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Research article

Diagnosis of pregnancy in Southern lechwes (*Kobus leche*) using a bovine assay for pregnancy-associated glycoproteins

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Abstract

Pregnancy diagnosis is an important part of reproduction management of wild ruminants involved in free-ranging and captive programmes. Pregnancy-associated glycoproteins (PAGs) are a polymorphic family of placenta-expressed proteins, which are released into maternal blood circulation where they can be assayed. These tests have been developed and validated in domestic species; PAGs have also been isolated in a few non-domestic species of the Bovidae and Cervidae families. Assays have been used in many cervid species, but in very few wild bovid species, with high specificity. One hundred and twenty serum samples collected from 79 mature female Southern lechwes (*Kobus leche*) were tested using a commercial enzyme-linked immunosorbent assay (ELISA) test (IDEXX Bovine Pregnancy Test Kit, IDEXX Europe BV, Hoofddorp, The Netherlands). Pregnancy was determined either at necropsy (n=7), by transabdominal palpation in late pregnancy (n=3), or by visualisation of birth (n=32). The other sera were controls from known non-pregnant females (n=78). Following the range of use, recommended by the manufacturer in domestic bovids, three false positive and two false negative results were identified. These tests provide high sensitivity and specificity (95.2 and 97.4%, respectively), and high positive and negative predictive values (95.2 and 97.4%, respectively) for pregnancy diagnosis in Southern lechwes. Using an ELISA validated in domestic species for the detection of PAGs appears to be a rapid, inexpensive and accurate test for pregnancy diagnosis in Southern lechwes. Nevertheless, more samples are needed to determine a better range of use of this test in this species.

Introduction

A blood test for detecting pregnancy is particularly suited for ecological studies when animals are captured or handled for other purposes, allowing determination of pregnancy rates in demographic studies (Haigh et al. 1991, 1993). This approach is especially useful for the management of captive ungulate populations, or population dynamics in free-ranging endangered species involved in conservation programs. Analysis of serum for chemical indicators of pregnancy provides a means of detecting pregnancy without having to perform palpation per rectum, or to sacrifice animals (Haigh et al. 1991, 1993).

Pregnancy-associated glycoproteins (PAGs) are a polymorphic family of placenta-expressed proteins, which are released into maternal blood circulation where they can be assayed by different radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) systems; their detection is used as a diagnostic technique of pregnancy in domestic ungulates (Eckblad et al. 1985; Reimers et al. 1985; Sousa et al. 2006). The presence of PAG during pregnancy and their use as a means of pregnancy testing have been demonstrated in a number of wild ungulates, mainly Cervidae (Haigh et al. 1988, 1991, 1993; Houston et al. 1986; Huang et al. 1999, 2000; Kiewisz et al. 2008; Lamglait and Rambaud 2016, 2017; Osborn et al. 1996; Ropstad et al. 1990; Rowell et al. 1989; Russel et al. 1998; Willard et al. 1994a,b,c, 1996; Wood et al. 1986).

The Southern lechwe (*Kobus leche*, Gray, 1850) is a golden-brown medium-sized antelope species native to swamp and floodplain margins in South-Central Africa. This species remains widespread, especially in extensive wetlands in Botswana, but has suffered from poaching, and is affected by the disruption of natural flooding regimes. Southern lechwes are currently classified as Least Concern on the International Union for Conservation of Nature Red List (IUCN 2008). Nevertheless, the distribution of the lechwe is discontinuous, and effective protection and management of its remaining populations and their wetland habitats in a few critical areas are necessary for its long-term survival (IUCN 2008). The objective of this study is to determine if the PAG ELISA appears to be a reliable indicator of pregnancy in captive Southern lechwes.

