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Single closed-tube quantitative real-time PCR assay with dual-labelled probes for improved sex determination of equine embryos

T. De Coster [a,d,*,1], M. Van Poucke [b,1], O. Bogado Pascottini [a], D. Angel-Velez [a,e], E. Van den Branden [c], S. Peere [c], M. Papas [c], I. Gerits [c], J. Govaere [c], L. Peelman [b], J.R. Vermeesch [d], A. Van Soom [a], K. Smits [a]

[a] Reproductive Biology Unit, Department of Internal Medicine, Reproduction and Population Medicine, Ghent University, 9820 Merelbeke, Belgium
[b] Laboratory of Animal Genetics, Department of Veterinary and Biosciences, Ghent University, 9820 Merelbeke, Belgium
[c] Clinic of Large Animal Reproduction, Department of Internal Medicine, Reproduction and Population Medicine, Ghent University, 9820 Merelbeke, Belgium
[d] Laboratory for Cytogenetics and Genome Research, Department of Human Genetics, KU Leuven, 3000 Leuven, Belgium
[e] Research Group in Animal Sciences – INCA-CES, Universidad CES, Medellin, Colombia

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ABSTRACT

In addition to fulfilling many breeders' curiosity, equine embryonic sex determination can have a profound commercial impact. However, the application of currently described assays for equine embryonic sexing has rendered variable diagnosis and validation rates, with sensitivity being the main problem. In addition, while pregnancy results of *in vivo*-flushed equine embryos following a needle aspiration biopsy equal those of non-biopsied embryos, the effect on *in vitro*-produced embryos is unknown. Here, we aimed to develop a highly sensitive and specific assay for equine sex determination that can be directly performed on few embryonic cells, and to test the effect of a needle aspiration biopsy on the viability of the *in vitro*-produced embryo. To this end, a multiplex quantitative real-time PCR (**qPCR**) assay with dual-labelled probes was designed to allow the simultaneous generation of both male-specific and control fragments in a single closed-tube reaction, avoiding potential sample loss or contamination. To improve sensitivity, multicopy and polymeric genes were chosen to be specifically amplified, i.e., eight copies of Y-chromosomal *ETSTY5* as male-specific and four autosomal *UBC* monomers as control fragment. Specificity was enhanced by the equine-specific character of *ETSTY5* and by using dual-labelled probes. The assay was optimised with equine male and female genomic DNA and demonstrated a 100% accuracy and a >95% qPCR efficiency down to 10 pg of DNA. The assay was subsequently applied to determine the sex of 44 *in vitro*-produced embryos, collecting trophectoderm biopsies by means of a needle aspiration biopsy and herniating cells. Of all trophectoderm biopsies and herniating cell samples (n = 54), 87% could be diagnosed. Assay results were validated on a second sample obtained from the biopsied embryo (n = 18) or, by ultrasound-based sex determination of the foetus (n = 7) following the transfer of the biopsied embryo to a recipient mare, with about half of the embryos being fillies and colts. The needle aspiration biopsy procedure did not impair initial pregnancy rate or early pregnancy losses as compared to non-biopsied embryos. In conclusion, we report a safe, reliable, fast, and cost-effective assay for equine sex determination which was validated for the sex determination of *in vitro*-produced embryos based on few embryonic cells, and needle aspiration biopsy did not impair the embryo's viability. The assay and safe biopsy strategy hold potential for other applications.

Implications

In this study, a highly sensitive and specific quantitative real-time PCR assay for equine sex determination was developed, which allowed reliable sexing of equine *in vitro*-produced embryos based on a needle aspiration biopsy, without an observed effect on embryo viability. Increased application of sexing at the preimplantation stage of both *in vitro-produced* and *in vivo*-flushed embryos will facilitate selection and reduce abortion rates. The assay may

* Corresponding author at: Reproductive Biology Unit, Department of Internal Medicine, Reproduction and Population Medicine, Ghent University, 9820 Merelbeke, Belgium.
E-mail address: tine.decoster@ugent.be (T. De Coster).
[1] These authors contributed equally.

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also facilitate other applications which require a robust method for equine sex determination, such as non-invasive prenatal sexing. The availability of a biopsy strategy that does not compromise the viability of embryos enables the wide application of preimplantation genetic testing in horses.

Introduction

Due to the application of embryo transfer and the growing popularity of *in vitro* embryo production by ovum pick-up, intracytoplasmic sperm injection (**OPU-ICSI**) and subsequent embryo cryopreservation, an increasing number of equine embryos are produced, transported and traded around the globe (Lazzari et al., 2020). The value of those embryos is often high, based on their extraordinary pedigree and the use of sophisticated, exclusive assisted reproductive technologies. Yet, sound information regarding their genetic make-up is generally missing.

Preimplantation genetic testing involves the sampling of embryonic material for genetic profiling prior to embryo transfer and provides the opportunity to select for genetic traits and diseases. One of the traits of interest for many horse breeders includes the sex of the future foal. The preferred sex depends on the purpose of the horse. For Thoroughbred horses, colts are preferred for their better sport performances, while for Polo sport horses, fillies are preferred because they are more docile and thus easier to handle in a team. Aside from fulfilling breeders' curiosity, sexing may also have a profound commercial impact and direct future breeding strategies. In contrast to ultrasound-based sex determination of the foetus *in utero* (Curran and Ginther, 1989; Van de Velde et al., 2018) and elective abortion of the foetus of unwanted sex, sex determination of embryos allows the marketing of male and female embryos *ex utero* and holds the potential to reduce abortion rates and thus improve animal welfare.

Research on preimplantation genetic testing in equine embryos resulted in an optimised biopsy procedure for *in vivo*-derived embryos. Initially, an invasive microblade-based biopsy was applied with a destructive effect on a large area of the capsule and on subsequent pregnancy rates (Guignot et al., 2015; Huhtinen et al., 1997; McKinnon et al., 1989; Müller and Cikryt, 1989; Seidel et al., 2010; Skidmore et al., 1989). Later, needle aspiration-based procedures were introduced, which resulted in pregnancy rates similar to those of non-biopsied embryos (~60–70% around day 25; Choi et al., 2010; Seidel et al., 2010; Guignot et al., 2012, 2015; Herrera et al., 2012, 2014; Riera et al., 2012, 2019). More recently, biopsies have been performed on *in vitro*-produced embryos too (Choi et al., 2015; Herrera et al., 2015; Lazzari et al., 2020), but the effect of a needle aspiration biopsy on the viability of the embryo following transfer remains unclear. Only one study published initial pregnancy rates on biopsied *in vitro*-produced embryos, which were similar to those of non-biopsied embryos (Lazzari et al., 2020). However, that study analysed cells that spontaneously herniated from an ICSI-derived hole were collected, which avoids invasive puncturing of the embryos. Yet, such cells are not always present, necessitating the aid of needle aspiration biopsy to obtain an embryo sample. Also, sampling herniating embryonic cells might involve more risk for contamination by embryonic material from distinct embryos or by remnant cumulus cells when cultured in a group. Performing a needle aspiration-based procedure on *in vitro*-produced embryos would, in theory, be less traumatic than for *in vivo-flushed* embryos, since the confluent capsule is absent *in vitro* (Tremoleda et al. 2003). On the other hand, *in vitro*-produced embryos are smaller and of lower quality (Pomar et al., 2005; Tremoleda et al., 2003) than *in vivo*-flushed embryos, and the inner cell mass cannot be visualised. This increases the risk of damaging or aspirating too many cells or cells which will give rise to the embryo proper, potentially lowering the viability and thus pregnancy results.

