NON-INSTRUMENTED IMMUNOASSAY FIELD TESTS FOR PREGNANCY DETECTION IN FREE-ROAMING FERAL HORSES

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Abstract. We evaluated 2 rapid non-instrumented field tests for pregnancy on feral horses (*Equus caballus*) because previous techniques required sophisticated and expensive instrumentation that limited their usefulness to field researchers. The measurement of urinary estrone conjugates (E_1C) by an enzyme immunoassay and based on observable color changes was 100% accurate when compared with instrumented spectrophotometrically measured tests for E_1C. A non-instrumented "dipstick" enzyme immunoassay for equine chorionic gonadotropin-like (eCG) molecules was 93% accurate in diagnosing mare pregnancies when compared with the results of the instrumented test for E_1C. The decrease in accuracy using the non-instrumented eCG test resulted from a time period of 40–140 days when eCG was measurable, whereas E_1C elevations were measurable after day 35 of a mare's pregnancy. Our results indicate that it is possible to detect pregnancy in free-roaming horses under field conditions and without instrumented assays; such field tests provide opportunities to study fecundity and fetal loss in a variety of free-roaming animals.

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The ability to diagnose pregnancy remotely in free-roaming wildlife provides the field research biologist with methods for studying fecundity and fetal loss without the stressors and costs of capture and immobilization. Recently, pregnancy in uncaptured feral horses has been diagnosed by means of urinary estrone conjugates (E_1C), urinary non-specific immunoreactive pregnanediol-3-glucuronide (iPdG), and fecal total estrogens (Kirkpatrick et al. 1988, 1990*a*,*b*,*c*; Daels et al. 1991). Urinary and fecal analysis of steroids or their metabolites also have been used to diagnose pregnancy successfully in free-roaming North American bison (*Bison bison*), caribou (*Rangifer tarandus*), and muskoxen (*Ovibos moschatus*) (Desaulniers et al. 1989, Messier et al. 1990, Kirkpatrick et al. 1991*a*).

The diagnostic methods used to date for pregnancy determination in free-ranging animals have employed radioimmunoassay (RIA) or enzyme immunoassay (EIA), both of which require sophisticated and expensive instrumentation when used as precise quantitative assays. These methods also require the preservation of samples in the field and their transport to the laboratory. These requirements limit the usefulness of previously described remote pregnancy testing for wildlife researchers.

The measurement of urinary E_1C as a test for pregnancy in mares is highly accurate (Evans et al. 1984, Kasman et al. 1984, Kirkpatrick et al. 1990*b*). As pregnancy proceeds in the mare, serum estrone concentrations increase (Cox 1975) and estrone is conjugated to both the sulfate and glucuronide forms that are primarily excreted in the urine. This particular metabolic pathway forms the basis for determining pregnancy in free-ranging mares.

Although equine chorionic gonadotropin (eCG) apparently is first detected in the serum of pregnant domestic mares between days 37 and 42 (Ginther 1979), it was thought that little, if any, eCG in blood was excreted into the urine. However, Roser and Lofstedt (1989), recently demonstrated that eCG can be detected in the urine of domestic mares by RIA and by a simple dipstick enzyme immunoassay during the first trimester of pregnancy.

The recent adaptation of a quantitative EIA that requires the use of spectrophotometric measurements (Lasley et al. 1991) appeared to lend itself to field application of pregnancy detection in free-ranging mares. Similarly, the simplicity and ease of execution of the eCG dipstick enzyme immunoassay test also appeared well-suited for field application. Herein, we evaluate the usefulness of field tests requiring no spectrophotometric measurements in detecting urinary E_1C and eCG and hence pregnancy in uncaptured feral horses.

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METHODS AND MATERIALS

Sample Collection

We tested 65 horses that inhabited Assateague Island National Seashore, Maryland, and Chincoteague National Wildlife Refuge, Virginia, for pregnancy during August–October 1989. Mares were identified by unique markings and were observed at 50–100 m. Immediately after micturition, urine was extracted and collected as described by Kirkpatrick et al. (1988). We aspirated urine directly from pools on the ground, or placed wet sand in a gauze bag and hand-centrifuged it inside a plastic bag. The urine that collected in the bottom of the plastic bag then was poured off into a collection vial. We also quickly collected samples from shallow pools of marsh water before the urine foam disappeared. Samples were immediately transferred to plastic vials and placed on ice. Previous experience with quantitative assays indicated that a wide range of temperature fluctuations over the course of a day would not interfere with the accuracy of the E_1C assay (J. F. Kirkpatrick, unpubl. data).