Methods

Southern lechwes included in this study were those being captured, handled or anesthetised for other purposes (transfer to other enclosures, preshipment health examination, vaccinations, hoof trimming, medical problem). On each of these occasions, blood samples were collected. Blood samples were also collected during necropsy of freshly dead individuals. One hundred and twenty samples were opportunistically collected over a 10-year period from 79 mature females kept under captive management at the Réserve Africaine de Sigean, France. Pregnant females were only sampled once during their gestation period. The samples were allowed to clot, and the serum was harvested and frozen at −20°C. Serum samples were then sent to the Laboratoire Agro-Vétérinaire de Seine-Maritime (Rouen, 76175, France), where they were assayed without any key to indicate reproductive status. The test used is an ELISA developed for the detection of early PAGs in serum or plasma of domestic bovids as a marker for pregnancy (IDEXX Bovine Pregnancy Test Kit, IDEXX Europe BV, Hoofddorp, The Netherlands). This test offers a laboratory-based method for accurate detection of pregnancy as early as 28 days post-breeding in cattle. Briefly, a microtiter plate format was configured by coating an anti-PAG antibody onto the plate. After dilution and incubation of the test sample in the coated well, captured PAG was detected with a PAG-specific antibody (detector solution) and horseradish peroxidase conjugate. Unbound conjugate was washed away and TMB substrate added to the wells. Colour development was proportional to the amount of PAG in the sample. For the purposes of this study, the assays were made following the manufacturer's instructions, and conclusions were drawn using the threshold values established in domestic species (positive result if optical density over 0.3). Results comprised numbers calculated using a manufacturer-supplied formula, and were expressed as optical densities. Assay data were then provided to those who collected the field data, and compared to the pregnancy status. Pregnancy—used as the gold standard—was confirmed either at necropsy (n=7), by transabdominal palpation in late pregnancy (n=3), or by visualisation of parturition (n=32). Mature females that had not been exposed to males (n=12), had not given birth 8 months after sampling (n=51) or were not gravid at necropsy (n=15) were used as negative controls.

Results

The results obtained for the determination of PAGs by an ELISA developed for domestic ruminants in Southern lechwes are compiled in Tables 1 and 2. The following intrinsic and extrinsic values were then calculated: sensitivity=95.2%, specificity=97.4%, positive predictive value=95.2% and negative predictive value=97.4%. Two false positive and two false negative results

Table 1. Detection of pregnancy by serum pregnancy-associated proteins in captive Southern lechwes (*Kobus leche*).

Animals	Sample size	Number PAG	
		Positive	Negative
Negative controls (females not explosed to males)	12	0	12
Necropsied females	12	0	12
Pregnant	7	5	2
Non-pregnant	15	0	15
Potential dams			
Pregnant	35	35	0
Non-pregnant	51	2	49

Table 2. Repartition of positive and negative results for pregnancy diagnosis by the detection of serum pregnancy-associated proteins in captive Southern lechwe, compared to the gold standard (foetal presence at parturition or palpation, or necropsy).

		Foetal presence		
		Positive	Negative	Total
PAG ELISA	Positive	40	2	42
	Negative	2	76	78
	Total	42	78	120

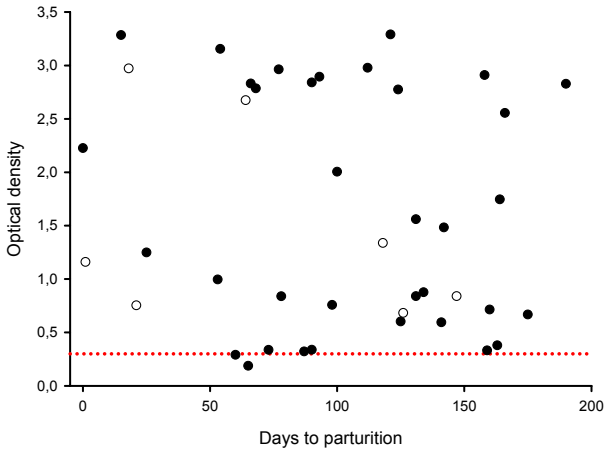

Figure 1. Graphic representation of optical density results using a commercial ELISA test for detection of pregnancy-associated glycoproteins (IDEXX Bovine Pregnancy Test Kit, IDEXX Europe BV, Hoofddorp, The Netherlands) in Southern lechwe (*Kobus leche*). The red dotted line represents the positive threshold value as established by the manufacturer in domestic species. White dots correspond to samples collected on dead animals.

were identified. The false positive results (optical density of 0.3360 and 0.3370) were very close to the positive threshold value (0.3); on the other hand, they consisted of early diagnoses (54 and 67 days post-breeding). The two false negative results were from serum sampled from freshly dead animals; in both cases, foetuses were in the last third of pregnancy. The results obtained for these cases were 0.2895 and 0.1875, therefore close to the positive threshold value.