The first technique described for sexing of equine embryos was karyotyping of whole embryos (Murer-Orlando et al., 1982; Romagnano et al., 1987, 1985; Wood et al., 1988). This technique is accurate but requires many cells and extended culture periods to obtain enough cells with readable metaphase plates, necessary for diagnosis. Even when sacrificing whole embryos, which renders the embryos per definition lost for transfer, it is challenging to obtain enough cells (56–100% readable karyotypes in Murer-Orlando et al. 1982) and therefore, karyotyping is considered not efficient enough for the sexing of embryonic biopsies containing only a few cells. Later, non-invasive immunological detection of male-specific H-Y antigens was attempted, but that technique proved insufficiently accurate (82% accuracy in Wood et al. 1988).

Through the development of PCR methods, molecular detection of the embryonic sex became possible, being fast, accurate and requiring only a little DNA (Peippo et al. 1995). Sexing of equine embryonic biopsies has been explored by several groups applying conventional PCR amplification of the Y-specific *ZFY* gene and its homologous *ZFX* gene located on the X chromosome, followed by restriction enzyme digestion of the amplified products (Huhtinen et al., 1997; Peippo et al., 1995) or, by amplification of Y-chromosome-specific genes (e.g., *SRY*, *RBMY*, *TSPY*, *ETY1* and *CL007*), alone or multiplexed with other target or control gene(s) (Choi et al., 2015, 2010; Guignot et al., 2015; Herrera et al., 2015, 2014, 2012; Jarazo et al., 2012; Lazzari et al., 2020; Troedsson et al., 2010). Two distinct studies aimed for sex diagnosis under field conditions, bypassing the requirement of sophisticated laboratory equipment by the application of loop-mediated isothermal amplification (Dini et al., 2016; Riera et al., 2019). The initial analyses of embryo biopsies seemed promising, with 100% diagnosis and validation rates in small-scale, laboratory-based studies (Choi et al., 2010; Troedsson et al., 2010). However, later studies, including three large-scale studies within embryo transfer programmes, found variable diagnosis (52–96%) and validation (84–100%) rates (Choi et al., 2015; Dini et al., 2016; Guignot et al., 2015; Herrera et al., 2015, 2014; Jarazo et al., 2012; Lazzari et al., 2020; Riera et al., 2019). Non-diagnosis of the embryonic sex and the validation of results being more often biased towards male embryos, pointing out the insufficient sensitivity of the applied assay (Herrera et al. 2014; Herrera et al. 2015; Riera et al. 2019). Because of the low amount of DNA within the embryo biopsy, efforts to improve the sensitivity of the assay were focused on sample lysis and upfront preamplification with one or two rounds of targeted PCR or whole-genome amplification (Choi et al., 2015, 2010; Guignot et al., 2015; Herrera et al., 2015, 2014). However, preamplification is at the expense of specificity as it increases the risk of picking up contamination. Also, whole-genome amplification is costly and therefore only executed when aiming for the simultaneous analysis of multiple traits (Choi et al., 2015, 2010; Guignot et al., 2015). Further attempts to increase both sensitivity and specificity included nested PCR (Jarazo et al., 2012) and the use of Y-specific multicopy genes (Herrera et al., 2015). Quantitative real-time PCR (**qPCR**) has the potential to offer faster, safer and more reliable detection of target DNA (Bastien et al., 2008; Staggemeier et al., 2015), but has not been applied yet for embryonic sex determination in horses.

Here, we developed a single closed-tube multiplex qPCR assay with dual-labelled probes, targeting the polymeric autosomal *UBC* gene and the multicopy Y-specific *ETSTY5* gene, for improved equine sex determination. With the objective to develop an in-house methodology for sexing equine embryos in a commercial embryo production programme, the assay was subsequently applied to herniating cells and to trophectoderm biopsies taken from *in vitro*-produced embryos by needle aspiration biopsy, and

the effect of the latter procedure on embryo viability was determined by transferring the embryos. Preliminary results have been published earlier in an abstract form (De Coster et al., 2023).

Material and methods

Assay design and optimisation

The equine reference sequence of Ubiquitin C (***UBC***), a polymeric autosomal housekeeping gene, consists of four conserved 228-base pair (**bp**) monomers (Acc. No. NC_009151.3; Van Poucke and Peelman, 2017). ClustalW (Madeira et al., 2022) was used to explore its sequence conservation, as all monomers would be targeted as autosomal control regions.

Paria et al. (2011) described Equine Testis-Specific Transcript on Y (***ETSTY****) 1* (Acc. No. EU687549.1), *2* (Acc. No. EU687550.1), *3* (Acc. No. EU687551.1), *4* (Acc. No. EU687552.1), *5* (Acc. No. EU687553.1), and Equine Transcript on Y *(**ETY**) genes 1* (Acc. No. EU687555.1) and *4* (Acc. No. EU687558.1) as equine-specific Y-borne multicopy genes. They were aligned to the equine reference genome (assembly EquCab3.0) and the Y-chromosome sequence (Acc. No. MH341179.1; Janecka et al. 2018) via NCBI Blast (Johnson et al., 2008), to select the longest Y-specific region with the highest copy number and the highest conservation as Y-specific target regions.

A multiplex qPCR assay with dual-labelled probes was designed with Beacon designer™ (Premier Biosoft, USA) to detect both the four conserved *UBC* monomers (as autosomal target) and the eight conserved *ETSTY5* gene copies (as Y-specific target). Both targets should be detected in male samples, while in female samples, only the autosomal target should be detected. The generated amplicons were smaller than 150 bp to allow a quick and efficient amplification/detection, even on degraded DNA. Primers were designed at a melting temperature of 65 °C to minimise secondary structures in the template (detected by mfold; Zuker, 2003), while the probes were designed at 70 °C and at least 10 bp away from the primers to assure binding before elongation starts. Probes were directed against the + or the − strand based on the minimal number of guanine (**G**) nucleotides in the probe sequence and did not contain more than three consecutive Gs nor a G at the 5′-side, in order to minimise unintended G:G mispairing and quenching of the fluorophore. The *UBC*-probe was labelled with 6-carboxyfluorescein (**FAM**) on the 5′ end and Black Hole Quencher (**BHQ**)-1 on the 3′ end, while the *ETSTY5*-probe was labelled with Texas Red (**TxRd**) on the 5′ end and BHQ2 on the 3′ end (Merck KGaA, Darmstadt, Germany). Primers and probes were also checked with Primer-BLAST (Ye et al., 2012) not to contain known single nucleotide variants and to specifically amplify and bind the intended regions.

