Animal Reproduction Science 165 (2016) 56–68

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Early sex determination in the canine foetus by ultrasound and PCR

Camille Prugnard[a], Amirat-Briand Lamia[a], Yannick Cherel[b], Candice Babarit[b], Claude Guintard[c], Eric Betti[c], Daniel Tainturier[a], Djemil Bencharif[a,*]

[a] Laboratory of Biotechnology and Pathology of Reproduction, ONIRIS: College of Veterinary Medicine, Food Science and Engineering, Loire Atlantique, BP 40706, 44307 Nantes, France
[b] UMR 703 INRA/Oniris: College of Veterinary Medicine, Food Science and Engineering, Loire Atlantique, BP 40706, 44307 Nantes, France
[c] Service d'Anatomie Comparée Animale, ONIRIS: College of Veterinary Medicine, Food Science and Engineering, Loire Atlantique, BP 40706, 44307 Nantes, France

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
15 December 2015
Accepted 20 December 2015
Available online 28 December 2015

Keywords:
Canine foetus
Sex determination
Ultrasound
PCR
Early

ABSTRACT

Twenty bitches were seen in consultation at the Department of Reproduction at ONIRIS (College of Veterinary Medicine, Food Science and Engineering, Loire Atlantique, Nantes, France) between 25 and 50 days of gestation for early sex determination of the canine foetus using ultrasound. The genital tubercle is not visible before 26 days; between 26 and 30 days, it is visible between the pelvic limbs; between 33 and 50 days, the position of the genital tubercle enables sex determination as it migrates caudally in the female and cranially in the male. Good statistical concordance between sexing via ultrasound and sexing at birth has been established (kappa coefficient of 0.8). Macroscopic, microscopic, and histological examinations of the external genital organs were also performed on 10 foetuses at 35 days of gestation; a cartilaginous structure was visualized in the genital apparatus of the male but also in half of the females. Finally, the development of a PCR technique on the SRY gene using formaldehyde-preserved tissues has been described for the first time in this study. It served as a reference for sexing canine foetuses.

1. Introduction

In utero sexing is now an integral part of ultrasound pregnancy diagnosis in large animal veterinary medicine. The ever-increasing popularity of pure-bred dogs has led to a growing demand from breeders to determine the sex of the puppies before birth. Sexing should therefore be integrated into a standard pregnancy monitoring programme using ultrasound. There are very few studies in the dog into sexual differentiation and the development of the genital organs (Evans and Christensen, 1993; Barone, 1978; Evans, 1986), whereas precise data about ultrasound criteria and periods of sexing have been published for ruminants (Stroud, 1996; Bucca, 2005). Sexing via ultrasound is principally based on the position of the genital tubercle, a foetal structure that develops into the penis in the male and the clitoris in the female. In the male bovine foetus, the genital tubercle migrates cranially towards the umbilical cord, whilst in females, it moves caudally towards the tail. On ultrasound, it has a characteristic hyperechoic, bilobate appearance, which appears on the screen as a horizontal "equals" sign (Tainturier et al., 2004).

* Corresponding author at: Laboratory of Biotechnology and Pathology of Reproduction, ONIRIS: The National Veterinary, Food Agriculture and Food Hygiene School of Loire Atlantique, BP 40706, 44307 Nantes, France. Fax: +33 2 40687748.
E-mail address: djemil.bencharif@oniris-nantes.fr (D. Bencharif).

http://dx.doi.org/10.1016/j.anireprosci.2015.12.007
0378-4320/

There are very few studies in the dog into embryonic development and in particular sexual differentiation, and the macroscopic, histological, and ultrasound development of the genital organs. The only study into late ultrasound examination for sexing canine foetuses, which was conducted by Gil et al. (2015), reported a precision rate of 62.24% for the determination of females and 65.48% for male foetuses from 55 to 58 days of gestation. However, the latter estimated that the success rate is around 100% when the bitches are carrying 1 or 2 foetuses, but only 66.7% when the bitch is pregnant with 3 or more foetuses (Gil et al., 2015).