Sample Preparation

We prepared Whatman No. 1 filter paper disks (7.0-mm diam) with a paper punch prior to fieldwork (Lasley et al. 1991). Three to 4 disks were placed in each urine sample immediately after collection, where they remained for 1 to several hours. They were removed with forceps at the end of each day, and air-dried on a non-absorbent surface. The remaining urine samples were stored frozen at −5 C with no preservatives. We collected all samples in late August or early October, which corresponded to the first trimester of pregnancy. We tested all samples for pregnancy on Assateague Island by the 2 field methods, and later by means of an instrumented assay for E_1C in a laboratory in Billings, Montana.

Estrone Conjugate Non-instrumented Enzyme Immunoassay (E_1C)

We used the non-instrumented field test for E_1C described by Lasley et al. (1991), and prepared it for use in the field by coating tubes, preparing standards, and packaging buffers. The only instruments required in the field were forceps and a 100- to 1,000-µl micropipet. The estrone conjugate–horse radish peroxidase conjugate (E_1C-HRP), H_2O_2, and 2,2'-azino-bis (3-ethylbenz-thioazol-6-sulfonic acid) (ABTS) required refrigeration and had a shelf life of up to 1 year. Results were obtained the same day as the urine collection. Before field testing, NUNC Maxisorp star tubes (No. 470319) were coated with 500 µl of a 1:1,500 dilution of anti-estrone conjugate antibody R583 (Duels et al. 1991) in a pH 9.6 carbonate buffer (1.59 g Na_2CO_3; 2.93 g $NaHCO_3$; 1 L dH_2O), and were incubated overnight at 5 C. Tubes were emptied and washed 5 times with 0.5 ml of a 1:100 dilution of TWEEN-20® (Sigma Biochemicals, St. Louis, Mo.), tapped dry, and stored refrigerated until use. At the time of assay, we dropped 2 filter paper disks into each tube and added 500 µl of a 1:5,000 dilution of E_1C-HRP conjugate (diluted in 0.1 M, pH 7.0 carbonate EIA buffer) to each tube. Preparation of the E_1C-HRP conjugate has been described by Munro and Lasley (1988). Eight additional tubes were used to generate a standard curve. Duplicate tubes used for the standard curve received 100 µl of estrone sulfate (E_1S) (Sigma Biochemical) and 500 µl of E_1C-HRP. Standards were equivalent to 0, 10, 100, and 1,000 ng/ml and were prepared in distilled water before fieldwork. We chose the top standard, 1,000 ng/ml, because that concentration distinguishes pregnant from non-pregnant mares (Kirkpatrick et al. 1990a). Samples were swirled gently by hand, incubated at room temperature for 2 hours, emptied, washed 5 times with 0.5 ml of TWEEN-20®, and tapped dry. Each tube then received 500 µl of substrate (from a stock solution of 4 mL 0.5 M H_2O_2 and 12.5 ml 40 mM ABTS). After a maximum incubation of 1 hour at room temperature, we compared color changes in sample tubes to color changes in the standard curve tubes. Samples that were as light or lighter in color than the 1,000 ng/ml tubes indicated a pregnancy.

Equine Chorionic Gonadotropin Assay (eCG)

We employed a dipstick enzyme-linked immunospecific assay (Mare Check®; MAB Inc., Mountain View, Calif.) to measure a urinary equine chorionic gonadotropin-like (eCG-like) molecule (Roser and Lofstedt 1989) present in mare urine. Each kit contained a vial of assay

buffer, an anti-eCG coated plastic tube, a plastic dropper, and a plastic dipstick coated with anti-bovine LH antibody and substrate. Assay buffer was added to the tube containing a lyophilized monoclonal antibody, conjugated to alkaline phosphatase, against the alpha subunit of luteinizing hormone (759G_{10}, eLH). The anti-eLH-alpha antibody cross-reacts with the eCG-like molecule. Three drops of raw urine were added to the tube and mixed gently by hand. If eCG is present, it will bind to the anti-eLH-alpha antibody in the tube. The plastic dipstick coated with a monoclonal antibody against bovine LHβ (518B_5, beta-LH) was placed in each tube and moved vigorously up and down for 1 minute. The 518B7 antibody has been shown to cross-react with serum eCG (Matteri et al. 1986) and urinary eCG (Roser and Lofstedt 1989), causing a "sandwich" of the eLH-alkaline phosphatase/eCG/bovine LHβ on the dipstick. The dipstick was left in the tube for 1 hour, after which it was removed and rinsed gently in cold tap water for 2–3 seconds. The dipstick then was placed in a tube containing the substrate bromo-chloro-indolyl phosphate for 15 minutes and rinsed in tap water for a second time. A blue color resulted in the presence of the alkaline phosphatase and, regardless of intensity, was an indicator of the presence of eCG-like molecule and indicated pregnancy.