Assays were performed with TEMPase Hot Start DNA polymerase (VWR International, Radnor, PA, USA) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in semi-frosted PCR tubes. All products were aliquoted for a single run use and only opened after a quick spin-down. Furthermore, all reaction tubes were immediately and individually closed after adding the template and/or the PCR mix. Negative and no template controls were always included to check for possible cross-contamination.

Assay optimisation was performed with genomic DNA of a stallion and a mare, isolated from EDTA-blood via proteinase K digestion, phenol/chloroform extraction and ethanol precipitation (Maniatis et al., 1982). To assess the possible influence of human contamination, the assay was tested on human genomic DNA, isolated in the same way from cultured HapMap Epstein-Barr virus-transformed lymphoblastoid cells (GM12891; obtained from NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research for Individuals). The purity and concentration of the DNA were measured with Nanodrop (Isogen, Utrecht, The Netherlands). The optimal annealing temperature was determined via gradient-qPCR (range: 64–72 °C), and PCR efficiency and sensitivity via a 1/10 dilution series (range: 10 ng–10 pg genomic DNA). In addition, the same dilution series was used to assess if opaque PCR tubes enhance the sensitivity of the assay. qPCR amplification products were analysed via agarose gel electrophoresis. Details on the optimised assay can be retrieved in Table 1.

Application on equine embryos

To test if the qPCR assay can be applied to sex equine embryos, equine embryos were produced *in vitro* and herniating cells and trophectoderm biopsies were analysed (Fig. 1).

In vitro embryo production

Embryos were produced *in vitro* from oocytes collected *post mortem* and from *in vivo*-collected oocytes. Immature oocytes, collected *post mortem*, were recovered from equine slaughterhouse-derived ovaries and matured as cumulus-enclosed non-vitrified oocytes under conditions as described by Angel-Velez et al. (2021). *In vivo*-recovered immature oocytes were obtained in 14 commercial OPU sessions from nine different warmblood mares using transvaginal ultrasound-guided follicle aspiration and matured as described by Papas et al. (2021). Mares were presented in the clinical OPU-ICSI program at Ghent University during the 2020/2021 and 2021/2022 OPU-ICSI seasons by different stud farms that requested sexing of *in vitro*-produced embryos. After maturation, all oocytes were denuded by successive pipetting in 0.1% bovine hyaluronidase (H3506-1G; Sigma-Aldrich, Saint Louis, MO, USA) in handling medium (Tissue Culture Medium (**TCM**)-199 with Hank's salts (22350-029; Thermo Fisher Scientific, Waltham, MA, USA) with 10% (v/v) foetal bovine serum (**FBS**; 10082147; Thermo Fisher Scientific, Waltham, MA, USA)) and washing in handling medium, using a STRIPPER pipettor and 170 µm and 135 µm capillaries (MXL3-STR-CGR, MXL3-175 and MXL3-135; Cooper Surgical, Trumbull, CT, USA). Mature oocytes selected based on the identification of a polar body were fertilised by ICSI, as described by Papas et al. (2021). Mature *post mortem*-collected oocytes were fertilised with frozen-thawed semen (38 °C water bath, 1 min) from two stallions with proven fertility, prepared upfront as described by Angel-Velez et al. (2021). For *in vivo*-recovered mature oocytes, fertilisation as described by Papas et al. (2021) was performed with commercially available frozen-thawed semen from seven different stallions. Embryo culture occurred as described previously by Papas et al. (2021). Blastocyst development was monitored daily from day 6 until day 13 *post*-ICSI.

Collection and analysis of trophectoderm cells and herniating cells

Morphologically normal blastocyst-stage embryos (grades 1 and 2 as determined by Carnevale and Metcalf (2019) and Carnevale and Hatzel (2020)) were biopsied. To this end, embryos were immobilised by suction of a holding pipette (MPH-MED-35; Cooper Surgical, Cooper Surgical, Trumbull, CT, USA) in 5 µL droplets of handling medium under oil (ART-4008-5PA; Cooper Surgical, Trumbull, CT, USA) on a heated stage (39 °C) of an inverted microscope (Olympus IX73; Olympus Shinjuku, Tokyo, Japan) equipped with a Research Instruments micromanipulation system (Cooper Surgical, Trumbull, CT, USA). Ten to 20 cells from the trophectoderm layer were aspirated using a bevelled biopsy micropipette (MPB-BS-30; Cooper Surgical, Trumbull, CT, USA) and placed in a 2.5 µL droplet of Ca^{2+}/Mg^{2+}-free phosphate

Table 1
Details on the quantitative real-time PCR assay for determination of equine sex.

Target gene (amplicon length)	Primer/probe	Sequence (5′ → 3′)	
UBC (147bp)	F-primer	CGAGAACGTCAAGGCCAAGATCCAG	
	R-primer	CGCAGGACCAGGTGCAGAGT	
	Probe	FAM-CCGACCAGCAGAGGCTCATCTTCGCC-BHQ1	
ETSTY5 (141bp)	F-primer	ACGGACGGGACCGGCAAATAAATAC	
	R-primer	GCGTTGACTTTGTGAGTGATGATGTGTGA	
	Probe	TxRd-CTTCTTGTCCGTCTCTTGGTCCTCCTCTTGGT-BHQ2	
Cycling programme			
14′40″	95 °C		Polymerase activation and denaturation of DNA
00′20″	95 °C	]	Denaturation of DNA
00′40″	68 °C	] × 50	Annealing, extension and signal detection
PCR mix (VWR International, Radnor, PA, USA)			
1.0 µL	10× Key Buffer		
1.0 µL	10 µM *UBC* primers (5 µM of each primer)		
1.0 µL	10 µM *ETSTY5* primers (5 µM of each primer)		
0.7 µL	10 µM *UBC*-probe		
0.3 µL	10 µM *ETSTY5*-probe		
0.2 µL	40 mM dNTPs (10 mM of each nucleotide)		
0.1 µL	5 U/µL TEMPase Hot Start DNA Polymerase		
	Template[1]		
	H_2O to make 10 µL		
10.0 µL	Total volume		

Bp = base pairs, *ETSTY5* = Equine Testis-Specific Transcript Y5, F-primer = forward primer, R-primer = reverse primer, *UBC* = Ubiquitin C.
[1] Template = 2 µL Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) with 1% (v/v) polyvinylpyrrolidone (PVP) containing the trophectoderm biopsy, the herniated cells or the whole biopsied embryo; 1 µL containing 5% of the whole biopsied embryo, 2 µL control template (mare, stallion or human DNA), 2 µL Ca^{2+}/Mg^{2+}-free PBS with 1% PVP or, 1 µL handling medium (Tissue Culture Medium – 199 with Hank's salts with 10% (v/v) foetal bovine serum.

