# Anti Early Embryonic Protein (EEP) for Pregnancy Test by Microtiter Strip in Dairy Cows

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# Anti Early Embryonic Protein (EEP) for Pregnancy Test by Microtiter Strip in Dairy Cows

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**Abstract**

The purpose of this study was to identify and isolate of EEP from cotyledons of pregnant dairy cow for detecting early pregnancy by microtiter strip method in post-inseminated cows at 28 day, including confirmation by serum progesterone and utrasonography. The EEP with a molecular weight of 59.88 kDa could induce the onset of anti-EEP, which can be used for pregnancy test. The EEP microtiter strip test with blood serum progesterone and optimum USG levels was found sensitivity and specificity, as well as had positive predictive values and negative predictive values of 93.33%, 100%, 100% and 83.33%, respectively.

**Keywords** : early embryonic protein (EEP), Pregnancy test, Microtiter strip, Dairy cow

An early pregnancy diagnosis is a necessary step for identification to reduce the loss of production time as a result of infertility. This can be conducted in two ways: specifically by detecting specific substances contained in the parent's blood such as Early Embryonic Protein (EEP) (El-Amiri *et al*., 2004) and by detecting non-specific substances in blood, urine or milk during pregnancy such as progesterone, estrone sulphate (Hafez, 2000).

## Materials and Methods

The method of this research was used 6 stages : 1. Isolation EEP from cotyledon 60 days pregnant cattle and characterization by SDS-PAGE method, 2. Purification with Elution method (Hermadi *et al*., 2018), 3. The making anti EEP in 6 male rabbits were injected subcutaneously with 150 µg of EEP isolates in *complete adjuvant Freunds*. The next booster was carried out 4$^{th}$ weeks after the initial injection with 100 µg of EEP isolates in *Freund's incomplete adjuvant*. 5 cc of blood was taken from each rabbit after 4$^{th}$ week, 4. The anti EEP was analyzed using indirect ELISA, 5. Anti-EEP was purificated using the Saturated Ammonium Sulphate (SAS) method, 6. The use of Anti-EEP for cow pregnancy diagnosis by microtiter strips method were designed based on the ELISA sandwich in post inseminated cows at day 28 including confirmation by serum progesterone by enzyme Immunoassay (EIA) and USG at 30$^{th}$ day after insemination.

## Results and Discussion

After isolation and identification of EEP protein from cotyledon of pregnant dairy cows with immunohistochemistry, SDS-PAGE and western blotting, the molecular weight of EEP protein 59.88 kDa was obtained, as shown in Fig 1.

The results of the examination of EEP protein levels after artificial insemination can be seen in Table I. The results of the pregnancy examination using an USG can be seen in Fig 2.

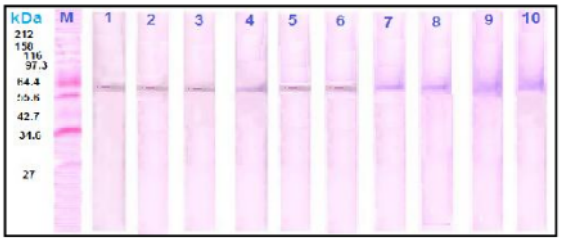


**Fig 1**. Results of Western Blot EEP Protein in Pregnant Dairy Cattle Cotyledons

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**Table I**. EEP protein levels of blood in serum dairy cow after artificial insemination.

| Cow | Days after Artificial Insemination | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 7 | | 14 | | 21 | | 28 | |
| | Abs | PL | Abs | PL | Abs | PL | Abs | PL |
| 1 | 0.313 | 6260 | 0.470 | 9400 | 0.783 | 15660 | 0.563 | 11260 |
| 2 | 0.346 | 6920 | 0.519 | 10380 | 0.692 | 13840 | 0.554 | 11080 |
| 3 | 0.332 | 6640 | 0.532 | 10640 | 0.796 | 15920 | 0.598 | 11960 |
| 4 | 0.257 | 5140 | 0.463 | 9260 | 0.720 | 14400 | 0.514 | 10280 |
| 5 | 0.284 | 5680 | 0.568 | 11360 | 0.738 | 14760 | 0.483 | 9660 |
| Average | 0.306 | 6128 | 0.510 | 10208 | 0.746 | 14916 | 0.542 | 10848 |

Abs: Absorbance; PL: Protein Level (µg / ml)

