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Acknowledgement Letter # 78/19

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ACKNOWLEDGEMENT

Reg. No: 78/19 Dated : 8/03/2019

Dear Dr. Erma Safitri,

We acknowledge the receipt of the following articles entitled “Anti Early Embryonic Protein (EEP) for The First Pregnancy Test in Microtiter Strip in Dairy Cow” (Erma Safitri, et al.).

For any further correspondence, please always quote the Registration Number of the Article.

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Article # 78/19 for revision & Referee comments & IVJ revised guidelines attached

From: Ind Vet Journal (ivj83@yahoo.com)

To: rma_fispro@yahoo.com

Date: Sunday, March 31, 2019 at 09:50 PM GMT+7

Sir / Madam,

Revise the paper according to the referee's comments and corrections marked on the manuscript and resubmit the revised article as per IVJ format for further process.

Sincerely

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THE INDIAN VETERINARY JOURNAL

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Dr. S. SUKUMAR
MANAGING EDITOR

No.11, Chamiers Road, Nandanam
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ARTICLE NO: 78/19

Date : 26.3.19

Author is requested to note :

- Revise the paper according to the referee's comments and corrections marked on the manuscript.
- Return the original manuscript and the referee's comments sent herewith.
- Resubmit the revised article as per IVJ format – one hard copy and one soft (CD) for each article separetely.

EDITOR's COMMENTS

1) Title of the artick requires slight alteration.
2) Abstract should not exceed 100 words.
3) Material and Method may be limited to 10 lines, giving only the important steps of procedure.
4) Clear Fig. 1 may be provided.
5) Result and Discussion to be presented in a Concised manner.
6) A Short summary of 3-4 lines may be provided.
7) References should strictly follow IVJ format. Volum no. pg no. etc. for Garbayo et al (1998) may be furnished.
8) Responding to the queries raised by the referee and carrying out all corrections pointed out, revised artick and a soft copy may be submitted as full research article of 5-6 pages, inclusive of table, after going thro' the IVJ guidelines enclosed for further action

To
Dr. Erma Sabitri
.....
.....
.....

Managing Editor

RETURN THIS PAPER WITH YOUR REPLY WITHIN 90 DAYS

78/19

Comments on Article A-78/19

"Anti early embryonic protein (EEP) for pregnancy test by microtiter strip in dairy cows"

By Abdul Samik and Erma Safiri from Surabaya, Indonesia

1. The manuscript describes steps in isolation, purification and quantification of EEP from cotyledons of early pregnant cows and raising anti-EEP asntibodies in rabbits and then its use to detect early pregnancy by microtitre strip method in post-inseminated cows at day 28 including confirmation by serum progesterone and ultrasonography. The approach is novel but following queries need to be met with.

2. Title need to be refined as above. English language throughout the manuscript need to be corrected as past tense. Some of the local terms cited are not understandable, some statements are not clear. These are pointed out in the manuscript

3. In M&M, what were the actual gestation days of cows when cotyledons were collected for EEP? What was the actual dose of EEP used in rabbit 150 µg or 100 g? need to be clarified, including interval between first and second EEP injection in rabbit. Microtitre strip test procedure need not be repeated for progesterone levels again if it was similar.

4. In R&D, Figure 1 is not visible, need to improve it. Other corrections/editing required are indicated on the manuscript itself. The conclusion drawn based on findings is valid.

5. The revised improved manuscript may be considered to publish in the IVJ.

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Anti Early Embryonic Protein (EEP) for The First Pregnancy Test in Microtiter Strip in Dairy Cow

Abdul Samik and Erma Safitri*

Faculty of Veterinary Medicine, UniversitasAirlangga, Surabaya, Indonesia

Abstract

The study purpose was to identify and isolation of Early Embryonic Protein (EEP) from cotyledons of dairy cow pregnant to use for the first pregnancy test inmicrotiter strip.The research phase isolation and examination of EEP protein levels, create of Anti-EEP, estrus synchronization, artificial insemination, pregnancy test (by reacting EEP protein with the anti-EEP), measurement of progesterone levels, and pregnancy test with rectal palpation and ultrasound (USG). The results was obtained that EEP proteins with a molecular weight of 59.88 kDa could be isolated from pregnant dairy cotyledon of 6480 ug / ml in sediments and 13920 ug / ml in supernatants. EEP protein is able to induce the onset of anti-EEP, which can be used for early microtiter strip pregnancy test. The EEP microtiterstriptest with blood serum progesterone and optimum USG levels found sensitivity and specificity, as well as positive predictive values and negative predictive values of 93.33%, 100%, 100% and 83.33% respectively.

Keywords :Early Embryonic Protein (EEP), Pregnant 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, of which 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, estronesulphate (Hafez, 2000).

Materials and Methode

A total of 80 grams of cotyledon from pregnant cattle is used for the EEP characterization via the SDS-PAGE method, wherethe EEP protein specificity was tested with the Western Blot method and then isolated using the Elution technique.

Method of making Anti-EEP :6 *New Zealand* male rabbits were injected subcutaneously with 150 µg of EEP isolates in *complete adjuvant Freunds*. The next booster is carried out 4 weeks after the initial injection with 100 g of EEP isolates in *Freund's incomplete adjuvant*. Blood collection is carried out over weeks 1-10 after the first injection: the third injection is carried out during the eight week. 5cc of blood is taken from each rabbit

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through the *auricular vein* to be analyzed for antibody titter (anti-EEP) using indirect ELISA. Furthermore, Anti-EEP purification using the Saturated Ammonium Sulphate (SAS) method is carried out.