buffered saline (**PBS**; 14190-144; Thermo Fisher Scientific, Waltham, MA, USA) with 1% (v/v) polyvinylpyrrolidone (**PVP**; ART-4005; Cooper Surgical, Trumbull, CT, USA), of which 2 µL containing the biopsy was subsequently transferred to a DNase-free 0.2 mL tube on ice using a 135 µm capillary, and immediately stored at −80 °C. To avoid the collection of presumably degenerated cells, the embryos were positioned in such a way that visual cell debris was not aspirated (Supplementary Fig. S1). For embryos produced from *post mortem*-collected oocytes, herniating cells were collected as well when present. In the case of a few cells, the collection was performed identically to the trophectoderm biopsies. When a large clump of herniating cells was present, it was mechanically loosened from the embryo using a 135 µm capillary, washed two times in 1% (v/v) PVP in Ca^{2+}/Mg^{2+}-free PBS and tubed, stored and analysed as described for the biopsied cells. To avoid sample loss, micropipettes and capillaries were coated with 7% PVP (ART-4005; Cooper Surgical, Trumbull, CT, USA) prior to each procedure and tubing of the biopsied cells was performed under visual control through a microscope. To avoid cross-contamination, every embryo was washed in handling medium before the biopsy procedure and manipulated with a new set of micropipettes and capillaries. The outline of the biopsy plate, a protocol and a video of the biopsy procedure, can be retrieved in Supplementary Fig. S2 and Supplementary Materials S1 and S2, respectively.

Trophectoderm biopsies or herniating cells were thawed 10 min prior to analysis, spun down and opened only once to add the qPCR mix for analysis with the assay described in Section '*Assay design*

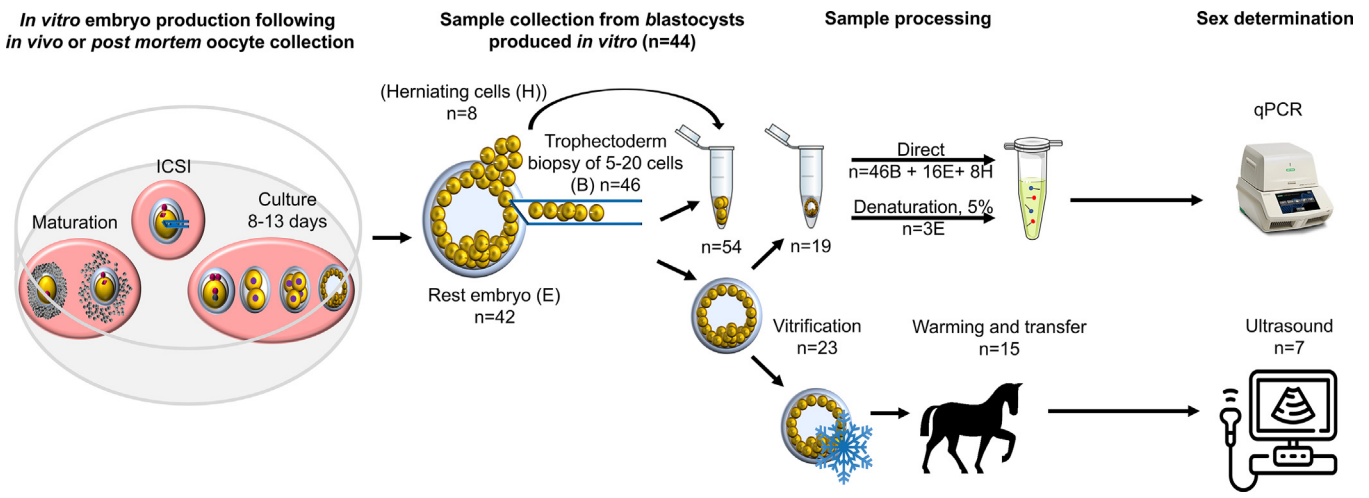

Fig. 1. Overview of samples and methodologies used to test the application of the quantitative real-time PCR (qPCR) assay for sex determination of equine embryos. ICSI: intracytoplasmic sperm injection.

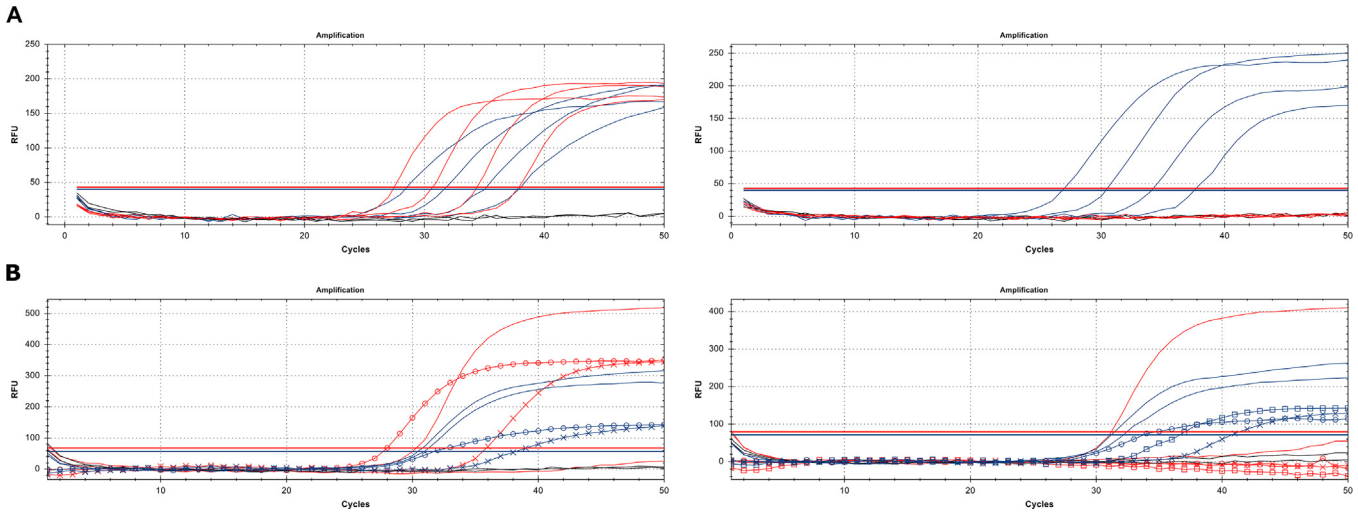

Fig. 2. Amplification plots of the quantitative real-time PCR (qPCR) for equine sex determination. Blue plots represent 6-carboxyfluorescein (FAM)-signals (Ubiquitin C *(UBC)* autosomal target) and red plots Texas Red (TxRd)-signals (Equine Testis-Specific Transcript Y5 *(ETSTY5)* Y-specific target). Horizontal lines are the threshold lines for both signals in their respective colour. Black plots are generated by the negative template control (NTC) and stay under the threshold lines. The X-axis denotes the cycle number and the Y-axis the relative fluorescence units (RFU). Male DNA is characterised by both FAM- and TxRd-signals, and female DNA only by a FAM-signal. (A) From left to right: qPCR amplification plots for 10 ng, 1 ng, 100 pg and 10 pg of stallion (left figure) and mare DNA (right figure). (B) qPCR amplification plots of the whole biopsied embryo (circled plots), the herniating cells (squared plots; right figure only) and the trophectoderm biopsy (crossed plots) of one equine embryo, diagnosed as male (left figure) or female (right figure). Full lines represent female, male and NTC control DNA amplification, respectively.

