times repeated for each sample).

Parameter	Number	Diluted time of sows' serum sample						Negative
		1/2	1/4	1/8	1/16	1/32	1/64	
Pregnancy	1	0.258*	0.380*	0.814	1.102	1.135	1.132	1.300
	2	0.146*	0.397*	0.468	0.561	0.562	0.874	0.772
	3	0.145*	0.183*	0.482	0.696	0.722	0.698	0.919
	4	0.148*	0.298*	0.692	0.52	0.644	0.897	0.735
	5	0.098*	0.440*	0.610*	0.704	0.835	1.021	1.321
	6	0.114*	0.297*	0.544	0.663	1.061	0.574	0.565
	7	0.125*	0.183*	0.388*	0.697	0.782	0.666	0.932
	8	0.188*	0.268*	0.792	0.631	0.844	0.837	0.766
	9	0.135*	0.480*	0.714	0.704	0.931	1.031	1.032
	10	0.146*	0.357*	0.631	0.769	0.665	0.672	0.874
	11	0.135*	0.283*	0.566	0.893	0.721	0.789	0.728
	12	0.178*	0.252*	0.591	0.722	0.746	0.863	0.639
Non-pregnancy	1	0.321*	0.438	0.603	0.794	0.761	0.862	0.835
	2	0.342*	0.533	0.692	0.834	0.996	0.822	0.921
	3	0.465	0.612	0.772	0.865	0.82	0.944	0.965
	4	0.372*	0.552	0.685	0.903	0.804	0.835	0.932
	5	0.467*	0.631	0.782	0.734	0.863	1.061	1.166
	6	0.494	0.611	0.612	0.734	0.892	0.782	1.032
	7	0.322*	0.461	0.722	0.776	0.7312	0.844	0.874
	8	0.479	0.490	0.694	0.811	0.904	0.931	0.728
	9	0.351*	0.589	0.731	0.921	0.869	0.665	0.939

* The OD values < 2.1 times of negative, means positive.

swine farms, the most common strategy for identification of non-pregnant females is detection of estrus via daily boar exposure from 17 to 23 days after breeding, followed by the use of ultrasound between 28 and 45 days of gestation (Almond et al., 1986; Zambelli et al., 2006). Although, this regimen is often used with a high

degree of accuracy, its usefulness is compromised by the inability to determine pregnancy status within 15 days after breeding (Shaw et al., 1980). Of course, the measurement of human chorionic gonadotrophin (hCG) was widely used for very early pregnancy diagnoses in human (Thomas et al., 1986), and the plasma hormone (including prostaglandin-F2, hCG, progesterone and estrone sulfate) concentrations have been reported in very early pregnancy diagnoses in swine (Almond et al.,1986; Krüger et al.,2002), these plasma hormones concentrations had obvious changes from a non-pregnant to pregnant swine, but there was no statistical difference due to the high variation of the hormone levels between individuals (Almond et al., 1987). Hence, these hormones were not having the cut-off value as a neutral standard concentration range for identified non-pregnant or pregnant sows. The use of hormones as a diagnostic method may not be applicable in commercial units because of the accuracy in reducing the test.

The earliest specific indication for fertilization and the continuing presence of a viable conceptus is a serum constituent, which was originally detected in mice (Morton et al., 1974). This substance is known as the EPF and has also been described in pigs (Greco et al., 1992, Koch et al., 1985). It has early appearance (within hours) after mating or insemination (Shaw et al., 1980), and rapid disappearance following induced death or removal of the embryos (Morton et al., 1987).These factors suggest that EPF may be the most useful tool for very early pregnancy diagnosis. The objectives of this study were to evaluate the effectiveness of the EPF test for detecting the pregnant sows, and to compare the reliability of RIT and anti-EPF antiserum blocking ELISA for very early pregnancy diagnosis.

The rosette inhibition titers of pregnant sows were significant higher than in non-pregnant gilts. This was consistent with the report of Koch (1985). The RIT test was based on the ability of ALG to inhibit the formation of spontaneous rosettes between human lymphocytes and sRBC. This means that the lymphocytes are spontaneously form rosettes, a flower-like arrangement in which a lymphocyte has several red blood cells attached to it. Lymphocytes from pregnant animals form fewer rosettes than those from non-pregnant animals. Therefore, with the aid of EPF diagnostics, a pregnancy can be detected at a very early stage. However, rosette inhibition test is not a quantitative assay, and the assay is not suitable for high-throughput diagnostic and commercial applications, and RIT proved cumbersome, time-consuming and was easy to disturb with a particular disadvantage which is the indirect character of the test and its possible interference by other substances (Koch et al., 1985). To replace the RIT with a more user-friendly assay, we developed an anti-EPF antiserum blocking ELISA for very early pregnancy diagnosis.

The production of anti-EPF antibody is indispensable for very early pregnancy diagnosis by anti-EPF antiserum blocking ELISA. To obtain anti-EPF antiserum of high titer and specificity, the antigenic peptide (36 to 55, IG_{20}) based on prediction of DNAMAN has been synthesized. Three BALB/c mice were immunized by IG_{20}-BSA, and the mouse anti-IG_{20} antiserum titers were 6.4×10^3. Using anti-IG_{20} antiserum, a blocking ELISA was constructed for very early pregnancy diagnosis in sows. When the porcine sera samples were 1/4 dilution, the pregnancy can be accurately determined (Figure 4). The concentration of EPF efficiently blocked the Ag-Abs reaction between IG_{20}-OVA and mouse anti-EPF antiserum by a blocking ELISA in pregnant serum samples. At the same time, the non-pregnancy ones were not obviously influenced by the OD values of blocking ELISA. The ones with pregnancy were positive and non-pregnant serum samples were negative, the pregnancy can be determined by the anti-EPF antiserum blocking ELISA.

However, some homologous substances of IG20 (HS-IG20) were in the porcine serum samples (Betsou et al., 2003), when the serum samples were 1/2 dilution, with the concentration increases of HS-IG20, the cross-reaction was enhanced between mouse anti-EPF antiserum and HS-IG20 (Leder et al., 1994), and it was blocking the reaction between antibody and antigen (the coating material, IG20-OVA). So, some non-pregnant serum samples were presented as false-positive. On the contrary, when the porcine sera samples were 1/8 to 1/64 dilution, the concentration of EPF was limited in serum samples, and it could not efficiently block the Ab-Abs reaction between IG20-OVA and mouse anti-EPF antiserum by blocking ELISA, the presence of EPF in these serum samples were not detected.

In conclusion, the synthetic segment including the residue 36 to 55 of EPF can be used as an antigen to produce the anti-EPF polyclonal antibody. Using this antibody, we developed an anti-EPF antiserum blocking ELISA for very early pregnancy diagnosis. When the test serum samples were in 1/4 dilutions, the very early pregnancy can be detected in sows.

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