Instrumented E_1C Enzyme Immunoassay for Pregnancy Confirmation

We confirmed pregnancy with the instrumented quantitative EIA of urinary E_1C described by Shideler et al. (1990) and validated in the horse by Kirkpatrick et al. (1990*c*, 1991*b*, 1992*a*). The E_1C concentrations were indexed to creatinine (Cr) to account for differences in urine concentration. We compared the results obtained from this highly accurate quantitative assay with those results obtained with the non-instrumented semi-quantitative field test. Approximate birth dates for foals were collected with the aid of National Park personnel during the spring months of 1990, and each mare was located again during June 1990 to determine if a foal was present.

RESULTS

On the basis of the instrumented E_1C assay, we determined that 42 mares were not pregnant and 23 were pregnant. Non-pregnant mares had E_1C concentrations that ranged from 0 to 0.833 μg/mg Cr ($\bar{x}$ = 0.14, SE = 0.03). Pregnant mares had E_1C concentrations that ranged from 1.02 to 14.2 μg/mg Cr ($\bar{x}$ = 7.43, SE = 1.51). None of the 42 mares with urinary E_1C concentrations of <1.0 μg/mg Cr had foals with them during the following summer. Twenty-one of the 23 mares with urinary E_1C concentrations >1.0 μg/mg Cr had foals with them during the following summer.

Color changes in the standard curve tubes for the non-instrumented E_1C field test ranged from a dark green for 0 ng E_1S/ml to almost clear for the 1,000 ng/ml. (*A color reproduction of the color changes for the standard curve and for 5 representative pregnant and for 5 representative non-pregnant mares will be forwarded upon request.*) The agreement between the results of the instrumented E_1C EIA and the non-instrumented E_1C field test was perfect.

The eCG dipstick assay revealed 19 pregnancies, all among the 23 with urinary E_1C concentrations >1.0 μg/mg Cr, and failed to detect 4 pregnancies. All 4 of the pregnancies missed by the eCG dipstick test resulted in foals before 10 May of the subsequent spring, whereas all other pregnancies detected by the eCG test resulted in foals born after 17 June (*A color reproduction illustrating representative samples of dipsticks from pregnant and non-pregnant mares will be forwarded upon request*).

DISCUSSION

Estrone Conjugate (E_1C) Immunoassay

The clear differences between urinary E_1C concentrations in the pregnant and non-pregnant mares permit the use of a subjective, non-quantitative colorimetric test based on the adaptation of an E_1C EIA. Assuming no pregnancy losses, the application of this test exhibited sensitivity and specificity of >90%. The highest E_1C concentrations in non-pregnant mares never exceeded the lowest concentrations in pregnant mares. The 2 mares that tested positive for pregnancy, but did not have foals at their sides the following summer, may have had fetal losses during the winter or neonatal losses. Fetal losses have been reported to be as high as 46.3% among 2-year-old feral mares on Sable Island (Lucas et al. 1991). One of the 2 mares that tested positive for pregnancy, but was without a foal in this study, was 2 years old.

The use of filter paper disks for sample storage and transfer rather than aliquots of whole urine offers great flexibility to the field researcher. Each disk absorbs approximately 10 μl of urine. This quantity is sufficient to discriminate among E_1C values when the combined absorbed volumes of 2 disks are assayed. Use of filter paper disks eliminates the need to transfer iced or frozen urine samples collected in the field over long distances to laboratories. In fact, as long as the air-dried disks are maintained in some manner that protects them from contamination or extraneous moisture, they can be sent from the field to the laboratory via ordinary first class mail.

The instrumented E_1C EIA used analysis of creatinine to account for differences in urine concentration; whereas the field test made no provisions for this variable. Nontheless, the field test provided an accurate indicator of pregnancy. Again, this emphasizes the large differences between the urinary E_1C concentrations of pregnant and non-pregnant mares. The field test can be converted to a somewhat more quantitative assay by transferring the contents of the tubes to microtiter plates and reading their optical densities in either a standard spectrophotometer or a microtiter reader at 405 nm. The major shortcoming to the E_1C field test is that urinary E_1C concentrations do not begin to increase dramatically until after day 35 of pregnancy (Daels et al. 1991); hence, the test is not useful before then.