and optimization' and in Table 1. Positive, negative and no template controls were always included to check on the performance of the assay and to position the signal thresholds. To avoid cross-contamination sample tubes were prepared first, followed by no template, negative and positive control tubes. A sample was diagnosed as male when both a FAM-signal for the *UBC* amplicon (autosomal target) and a TxRd-signal for the *ETSTY5* amplicon (Y-specific target) were detected, and as female when only the FAM-signal for the *UBC* amplicon (autosomal target) was detected (Fig. 2B). We also checked on potential DNA impurities in the Ca^{2+}/Mg^{2+}-free PBS with 1% (v/v) PVP in Ca^{2+}/Mg^{2+}-free PBS and handling medium used during the biopsy process, using 2 μL and 1 μL as a template, respectively.

Validation of analysis results and effect of the biopsy on embryo viability

For embryos produced from *post mortem*-collected oocytes, the result of the assay on the trophectoderm biopsies and, when present, herniating cells, was validated by the result of the assay (see Section '*Assay design and optimization*' and Table 1) on a second sample of the corresponding biopsied embryo (whole biopsied embryo, 5% of the whole biopsied embryo, herniating cells or second trophectoderm biopsy) (Fig. 1).

Initially, all biopsied embryos were collected for validation purposes by two washes and subsequent transfer to a 0.2 mL tube with 2 μL 1% (v/v) PVP in Ca^{2+}/Mg^{2+}-free PBS on ice using a 200 μm capillary and stored at −80 °C. In two embryos, the zona pellucida was removed by incubation in warm 0.1% (v/v) pronase (P8811; Merck KGaA, Darmstadt, Germany; with Hank's salts) dissolved in TCM-199 and subsequent washing in handling medium and Ca^{2+}/Mg^{2+}-free PBS with 1% (v/v) PVP, while removing the zona by pipetting with a 200 μm capillary. qPCR analysis was subsequently performed on whole biopsied embryos without upfront isolation of the DNA. Because such analysis was frequently unsuccessful due to inhibition (see Section '*Application on equine embryos*'), we continued validating the results of the trophectoderm biopsies and herniating cells with the assay result on either a second biopsy (taken at the same time as the first biopsy) or, on 1 μL of the whole biopsied embryo, lysed in 20 μL 1% (v/v) PVP in Ca^{2+}/Mg^{2+}-free PBS after a 15 min heat treatment at 95 °C (5% of the whole biopsied embryo).

For embryos produced from *in vivo*-collected oocytes, the result of the assay on the trophectoderm biopsies was validated by the ultrasound-determined sex of the foal after transfer (Fig. 1) and, by the phenotypical sex at birth. To this end, biopsied embryos were vitrified individually in a minimal volume on a Cryolock (CL-R-CT-C; Irvine Scientific, Santa Ana, CA, USA) and warmed as described by Choi et al. (2011) on a 39 °C heated stage and subsequently, transferred non-surgically into the uterus of day-four synchronised recipient mares during the reproductive seasons of 2021 and 2022. To determine the effect of the needle aspiration biopsy procedure on the viability of the embryos, pregnancy rate 7 days *post*-transfer and early pregnancy loss (detected between day 25 and 45 of gestation) following transfer of biopsied embryos were determined and compared to the results all non-biopsied embryos of the same age (day 8–11) and morphological grade, that were vitrified-warmed by the same protocol and produced, transferred and followed-up within the same period (reproductive seasons of 2020–2021 and 2021–2022) within the commercial OPU-ICSI program at Ghent University. Ultrasound-based sex determination was performed based on the location of the genital tubercle between 60 and 70 days of gestation (Curran and Ginther, 1989) and or on the identification of the reproductive organs between 120 and 210 days of gestation as described by Van de Velde et al. (2018). The operator was blinded to the results of the qPCR assay for the corresponding embryo biopsy.

Results

Assay design and optimisation

ETSTY5 was chosen as Y-specific target region, because it contains a 791-bp region in eight highly conserved copies on the Y-chromosome (Janecka et al., 2018), while no parts were matching with autosomal DNA. None of the other candidate genes contained such a long Y-specific sequence with so many conserved copies. For both the autosomal (*UBC*, four copies) and Y-specific

(*ETSTY5*, eight copies) target regions, a qPCR assay with a dual-labelled probe was designed with primers/probe matching 100% with all copies at a similar melting temperature and generating/detecting the intended amplicon of similar length, enabling multiplexing.

During optimisation with genomic DNA isolated from male and female blood samples, gradient-qPCR showed that an annealing temperature of 68 °C yielded optimal results. The autosomal target (*UBC*, FAM-channel) was detected in both male and female samples, while the Y-specific target (*ETSTY5*, TxRd-channel) was only detected in the male sample (no template controls (**NTCs**) were negative for both assays). Agarose gel electrophoresis confirmed that both assays only amplified the intended fragment of 147-bp (*UBC*) and 141-bp (*ETSTY5*). By performing the assay on a 1/10 dilution series of male and female DNA (range: 10 ng–10 pg), we showed that both assays are very sensitive, generating signals down to 10 pg of template DNA (Fig. 2A), with qPCR efficiencies higher than 95%. It is interesting to note that the TxRd-signal (Y-target) crosses the threshold a bit earlier (=lower quantification cycle (**Cq**)) than the FAM-signal (autosomal target), as it might help in calling signals at the detection limit. For samples containing ≥10 pg of purified DNA, signal calling is straightforward (both signals for a male and only the FAM-signal for a female). However, for samples containing less DNA or inhibitors lowering PCR efficiency, signals will be at the detection limit and should be interpreted with caution. To be able to interpret these signals better, the assay was performed for 50 cycles instead of the usual 40 cycles. Samples with only a TxRd-signal (Y-target) at the detection limit are suggestive of a male because the FAM-signal (autosomal target) should have crossed the threshold a bit later, but in this case, it did not because the detection limit was not reached. Samples with only a FAM-signal (autosomal target) at the detection limit are suggestive of a female because although the FAM-signal was reaching the detection limit, the male-specific TxRd-signal should have crossed the threshold before the FAM-signal did. Final relative fluorescence unit (**RFU**) signals were higher when using opaque tubes, but because Cq-values were similar, they did not make the assays more sensitive. The assay was also performed on 1 ng of human DNA, but no signals were generated as predicted by Primer-BLAST.