Sexing can also be accomplished via fluorescence in situ hybridization (FISH) on the Y chromosome. This is a molecular biology hybridization technique using probes labelled with a fluorescent marker to reveal specific intracellular elements, but there are no commercial probes for the canine genome; they will need to be synthesized. An alternative molecular biology technique for sexing foetuses is in situ hybridization (HIS) to detect the expression of the SRY gene. This involves locating a known sequence of nucleotides (RNA) on a histological section using a labelled probe. This method is also long and involved, and the SRY gene is only expressed in certain cells and in a precise spatiotemporal window. The third and final technique is PCR on the SRY gene. This method has already been used for other studies in the dog and several sequences of primers have been published enabling the amplification of different segments of the canine SRY gene (Nakayama et al., 2010; Olivier et al., 1999). The primer pairs for this gene are therefore readily available commercially. However, published PCR results were obtained on blood samples rather than formaldehyde preserved tissues.

Formaldehyde can degrade RNA and DNA, thus complicating the development of molecular biology techniques, and in particular PCR; it destabilizes the hydrogen bonds between nucleic acids, denaturing the double helix of the DNA (Douglas and Rogers, 1998; Soullier et al., 1999). It also provokes scission of the phosphodiester backbone of DNA, which cleaves the double helix into low molecular weight elements (between 100 and 200 bp) (Douglas and Rogers, 1998). Formaldehyde acts on the interaction between proteins and nucleic acids and provokes the formation of protein/DNA bonds. The digestion of these complexes by non-specific proteinases does not produce pure DNA (Plenat et al., 2006). In summary, PCR on formaldehyde preserved tissues is delicate to perform due to the impregnation of the formaldehyde and its de-structuring effect on DNA: there is less DNA and latter is “cut” into small sequences (Lehmann and Kreipe, 2001; Curran and Ginther, 1991). It is therefore essential to adapt molecular biology techniques, including PCR, to enable the demonstration of the SRY gene in canine foetuses preserved in a solution of formaldehyde.

This study presents the development of the external genital organs on ultrasound and describes the macroscopic appearance of these structures at an early stage of development. We also developed a PCR technique on the SRY gene using tissues preserved in formaldehyde, which had been recovered by caesarean section.

2. Materials and method

2.1. First study: ultrasound

2.1.1. Selection of the animals

The experimental protocols were approved by our local ethical committee. Written informed consent was obtained from the owner of each animal included in the study.

The ultrasound examinations were performed on 25 bitches from 2 to 11 years of various breeds (Beagle, Chihuahua, West Highland White Terrier, Doberman, Poodle, Jack Russell, Dachshund, Spaniel, French bulldog, and crossbreds). The animals were monitored from the start of their cycle via vaginal smears and/or blood progesterone assays to determine ovulation and the ideal timing of natural mating or artificial insemination (AI) with fresh semen. The latter were performed twice at 48-h intervals when the progesterone was higher than or equal to 10 ng/ml. The start of gestation, i.e., D0, was fixed as the day of the second natural mating or AI. Certain gestations were stopped at different stages (between 30 and 40 days), either medically (two injections of algepristone, ALIZINE® subcutaneously, 24 h apart, 10 mg/kg, i.e., 0.33 ml/kg/injection) or surgically (caesarean section). For all of the bitches, the puppies were sexed by ultrasound between 30 and 35 days of gestation. Some of the bitches were regularly monitored with ultrasound (4 or 5 times between 15 days and 45 days of gestation). A second sexing was performed on the same puppies either at birth (natural delivery or caesarean section) or on aborted foetuses, to verify the results of the ultrasound sexing.

2.1.2. Examination procedure

The ultrasound examinations were performed by a single operator on conscious animals in dorsal decubitus using a linear 12.5 MHz probe. The entire uterus was scanned; transverse and sagittal sections of the foetuses were performed to visualize and distinguish the different foetal structures. The ultrasound criteria used to sex the foetuses were chosen arbitrarily as a function of those described for other species, notably the relative position of the genital tubercle (Stroud, 1996; Nakayama et al., 2010): on a frontal slice, if the genital tubercle is cranial to the pelvic limbs and just behind the umbilical cord, it is a male; if it is caudal to (or between) the pelvic limbs and above all close to the tail, it is a female.

2.1.3. Statistical analysis

To determine the reliability of the ultrasound technique for the early sexing of canine foetuses, the Kappa coefficient was calculated, and revealed the concordance between ultrasound sexing and sexing at birth or on aborted foetuses, corrected with respect to chance alone.

For all statistical tests, a significant difference was retained for a first error rate of less than 0.05.

2.2. Second study: histology

2.2.1. Macroscopic examinations

Macroscopic examinations of the external genital organs were performed on ten 35-day foetuses that had