Cow Pregnancy Diagnosis with EEP MicrotiterStrip :*Microtiter strips* are based on the ELISA Sandwich using following methods: the micro plate 96 is well coated with 100 µl anti-EEP and then incubated at 4 0C for 24 hours. After, it is washed with 0.05% PBS-Tween 20 6 times and blocked with BSA grade 5 with a concentration of 1%. Again, it is then washed with 0.05% PBS-Tween 20 another 6 times before being reacted with 100 µl EEP (blood serum from cattle suspected to be pregnant) and incubated at 37 0C for 1 hour. This is washed 6 more times, reacted with alkaline phosphatase conjugate antibodies and incubated at 37 0C for 1 hour, then washed. PNPP substrate was added, and if the color in the control had turned yellow, the reaction was stopped. OD results are read on the ELISA reader of the BIO-RAD system at a wavelength of 405 nm.

Pregnancy Diagnosis of Cows by Measuring Blood Serum Progesterone Levels is similar to using the EEP microtiter strip. The framework for the ELISA Sandwich is used: the microplate 96 is again coated with 100 µl anti-EEP l and incubated at 4 0C for 24 hours. It is then washed with 0.05% PBS-Tween 20 6 times and blocked with BSA grade 5 with a concentration of 1%, then the wash is repeated another 6 times This is then reacted with 100 1001 EEP (blood serum. from cattle suspected to be pregnant) and incubated at 37 0C for 1 hour. It is washed and reacted with alkaline phosphatise conjugate antibodies, then incubated at 37 0C for another hour before being washed again. After that PNPP substrate was added, the color was tested: if the control had turned yellow, the reaction was stopped. OD results are read on the ELISA reader of the BIO-RAD system at a wavelength of 405 nm.

Blood serum Progesterone levels were measured via Enzyme Immunoassay (EIA). In each well 25 µl of standard, sample and control solutions were included. This was then mixed with 100µl *conjugate Progesterone-HRP reagent* and each 50 µl of anti-*progesterone rabbit reagent* was added to each well. The absorbance value was read on the ELISA reader after 15 minutes with an absorbance of 450 nm.

Pregnancy diagnosis with *Ultrasonography* is established on the 30th day after consummation. The researcher's left hand is protected by the EEP tik glove and inserts the ultrasound probe into the cow's rectum to look for the uterus. The probe is placed on top of the enlarged uterine cornua, where the embryo appears, and the heartbeat, spine, legs, fluid, amniotic sac and eyes can be observed.

Results and Discussion

After 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 is obtained, as shown in Figure 1.

Figure1. Results of Western Blot EEP Protein Pregnant Dairy Cattle Cotyledons

The results of the examination of EEP protein levels after artificial insemination can be seen in table 1. The results of the pregnancy examination using an USG can be seen in Picture 2

Table 1. EEP protein levels of dairy cow blood serum after artificial insemination

Cow	After Artificial Insemination							
	7 days		14 days		21 days		28 days	
	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

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

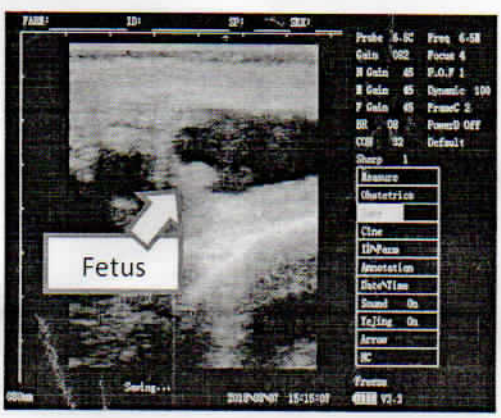

Figure 2. Pregnancy ultrasound results in 28-day pregnant dairy cows

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 Tables 2.

Table 2. 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%

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 can recognize EEP protein as a band with a molecular weight of 59.88 kDa. The protein band is in accordance with molecular weight early embryonic protein (EEP), known as Pregnancy Associated Glycoprotein (PAG) in ovine placenta, 60-100 days of gestation found by El-Amiri *et al.* (2004) range 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 is 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 increasing in the third week after the booster.

Pregnancy diagnosis with EEP microtiter strips were carried out on the 28th day after artificial insemination. To strengthen the success of the pregnancy diagnosis, blood serum progesterone levels were measured, and an ultrasound was carried out. On the 28th day after artificial insemination, the results of each of the 14 microtiter strips from 20 cows were positive for pregnancy. Xie *et al.* (1996) detected EPF in goats three weeks into the pregnancy. EPF concentration is 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 three to

four weeks into 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 is 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 it 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 found sensitivity, specificity, positive predictive value and negative predictive value at rates of 93.33%, 100%, 100% and 83.33% respectively.

The conclusion, Pregnancy testing with a microtiterstrip, 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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“Anti Early Embryonic Protein (EEP) for Pregnancy Test by Microtiter Strip
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Sir / Madam,

The following article has been accepted and will be published in SEPTEMBER, 2019 issue of Indian Veterinary Journal.

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Dated : MAY 18, 2019

ACCEPTANCE LETTER

The following article has been accepted and will be published in **SEPTEMBER, 2019** issue of Indian Veterinary Journal.

Article No.	Title	Author (s)
78/19	Anti Early Embryonic Protein (EEP) for Pregnancy Test by Microtiter Strip in Dairy Cows	Abdul Samik **Erma Safitri**

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