Application on equine embryos

A total of 73 samples (trophectoderm biopsies, cells herniating from the ICSI hole or biopsied whole embryos) were collected from 44 *in vitro*-produced equine blastocysts (Fig. 1). Samples were analysed by qPCR, either directly (n = 70) or following heating and dilution to 5% (three biopsied whole embryos).

The validation rate of the qPCR assay was 100%, with sex results of all trophectoderm biopsies and herniating cells being correct when validation was possible. When qPCR was performed on trophectoderm biopsies, six out of 46 did not generate a signal. For 25 embryos, the sex determined by the qPCR assay on the trophectoderm biopsy was confirmed by the result of the remaining whole biopsied embryo (n = 10), the corresponding whole biopsied embryo and the herniating cells (n = 5), herniating cells only (n = 2), a second trophectoderm biopsy (n = 1) or ultrasound-based sex determination (n = 7). For the remainder of 13 embryos for which the sex was determined by qPCR analysis of the trophectoderm biopsy (for two embryos, a second trophectoderm biopsy was taken for validation of the result), the result could not be validated, either because the qPCR assay did not generate a signal for the corresponding whole biopsied embryo (n = 2, see section below), because the corresponding biopsied embryos were not transferred yet or sold (n = 6) or, because pregnancies were lost following embryo transfer before the first window in which validation by ultrasound-based foetal sexing could be performed (n = 5). When qPCR was performed on the eight herniating cell samples, one out of eight remained undiagnosed. In the latter case, the number of herniating cells was limited and the sample showed a low-intensity signal after more than 45 cycles which was doubtful as a stand-alone result. For seven embryos, the sex determined by the qPCR assay on herniating cells was confirmed by the result of the corresponding whole biopsied embryo and the trophectoderm biopsy (n = 5) or the trophectoderm biopsy alone (n = 2). The diagnostic and validation rates of the assay on trophectoderm biopsies and herniating cells are shown in Table 2. Ca^{2+}/Mg^{2+}-free PBS and handling media used during the biopsy process did not generate a qPCR signal.

Table 2
Diagnostic rate and validation of the qPCR assay when applied on equine trophectoderm biopsies and herniating cells.

Sample	n	Diagnosis	Validation
Trophectoderm biopsy	46	40 (86.9%)	25/25 (100%)
Herniating cells	8	7 (87.5%)	7/7 (100%)

Diagnostic rates are presented as samples with a sex diagnosis on the total number of samples. Validation rates are presented as samples with a correct sex diagnosis on the total number of samples that could be diagnosed and validated by analysis of at least one other sample of the same embryo (18 trophectoderm biopsy and seven herniating cell samples), or by ultrasound-based sex determination of the foetus following transfer of the biopsied embryo to a recipient mare (seven trophectoderm biopsy samples). n = number of samples analysed.

When applying the qPCR assay directly on whole biopsied embryos for validation of the results of the trophectoderm biopsies or herniating cells, three out of 16 whole biopsied embryo samples did not generate a qPCR signal. The sex of one additional sample could not be determined because a low-intensity signal presented after more than 45 cycles, which was considered too doubtful as a stand-alone result. For all four whole biopsied embryos of which the sex could not be determined, qPCR analysis of both the trophectoderm biopsy and when present, herniating cells (n = 2) did generate a signal. In addition, for two out of 12 embryos with sex diagnosis, amplification of the trophectoderm biopsy and herniating cells (n = 1) or the herniating cells alone (n = 1) occurred faster, notwithstanding they contain fewer cells. Non-amplification also occurred for one out of two embryos from which the zona pellucida was removed, excluding it as the sole reason for non-amplification. In subsequent experiments, we found inhibition of the amplification reaction when too much embryonic material was used as a template (25–75% of denatured embryonic material), which could be solved by dilution of the sample (to 2.5–10% of denatured embryonic material; results not shown). Therefore, we decided to continue validating the biopsy results on a second biopsy of the same embryo (n = 2) and later on 5% of the whole biopsied embryo, which all generated a result (n = 3).

About half of the embryos for which the sex of the trophectoderm biopsy could be validated were female and male (Fig. 2B, Table 3). The same was observed for the sex distribution of all embryos of which the sex could be determined based on qPCR of at least one sample or following transfer and ultrasound (41 out of 44, Table 3).

Viability of biopsied embryos

Pregnancy results of biopsied and non-biopsied *in vitro*-produced embryos are shown in Table 4. Out of 23 embryos of which a trophectoderm biopsy was taken, eight were not transferred or sold. The initial pregnancy rate and early pregnancy loss of 15 transferred embryos were similar compared to embryos without trophectoderm biopsy. Additionally, we know of two pregnancies from a biopsied embryo that were lost at a later stage, one

Table 3
Sex ratio of biopsied equine embryos.

Embryo sex determination	n	Male	Female
Trophectoderm biopsy and validation	25	12 (48.0%)	13 (52.0%)
Trophectoderm biopsy and/or herniating cells and/or (transferred) whole biopsied embryo	41	19 (46.3%)	22 (53.7%)

n = number of embryos.

Table 4
Initial pregnancy rate and early pregnancy loss of equine *in vitro*-produced embryos that underwent or did not undergo a trophectoderm biopsy.

Embryo	Pregnancy rate	Early pregnancy loss
Trophectoderm biopsy	13/15 (86.7%)	3/13 (23.1%)
No biopsy	71/118 (60.2%)	17/57 (29.8%)

Pregnancy rate was determined as the number of pregnant recipients on the total number of transferred embryos on day 7 *post*-transfer. Early pregnancy loss was determined as the number of pregnancies lost as detected between 25 and 45 days of pregnancy on the total number of pregnant recipients of which a follow-up was performed.

between day 45 and day 60 of gestation, and one in the last trimester of gestation. However, as pregnancies are ongoing, data beyond 60 days of gestaion are incomplete at the time of writing. No twin pregnancies occurred. The sex of foals that were born already confirmed the result of the biopsy.

Discussion

In this study, a highly sensitive and specific equine sex determination assay was developed, allowing for reliable sex determination of embryos based on herniating cells or a trophectoderm biopsy. The collection of the latter type of sample by a needle aspiration biopsy procedure did not have an observed negative effect on the early pregnancy outcome of *in vitro*-produced embryos.