Pregnancy diagnosis by detection of PAGs in Southern lechwes was performed as early as 33 days post-breeding in the present study. Optical density results exceeding the positive threshold value were then encountered throughout the pregnancy period until the day of parturition (Figure 1); no distinctive pattern could be detected according to the stage of gestation.

Five dams that were not pregnant at the time of collection were sampled during their lactating period from 64–132 days after parturition; all tests were below the positive threshold value for these samples.

Discussion

The detection of pregnancy-associated proteins derived from binucleate trophoblast cells has allowed for the early detection of pregnancy in cattle (*Bos taurus*) (Eckblad et al. 1985; Reimers et al. 1985; Sousa et al. 2006). These proteins have also been demonstrated in pregnant elk (*Cervus elaphus nelsoni*), moose (*Alces alces*) (Huang et al. 1999), American bison (*Bison bison*) (Kiewisz et al. 2008) and muskoxen (*Ovibos moschatus*) (Rowell et al. 1989).

A high degree of cross-reactivity using assays for the detection of these proteins has been found in several Cervidae species (Haigh et al. 1988, 1993; Huang et al. 2000; Osborn et al. 1996; Ropstad et al. 1990; Russel et al. 1998; Willard et al. 1994a,b,c, 1996; Wood et al. 1986), and in a few wild Bovidae species (Haigh et al. 1991; Houston et al. 1986; Lamglait and Rambaud 2016, 2017). The detection of pregnancy-specific proteins in serum from ewes resulted in 97% accuracy at 55 days post-breeding (Ishwar 1995). Accuracy of RIA in elk and moose was 93% compared to calving observation (Huang et al. 2000). The specificity and sensitivity found in the present study are consistent with the high accuracy rates previously reported in other Cervidae and Bovidae species (Huang et al. 2000; Ishwar 1995; Lamglait and Rambaud 2016, 2017). The two false positive results detected in the present study may be due to embryonic death as pregnancy diagnoses were made early (54 and 67 days post-breeding). No stillbirth was detected in this population during the study period; nevertheless, early embryonic mortality can result in foetal resorption. The positive threshold value used in the present study was established by the test manufacturer for domestic ruminants; this value may not be adapted for pregnancy diagnosis in Southern lechwes. If the positive threshold value were to be changed from 0.3 to 0.4 in the present population, no false positive but three false negative results would arise. The resulting intrinsic and extrinsic values would therefore be: sensitivity=92.7%, specificity=100.0%, positive predictive value=100.0% and negative predictive value=96.3%. Depending on the user's aim, a positive threshold value of 0.4 may be preferable in order to benefit from higher specificity and positive predictive value.