The accuracy of the instrumented EIA or RIA for urinary E_1C as a test for pregnancy in domestic mares is 100% and has been documented previously by rectal palpation and by confirmation of pregnancy (Evans et al. 1984; Kasman et al. 1984, 1988); it also has been validated in feral mares (Kirkpatrick et al. 1990*b*). As pregnancy proceeds in the mare, estrone is conjugated to both the sulfate and glucuronide forms and excreted primarily in the urine. This particular metabolic fate forms the rationale for the urinary pregnancy test and explains the high degree of accuracy of the assay.

Equine Chorionic Gonadotropin (eCG) Immunoassay

Concentrations of urine eCG in both the RIA and dipstick assay rise between days 35 and 40, peak between days 50 and 90, and slowly reach baseline levels by days 130–140 of pregnancy. In our study, 19 out of 23 pregnant feral mares were positive for urinary eCG by the dipstick method, thereby demonstrating that the field test can be employed successfully to detect pregnancy in feral horses. The negative results for 4 pregnant mares may have been the result of mares being tested before day 35 of pregnancy or past 130–140 days when levels in the urine were too low to detect (Roser and Lofstedt 1989), or the pH of the urine interfered with the kinetics of the enzyme assay. Considering the approximate birth dates (early Mar to mid-Apr) for the 4 foals in question, the urine samples were collected after 140 days when eCG levels are too low to measure.

The E_1C and eCG tests can be performed at the same time to positively identify the rate of conception and pregnancy loss. The eCG test verified conception after 35–40 days but not necessarily embryo viability, since the endometrial cups formed during embryonic attachment continue to secrete eCG for 130–140 days even if the mare aborts (Ginther 1979). The E_1C assay confirms pregnancy after days 35–40 of pregnancy, but only when a viable embryo is present and secreting high levels of estrone into the blood and excreting it in conjugated forms in the urine (Kasman et al. 1988). Use of the 2 tests together, for example, where the E_1C test is negative but the eCG test is positive, will indicate that the mare conceived but subsequently aborted after 35 days. This information should help researchers to evaluate conditions surrounding embryonic loss in feral horses.

The application of these methods to feral horses on other types of terrain and to other species of ungulates is an important consideration. Collection of urine has been accomplished from feral horses inhabiting the Pryor Mountains in Montana and on the Challis, Idaho horse range (Kirkpatrick et al. 1988) where they seldom can be approached closer than 100 m, and from feral donkeys in tropical forest in Virgin Islands National Park (J. W. Turner, pers. commun.). More recently, one of the authors (J. F. Kirkpatrick) has recovered urine of bison from soil in Yellowstone National Park after observing micturition from >500 m, and urine of pronghorns (*Antilocapra americana*) on prairie habitat in Montana at 1,200 and 1,500 m by using a spotting scope and hand-held radios to guide the collector to the site of the urination. Urine samples also have been collected from

free-roaming gorillas (*Gorilla gorilla*) in tropical forests in Rwanda (N. Czekala, San Diego Zoo, Calif., pers. commun.). Because of the high grasses, marshes, and beach sand, the greatest difficulties have been encountered on Assateague Island.

Instrumented EIA's for E.C and a variety of other steroid metabolites, including pregnanediol-3-glucuronide and non-specific progesterone metabolites have been successfully used to determine pregnancy and detect ovulation in a variety of free-roaming species, and all of these assays can probably be put into a non-instrumented format. Additionally, water extracts of fecal samples from horses (Kirkpatrick et al. 1991*b*) and bison (Kirkpatrick et al. 1992*b*) can be used for pregnancy diagnosis. Non-instrumented assays can be developed for a variety of other species.

RESEARCH IMPLICATIONS

The costs (each Mare Check kit costs approx. $2.00; the cost of a single E.C test is approx. $3.00, excluding the cost of the micropipet) and the accuracy of our results indicate that it is possible to detect pregnancy in free-roaming feral horses under field conditions, and without instrumented assays. This should provide wildlife researchers the ability to study fecundity and fetal loss in a variety of free-roaming animals. We recommend that these methods be tested in a variety of physical terrains, with other species of animals, and with other existing enzyme immunoassays to broaden the use of this approach.

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