Equine sex determination was improved by optimising both assay design strategy and methodology. Thanks to the availability of the equine Y-chromosome sequence (Janecka et al., 2018), analysis of equine-specific multicopy genes from the male-specific region on the Y-chromosome (Paria et al., 2011) has become more accurate in terms of copy number and conservation rate. This allowed us to design an assay amplifying eight copies of a Y-specific region, making the assay eight times more sensitive compared to an assay targeting a single-copy gene. By combining this assay with an assay detecting an autosomal control region (present in both male and female samples), the presence and the amplifiability of the DNA (influenced by DNA concentration, DNA degradation and/or presence of PCR inhibitors) were tested. As such, an internal control was included in every assayed sample, without the need of splitting the sample as would be needed in two monoplex assays. By using the *UBC* monomers as autosomal target (four copies per chromosome), a similar sensitivity was achieved for both the autosomal and Y-specific assay. Using purified genomic DNA as a template, successful assays could be performed down to 10 pg, being the DNA equivalent of less than two cells (Fig. 2A). Although the actual *ETSTY5* copy may vary among individuals (Castaneda et al., 2022), we generally observed slightly lower Cq-values for the *ETSTY5* assay compared to the *UBC* assay in our male samples. Because both signals are generated in the same tube with similar PCR efficiencies, the number of targeted *ETSTY5* copies should be at least as high as the number of targeted *UBC* copies. Furthermore, even in the case of a potential halving of the *ETSTY5* copy number, the *ETSTY5* signal would only be delayed with one Cq, which would affect only male samples with signals at the detection limit.

Due to the high sensitivity of the qPCR-based assay, it is important to minimise the chance that accidental contamination can influence the outcome. To this end, we designed a qPCR assay with dual-labelled probes in order to detect both target regions in a single closed-tube assay without post-PCR manipulation. By performing sequence comparisons using *in silico* algorithms, we could design primers/probes that were unable to detect human DNA, which was experimentally confirmed with 1 ng of purified human DNA. This means that, when following good laboratory practices, operators should not be able to bias the results by accidental contamination with their own genetic material. Contamination was further avoided by aliquoting products for single run use, minimised opening of the tubes and by the inclusion of negative and no template controls. For the analysis of embryonic cells, the risk of contamination or sample loss was further reduced by the release of DNA from the cells during the first step of the qPCR assay (15′ at 95 °C), also avoiding additional manipulations, expenses and time required for DNA extraction. In addition, the possibility of contamination by the media used during embryo manipulation (Ca^{2+}/Mg^{2+}-free PBS and handling media) was assessed, and negative. The specificity of the qPCR assays was enhanced by designing primers/probes that were, according to sequence comparisons using *in silico* algorithms, able to exclusively amplify the intended equine regions, a specificity that was further enhanced by using dual-labelled probes. We indeed experimentally confirmed that both assays were highly specific, with PCR efficiencies >95%.

Applied for equine embryonic sex determination, the biopsy procedure and analysis with the qPCR assay could be performed in less than two hours. A validation rate of 100% was obtained, solving the problem of invalid, predominantly false negative results, reported by earlier studies (Herrera et al., 2014; Riera et al., 2019). Yet, despite the high sensitivity of the assay, part of the trophectoderm biopsies and herniating cells did not amplify and thus, remained undiagnosed. Tubing errors seem unlikely to explain all undiagnosed samples, as for some biopsies that subsequently failed diagnosis it could be visually confirmed that the biopsies went into the micropipette used for tubing. More likely, the qPCR assay may be inhibited by the presence of embryonic material other than DNA, including cellular compounds, intercellular matrix components and blastocoel fluid aspirated during the biopsy procedure. When analysing biopsied whole embryos with the qPCR assay, an amplification signal was lacking or delayed as compared to the trophectoderm biopsies or herniating cells from the same embryo in six out of 16 embryos, regardless of the presence of many cells and thus template DNA. The problem could be solved completely by upfront dilution of the sample. Whether the qPCR successfully amplifies the DNA in trophectoderm biopsies or herniating cells may therefore depend on the relative amount of embryonic material other than template DNA, which inhibits template DNA amplification and/or lowers the qPCR efficiency.

In future application, inhibition of the qPCR could be avoided by an optimised template, with five to 10 cells and a minimal amount of other embryonic material. Due to the simultaneous aspiration of embryonic material conjunctively with embryonic cells during a needle aspiration biopsy, it is difficult to determine the exact number of cells within the biopsy under the microscope. To increase the cumulative chance of successful sex determination and to verify signals at the detection limit, a biopsy of 10–20 cells, as we aimed for here and which did not impair early pregnancy outcomes, could be divided into two samples. Alternatively, some technical aspects of the qPCR reaction could be adapted. A first includes an increased qPCR volume (e.g., 20 µL), which would result in the dilution of inhibiting factors. A second includes a pretreatment of the sample. Here, an initial heating step was required to activate the poly-

merase. Such heating results in lysis of the cells resulting in an increased availability of the DNA, which has previously been shown to increase the diagnostic rate (Herrera et al., 2014). An upfront proteinase K treatment and DNA isolation, in which potentially inhibiting proteins are additionally lysed and removed, could be even more successful. Proteinase K treatment has been performed in other studies attempting equine embryonic sex determination, reporting a good diagnostic rate (Peippo et al., 1995; Huhtinen et al., 1997; Lazzari et al., 2020). However, upfront DNA isolation is more expensive and time-consuming, as it comes with additional manipulations, increasing the possibility of contamination and sample loss. This is also true for a preamplification step, which is necessary when multiple assays on the sample are intended, as performed in the studies of Choi et al. (2010, 2015) and Guignot et al. (2015).

Equine embryonic sex determination was performed on trophectoderm cells, collected via a needle aspiration biopsy and on cells, herniating from the ICSI hole. As far as the authors are aware of, this is the first study reporting pregnancy results of transferred vitrified-warmed *in vitro*-produced equine embryos that underwent a trophectoderm biopsy by needle aspiration. Similar to Lazzari et al. (2020), who collected herniating cells from *in vitro*-produced embryos, we did not find a negative effect on the initial pregnancy rate. Additionally, early pregnancy loss was similar to that of non-biopsied embryos. Furthermore, a 100% validation rate was retrieved for both the results of herniating cells and biopsy samples. Thus, both the collection of embryonic cells via a needle aspiration biopsy or, via the collection of herniating cells are possible to determine the equine embryonic sex and support pregnancy for *in vitro*-produced embryos. Therefore, the hole in the zona pellucida resulting from the ICSI or biopsy procedure does not prohibit the viability of the *in vitro*-produced embryos. This is different from the observation in *in vivo* embryos where damage to the capsule results in decreased pregnancy rates (Guignot et al., 2015; Huhtinen et al., 1997; McKinnon et al., 1989; Müller and Cikryt, 1989; Seidel et al., 2010; Skidmore et al., 1989; Stout et al., 2005). In *in vitro*-produced embryos, the confluent capsule is only formed after transfer to the uterus (Choi et al., 2009). Nevertheless, the number of transferred biopsied embryos was limited and therefore the detection of smaller effects on embryonic and foetal viability would require the transfer of a larger number of embryos. Also the potential effect on later pregnancy losses remains to be determined further. A potential risk of the hole in the zona, made by ICSI or the biopsy procedure includes the induction of twin pregnancies, which are largely incompatible with equine pregnancy (Jeffcott and Whitwell, 1973). An increased number of twin pregnancies has been reported for *in vitro*-produced equine embryos, which have hitherto been derived by ICSI (Dijkstra et al., 2020). Also in humans, where embryos are produced *in vitro* following both ICSI and regular *in vitro* fertilisation, twin pregnancies in humans may be related to the manipulation of the zona pellucida, although the evidence is controversial (Jauniaux et al., 2013; Lacey et al., 2021). Recent development of *in vitro* fertilisation in horses (Felix et al., 2022) generates the opportunity to produce embryos with an intact zona pellucida and may facilitate research regarding the effect of a biopsy- or ICSI-derived hole in the zona pellucida.