Alternative techniques for pregnancy diagnosis have been proposed in wild ungulates. Palpation per rectum has been used in the management of moose (Haigh et al. 1982, 1993) and bison (Haigh et al. 1991), and is considered an acceptable procedure for other large wild ruminants (Haigh et al. 1982). This technique often requires immobilisation, and, because of abdominal pressure in recumbent animals, can be difficult to perform. Furthermore, it is not sensitive, particularly in early pregnancies or for untrained practitioners. Finally, it provides an accuracy of 90% at 60 days of gestation in sheep, but it is unsuitable for goats because of the risk of trauma, abortion or death (Ruder et al. 1988; Ishwar 1995); rectal abdominal palpation is therefore not considered suitable for small- to medium-sized wild species, such as Southern lechwe. Ultrasonography appears to be a simple, reliable, non-invasive imaging technique for pregnancy diagnosis without side effects (Gonzalez de Bulnes et al. 2010). Transrectal ultrasonographic examination has been reported as a reliable method for early pregnancy diagnosis in ewes and does of several breeds, as early as 25 days post-breeding (Buckrell 1988; Gonzalez de Bulnes et al. 1998, 2010; Ishwar 1995; Martinez et al. 1988; Padilla-Rivas et al. 2005). Between 60 and 80 days of gestation, an A-scan instrument is approximately 95% accurate and a Doppler system up to 100% (Ishwar 1995). Nevertheless, transrectal ultrasonography requires special rectal probes and trained personnel. Transabdominal ultrasonography from the right inguinal region has been proposed as a tool for pregnancy diagnosis in moose (Haigh et al. 1993), mule deer (*Odocoileus hemionus*) (Smith and Lindzey 1982) and bighorn sheep (*Ovis canadensis*) (Harper and Cohen 1985). This non-invasive technique is preferred in small ruminants as it is less invasive (Gonzalez de Bulnes et al. 2010). Pregnancy can be diagnosed from 32–34 days with an efficiency reaching 85–100%, depending on operator experience. Ultrasonography has the advantage of not requiring immobilisation; nevertheless, it requires expensive material and trained personnel, and is not always suitable for field work. False negative diagnoses with this technique can be due to: first, the trophoblastic vesicle or the embryo not being detected; second, an intrauterine accumulation of fluids caused by a condition other than pregnancy; or third, the confusion of embryo vesicles with intestinal loops, blood vessels or pathological conditions (Buckrell 1988; Gonzalez de Bulnes et al. 2010). Serum progesterone assay has been reported as a means of determining pregnancy in ungulates and has been used in bighorn sheep (Brunige et al. 1988) and moose (Haigh et al. 1982). Determination of progesterone on Day 20 of gestation provides 90% accuracy in ewes and does (Ishwar 1995). Progesterone has the disadvantage that it is not specific for pregnancy, and is also present during the luteal phase of the oestrous cycle (Ishwar 1995). As a result, this technique requires repeated samples during a period longer than one cycle in order to show that concentrations of progesterone remain high (Haigh et al. 1993). Individuals have been sacrificed to infer pregnancy rates in wild populations (Haigh et al. 1991, 1993), but this cannot be applied in zoological institutions or for endangered populations under conservation management programmes. Finally, immunoreactive faecal progestins and oestrogens have been used as indicators of pregnancy in cattle, muskoxen (Desaulniers et al. 1987) and caribou (*Rangifer tarandus*) (Messier et al. 1990). They have the advantage over other methods in that their collection is non-invasive; however, their application in free-ranging ungulates or gregarious species under captive management appears challenging as repeated samples (at least 1 or 2 per week), and a prolonged elevated progesterone level beyond a single oestrous cycle length are required. Although several studies have been carried out in small ruminants to individually evaluate each of these different methods, it is difficult to establish adequate comparisons due to differences, such as the breed of goat or sheep, age and farming conditions. Transrectal ultrasonography and determination of PAG concentrations provide very accurate pregnancy diagnosis at 24–26 days post-breeding in goats (Gonzalez et al. 2004), cattle (Maurer et al. 1985) and reindeer (*Rangifer tarandus*) (Ropstad et al. 1990). As a result, the PAG assay appears to be an inexpensive and easy-to-operate tool for pregnancy diagnosis in ungulates, either free-ranging or under captive management (Lamglait and Rambaud 2016; Sousa et al. 2006).

The timing of appearance of PAGs after conception, or their disappearance after parturition or abortion, is unknown in Southern lechwes. Pregnancy-specific protein is present by 24 days post-conception in cattle, and 30 days in red deer (*Cervus elaphus*) (Haigh et al. 1993; Sasser et al. 1986). Pregnancy diagnosis in the present study was performed as early as 33 days post-breeding; more work on Southern lechwes is needed to establish the earliest day at which the PAG ELISA can be carried out. On the other hand, tests conducted on a few dams collected 64–132 days after parturition in the present study were under the positive threshold value; these results show that PAGs may have disappeared by 2 months after parturition in this species. After parturition, embryonal death, or abortion, PAGs have been shown to disappear by 60 days in cattle, and by 25 days in sheep (Haigh et al. 1993; Kiracofe et al. 1993; Ishwar 1995). The timing of post-partum disappearance of PAGs in the serum of Southern lechwe dams remains to be established. Ultrasonography comprises a good additional tool to assess embryonic presence or viability (Buckrell 1988; Ishwar 1995).

Conclusion

The PAG ELISA was shown to be a reliable indicator of pregnancy in Southern lechwe. This assay can assist in the management of this species under either wild or captive conditions. Using Southern lechwe as a model, these results may be extended to other Reduncinae species for which culling or palpation per rectum cannot be carried out, especially in endangered species of the *Kobus* genus, such as the Upemba lechwe (*Kobus leche anselli*), the Nile lechwe (*Kobus megaceros*) or the puku (*Kobus vardonii*). Further investigation is required to determine the time of PAG appearance in early pregnancy and disappearance after parturition.

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