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Early pregnancy diagnosis in cattle and buffaloes from milk

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ABSTRACT

An immunodiagnostic kit has been developed for the early pregnancy diagnosis in cattle and buffaloes, based on the detection of progestrerone in the milk by dipstick EIA method. Discriminatory level was placed at 5-ng/ml progesterone for pregnant animals and 1 ng/1ml for non-pregnant animals. The accuracy for positive diagnosis was 90-92% while non-pregnancy was diagnosed with 100% accuracy. The pregnancy can be diagnosed between 18-24 days of post insemination by this method. All the components in the kits are supplied in lyophilized form for a prolonged shelf-life and convenience for field application. Assay is well suited for routine testing particularly in cattle and buffaloes since milk sampling is easy and doesn't disturb the animal.

Key words : Pregnancy diagnosis, enzyme immunoassay, cattle, buffalo

Timely and accurate determination of pregnancy status is of considerable economic importance to the dairy farmer, as the managemental practices of pregnant animals differ from those of non-pregnant ones. Conventionally rectal palpation of the fetus has been most widely used method for pregnancy diagnosis in bovines but it is practicable only 45-60 days after insemination. Not only does it require the services of a highly skilled veterinarian, but also results in a loss of the valuable time before pregnancy status becomes known. Many times rectal palpation results in fetal losses (Alexander *et al.*, 1995). Other techniques like ultrasound scanning, radiography and vaginal biopsy have found little practical applications (Eckhardt, 1989) because of their requirement of skilled technicians and expensive nature of the tests.

For early pregnancy diagnosis, methods detecting progesterone the "pregnancy hormone" secreted by the corpus luteum of the ovary, have found wide application and is based on the cyclic nature of progesterone production during the bovine estrous cycle. The average length of estrous cycle in the cattle is 21 days thus an early diagnosis can be made by progesterone determination around this time i.e. 18-24 days after insemination (Shemesh *et al.*, 1983) when pregnant and non-pregnant animals exhibit marked difference in their blood progesterone levels.

Milk progesterone assays have been widely applied for pregnancy diagnosis in case of bovines as milk progesterone levels have been found to be highly co-relative with those of peripheral blood (Glencross and Abeywardene, 1983). A dipstick enzyme immunoassay for quantitative analysis of milk progesterone has been developed in this laboratory and validated for bovine pregnancy diagnosis using milk samples. The assay utilizes highly specific, affinity purified progesterone antibodies, a Penicillin progesterone conjugate and a substrate consisting of starch-iodine-penicillin. The test has been standardized with a discriminatory progesterone level of 5 ng/ml for pregnant animals and 1 ng/ml for non-pregnant animals.

MATERIALS AND METHODS

Production of progesterone antisera : Primary immunization of New Zealand white rabbits was carried out with 11-α-hydroxy progesterone hemissuccinate-BSA conjugate along with Freund's complete adjuvant. Subsequently three booster doses of antigen were given and rabbits were finally bled 14 days after the third booster immunization.

Purification of the antibodies : Antibodies were purified by affinity column chromatography using 6-amino hexanoic acid Sepharose 4-B conjugated with progesterone carboxymethyloxime. Elution was done by 0.1 M glycine buffer of pH 4.0 and 2.7.

Preparation of progesterone penicillinase conjugate and starch iodide penicillin substrate : 11-α-hydroxy progesterone hemisuccinate (Sigma-Aldrich, USA) was coupled covalently to Penicillinase (Hindustan Antibiotics Ltd., Pune) by carbodiimide condensation reaction. The conjugate was purified by gel exclusion chromatography using Sephadex G-50 column.

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Conjugate was lyophilized with 0.1% thiomerosal and 0.4% gelatin. Starch-Iodine penicillin substrate was lyophilized with 0.1% polyethylene glycol PEG-6000 to enhance the process of lyophilization.