The collection of herniating cells could be preferred over the needle aspiration biopsy, because puncturing of the embryo and thus, potential damage to the inner cell mass is avoided that way. This is especially true for *in vitro*-produced embryos in which the inner cell mass cannot be visualised and for which an upfront blastocoel collapse is not required prior to cryopreservation. Moreover, the risk of inhibiting the qPCR assay might be lower because these cells are most likely viable and isolated from the cell debris and blastocoel fluid within the zona pellucida. In one study, where many embryos were sexed by analysing herniating cells, a high diagnostic rate was reported (94%) (Lazzari et al., 2020). Here, the analysis of herniating cells was successful too, but this strategy could only be applied to one-third of biopsied embryos which presented them. Another, less invasive strategy allowing to minimise the aspiration of blastocoel fluid and applicable for embryos without herniating cells at the time of trophectoderm delineation, including also *in vivo*-produced embryos, involves the collection of trophectoderm from the periphery of the embryo (Choi et al., 2011). Yet, for this strategy, a piezo-drill system would be required and overall, our results indicate that the applied aspiration procedure did not reduce embryo viability.

Here, morphologically normal *in vitro*-produced blastocysts of all ages were biopsied on the day of trophectoderm delineation. Concerns have been raised about the biopsy effect on the viability of early-stage blastocysts, as these embryos contain fewer cells and consequently, a larger portion of cells is removed. *In vitro*-produced embryos, especially, could be more affected as they contain fewer and more apoptotic cells as compared to their *in vivo* counterparts (Pomar et al., 2005; Rambags et al., 2005; Smits et al., 2011; Tremoleda et al., 2003). Here, the biopsy procedure on *in vitro*-produced embryos did not impair early pregnancy rates. Extended culture or, alternatively, ICSI with a larger needle could increase the number of cells and embryos with herniating cells. However, we prefer to biopsy and vitrify the *in vitro*-produced embryos as soon as trophectoderm delineation becomes clear because prolonged culture may negatively affect the embryo viability (Ducheyne et al., 2019; Lewis et al., 2023) and, similar to what is observed for *in vivo*-derived embryos, a large volume of blastocoel fluid may compromise the vitrification process (Choi et al., 2011). Furthermore, younger blastocysts facilitate the biopsy procedure and might result in the collection of more cells and less inhibiting material. That is because less blastocoel fluid and intercellular connections are present in young blastocysts, and thus, cells are less firmly attached to each other. The same has also been postulated for the aspiration biopsy of *in vivo*-derived embryos (Huhtinen et al., 1997; Jarazo et al., 2012), implying an early embryo flush. The inclusion of blastocysts of all ages for sex determination in the present study may further explain why the sex of *in vitro*-produced embryos was not skewed towards male embryos, in contrast to earlier studies. Embryos developing at high speed are more frequently male (Claes et al., 2020) and aforementioned studies (Claes et al., 2020; Claes and Stout, 2022) reported results based on the number of fillies and colts born resulting from the transfer of exclusively young embryos (up to days 8 or 9 post-ICSI).

In conclusion, here we report a fast, safe, reliable, relatively non-invasive, and cost-effective method for sex determination of equine embryos based on a trophectoderm aspiration biopsy or herniating cells applying a qPCR assay. Future application in OPU-ICSI labs and large-scale embryo transfer programmes will point out if the assay results in improved sexing of *in vitro* and *in vivo*-derived equine embryos and if trophectoderm aspiration biopsy of *in vitro*-produced embryos affects the conceptus' viability later in gestation. In addition, our assay can be used for different applications of equine sexing, including non-invasive prenatal sexing through the detection of foetal-derived DNA in pregnant mare serum. Studies have been initiated in the horse (de Leon et al., 2012; Kadivar et al., 2021, 2016; Tonekaboni et al., 2020), based on the success of non-invasive prenatal testing in women (Ghiasi et al., 2023). Yet, clinical application in horses will require a robust

detection method, to which our assay, amplifying small sequences which typically make up the majority of circulating cell-free DNA (Chan et al., 2004; Fan et al., 2010), may contribute.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100952.

Ethical approval

Ethical approval was granted by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University for the collection of blood from mares (reference number 2021-094). Further ethical review and approval were waived for this study since embryonic samples are no subject to ethical approval and transfer procedures were executed as part of a clinical programme.

Data and model availability statement

None of the data was deposited in an official repository, but data are available upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Author ORCIDs

T.D.C.: https://orcid.org/0000-0002-0415-2744
M.V.P.: https://orcid.org/0000-0002-4316-8984
J.G.: https://orcid.org/0000-0002-0385-4170
O.B.P.: https://orcid.org/0000-0002-5305-2133
D.A.V.: https://orcid.org/0000-0001-8777-4065
M.P.: https://orcid.org/0000-0001-8610-2734
J.G.: https://orcid.org/0000-0002-0385-4170
L.P.: https://orcid.org/0000-0003-3853-5310
J.R.V.: https://orcid.org/0000-0002-3071-1191
A.V.S.: https://orcid.org/0000-0001-5010-6311
K.S.: https://orcid.org/0000-0002-8205-3725.

Author contributions

Tine De Coster: Conceptualisation, Methodology, Equine *in vitro* embryo production, Wet lab, Formal analysis, Writing and original draft preparation, Writing, review and editing. **Mario Van Poucke:** Conceptualisation, Methodology, Primer design, Wet lab, Validation, Writing, review and editing, Supervision. **Osvaldo Bogado Pascottini:** Writing, review and editing, Formal analysis. **Daniël Angel-Velez:** Equine *in vitro* embryo production, Writing, review and editing. **Emma Van den Branden:** Equine clinical work. **Sofie Peere:** Equine clinical work. **Marion Papas:** Equine clinical work, Writing, review and editing. **Ilse Gerits:** Equine clinical work. **Jan Govaere:** Equine clinical work, Writing, review and editing, Resources. **Luc Peelman:** Writing, review and editing, Resources. **Ann Van Soom:** Writing, review and editing, Resources. **Joris Robert Vermeesch:** Writing, review and editing, Resources. **Katrien Smits:** Conceptualisation, Equine *in vitro* embryo production, Writing, review and editing, Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of Interest

The authors declare no conflict of interest.

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