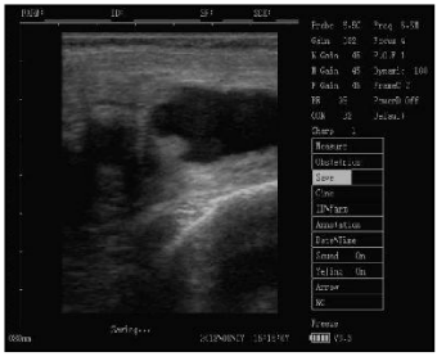

**Fig 2**. Pregnancy ultrasound results in 28-day pregnant dairy cow.

The results of the pregnancy diagnosis in dairy cows using the microtiter strip method, blood serum progesterone and ultrasound, were compared using the validity test (sensitivity and specificity) as shown in Table II.

The western blot results indicate that EEP molecules specifically bind to anti-EEP as primary antibodies and anti-rabbit IgG as secondary antibodies. Anti-EPF and anti-rabbit IgG could recognize EEP protein as a band with a molecular weight of 59.88 kDa. The protein band was in accordance with molecular weight of early embryonic protein (EEP), known as Pregnancy Associated Glycoprotein (PAG) in ovine placenta. 60-100 days of gestation found by El-Amiri *et al*. (*loc cit*) ranged from 55 to 66 kDa, whereas Garbayo *et al*. (1998) found the molecular weight of PAG in goat placenta was 55 kDa, 59 kDa and 62 kDa.

Austyn and Wood (1993) stated that to produce antibodies, repeat immunization can be carried out in rabbits. The lowest anti-EEP titre was obtained in week 5, which was increased after the booster. This was in line with the findings of Darnell *et al*. (1990) who stated that over a period of 2 weeks after injecting antigens, the concentration of IgG in serum began to rise, and quickly increased in the third week after the booster.

Pregnancy diagnosis with EEP microtiter strips were carried out at day 28 day after artificial insemination. To strengthen the success of the pregnancy diagnosis, blood serum progesterone levels were measured, and an

**Table II**. Pregnancy Diagnosis of Dairy Cows with EEP Strip Microtiter at 28 Post-IB Days and Progesterone Levels and USG at 28 Post-IB Days

| Progesterone | USG | Results of Strip Microtiter | |
|---|---|---|---|
| | | Yellow (pregnant) | Colorless (Not pregnant) |
| Pregnant | | 14 | 1 |
| Not Pregnant | | 0 | 5 |
| Total | | 14 | 6 |

*Sensitivity*: 14:15 = 93.33%; *Specificity*: 5:5 = 100%

*Positive predictive value*: 14: 14 = 100%; *Negative predictive value*: 5: 6 = 83.33%

ultrasound was carried out. At day 28 after artificial insemination, the results of each of the 14 microtiter strips from 20th cows were positive for pregnancy. Xie *et al.* (1996) detected EPF in goats three weeks into the pregnancy. EPF concentration was reported to reach optimal before birth. Gonzales *et al.* (2000) found that the goat EPF concentration reached a maximum at 8 weeks gestation and began to decline over the period of 12-14 weeks, which remained the constant until delivery. Meanwhile, Vandaele *et al.* (2005) succeeded in detecting this substance at three to four weeks of the pregnancy, the concentration of EPF was directly proportional to the breed, fetus number and weight, while the age of the parent and sex of the fetus did not affect the EPF content. It was also reported that EPF concentrations began to decline dramatically after four weeks post partum.

The results of the progesterone blood serum testing on 28 dairy cows after artificial insemination concluded that 15 of the sample had progesterone levels > 1 ng / ml and 5 tails had a progesterone level <1 ng / ml. The progesterone levels of the pregnant dairy cattle obtained in this study was between 7.18 - 7.75 ng / ml.

*Early embryonic protein* during pregnancy plays a role in maintaining the *corpus luteum* by stimulating the production of prostaglandin E2 (PGE2). Prostaglandin E2 will increase cAMP synthesis so that the progesterone production in the corpus *luteum* will also rise during pregnancy (Karen *et al.*, 2001).

The results of the blood serum progesterone test, ultrasound and EEP Microtiter strips revealed sensitivity, specificity, positive predictive value and negative predictive value at rates of 93.33%, 100%, 100% and 83.33% respectively, for pregnancy.

## Summary

The conclusion, Pregnancy testing with a microtiter strip, 28 days after artificial insemination is considered both valid and accurate because during that period of time, the placenta is formed, hence the EEP protein can be released into the maternal peripheral blood. It is therefore detected with an anti-EEP in the strip microtiter.

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