Pregnancy diagnosis for milk samples through Dipstick Enzyme Immunoassay (DEIA) utilizing lyophilized kit components : The standardized lyophilized substrate was utilized in the dipstick enzyme immuno-assay for pregnancy diagnosis from milk samples under field conditions. For present study, milk samples from cows and buffaloes were tested in the field conditions. Results were verified with the diagnosis made by the rectal palpation method in order to assess the accuracy of the lyophilized substrate for the DEIA.

Milk samples were diluted 1:3 with distilled water, Nitrocellulose dipsticks coated with progesterone antibodies were dipped in the diluted milk samples for one hour at room temperature and after washing were incubated with progesterone-penicillinase-conjugate for 30 min. Dipsticks were placed in the reconstituted lyophilized substrate solution for visual determination of non-pregnancy (decolorization of the substrates within 15 minutes) and pregnancy (no decolorization of the substrate within 15-20 minutes).

RESULTS AND DISCUSSION

Out of 82 milk samples tested in the field by the dipstick test, 70 cows were diagnosed as pregnant and 12 as non-pregnant. By rectal palpation also, same cows were diagnosed as non-pregnant while 63 cows were diagnosed as pregnant. Thus, the DEIA diagnosed seven cows as false positive. This gave an accuracy of 100% in diagnosis of non-pregnancy and about 92% in diagnosis pregnancy through the dipstick test utilizing lyophilized substrate. The results are summarized in Table No.1.

The assay, utilizing the principle of "residual binding method" involved the adsorption of progesterone antibodies onto the nitrocellulose paper dipsticks. When these dipsticks were exposed to test milk samples, pregnant animal milk containing high progesterone saturated all the antibodies, while in case of non-pregnant animals, little progesterone binds and hence most antibody sites remained free. When the dipstick was exposed to progesterone-penicillinase conjugate, the conjugate was bound by the antibodies. Higher the progesterone concentration in the milk, lower the binding of conjugate to the progesterone antibody.

Penicillinase enzyme system employed starch-iodine penicillin as the substrate. The blue coloured substrate solution, when exposed to conjugates bound dipsticks, was decolorised in case of non-pregnant by the action of Penicillinase on penicillin to produce penicilloic acid. Panicilloic acid withdraws iodine from the starch-iodine complex, resulting in decolorization. In case of pregnant milk samples, there was no decolorisation of the substrate as there were no free antibody sites available for the conjugate to bind.

To increase the sensitivity of the 6-aminohexyl Sepharose 4-B affinity matrix, progesterone-3-CMO was used as the ligand where the bridge is carboxy-methyloxime instead of hemisuccinate. Such an affinity matrix could not bind antibodies against the bridge and hence yielded antibodies specific to progesterone only. The conjugate was lyophilized with 0.1% thiomerosal and 0.4% gelatin. The prolonged stability of conjugate could be attributed to the antimicrobial effect of thiomerosal and a protective action of gelatin on the penicillinase (Manson and Pollock, 1953).

Towards achieving greater stability of the substrate, the starch-iodine-penicillin substrate solution was lyophilized. Upon reconstitution of the lyophilized substrate, the colour development was rapid. However, the original blue colour of the substrate was not reproducible in the reconstituted solution. For the convenient detection of penicillinase activity within 10-15 minutes threshold iodine concentration in case of lyophilized substrate was raised to 60 μM. Blue colour of the substrate solution is due to the complex between iodine and starch (Novick, 1962).

Table 1. Validation of early pregnancy diagnosis kit with field samples and comparison with rectal palpation method

Particulars	Dipstick enzyme immunoassay	Rectal palpation	% agreement
No. of animals tested	82	82	–
No. of animals diagnosed +ve	70	63	92
No. of animal diagnosed -ve	12	12	100

To increase the efficiency of lyophilization polyethylene glycol (PEG-6000) was added to the substrate solution. Time for lyophilization was scaled down by reducing the bulk of the solution to be lyophilized, thus substrate solution was prepared in 10X concentration. The standardized lyophilized substrate was found to remain stable for more than 6 months fulfilling the criteria of stability.

The practicability of the dipstick EIA using lyophilized substrate was demonstrated in detection of pregnant cows with 92% precision and non-pregnant cows with 100% precision.

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