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Review

Recent developments in embryo sexing and its field application

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Abstract — This review focuses on polymerase chain reaction (PCR) sexing of bovine embryos in commercial situations with emphasis on new developments. Simplifications of the biopsy technique is one of the major simplifications over the last few years. The stabilization of the embryo by means of protein-free medium or scratches produced on the bottom of the Petri dish makes it possible to perform a biopsy with a single microinstrument. The traditional PCR sexing approach utilizes electrophoresis, which involves the risk of deoxyribonucleic acid (DNA) contamination of subsequent assays. Such contamination, resulting in females misdiagnosed as males, is avoided efficiently by using a non-electrophoretic method in which the sex is determined based on fluorescence of unopened tubes. However, female samples cannot be distinguished from blank samples in the non-electrophoretic assay, which thus relies on accurate transfer of biopsy into tubes. Nevertheless, an accuracy of about 95 % can be reached with both approaches. High pregnancy rates (50–70 %) can be reached with biopsied Grade 1 embryos, but there is evidence that pregnancy rates with Grade 2 embryos is 15–20 % lower. Recent data indicate that pregnancy rates of 50 % can be achieved with frozen-thawed biopsied Grade 1 embryos. In conclusion, recent developments in biopsy techniques, detection systems and freezing should increase interest in PCR sexing. © Inra/Elsevier, Paris.

PCR / sex determination / bovine embryo / biopsy / freezing

Résumé — Récents développements du sexage d'embryons et applications sur le terrain. Cette revue porte sur le sexage des embryons bovins par PCR en situation de pratique commerciale, et en particulier sur les nouveaux développements. Les simplifications de la technique de prélèvement des biopsies sont l'une des améliorations majeures de ces dernières années. L'immobilisation de l'embryon dans un milieu sans protéines ou par rayures sur le fond de la boîte de Petri permet d'effectuer la biopsie avec un seul micro-instrument. La technique traditionnelle de sexage par PCR utilise l'électrophorèse, dont l'emploi implique le risque de contamination de l'ADN pour les essais suivants. Cette contamination, entraînant des faux positifs, est évitée en utilisant une méthode non électrophorétique dans laquelle la détermination du sexe est basée sur la fluorescence émise à travers des tubes fermés. Cependant, les biopsies femelles ne peuvent être distinguées des blancs par cette méthode, qui dépend donc d'un transfert précis des échantillons dans les tubes. Néanmoins, une précision

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d'environ 95 % peut être atteinte avec les deux méthodes. Le succès des gestations est de 50 à 70 % après biopsie d'embryons de qualité 1, mais il est inférieur de 15 à 20 % avec des embryons de qualité 2. Des résultats récents montrent qu'il est possible d'obtenir 50 % de gestations après biopsie et décongélation d'embryons de qualité 1. En conclusion, les développements récents des techniques de biopsie, de détection et de congélation devraient susciter un renouveau d'intérêt pour le sexage par PCR.

PCR / sevrage / embryon bovin / biopsie / congélation

1. INTRODUCTION

Several approaches have been used to determine the sex of bovine embryos prior to embryo transfer. One of the first was by 1) cytology, in which the gender was determined by observing the sex chromosomes from metaphase spreads of a biopsy. Although the accuracy of this method is virtually 100 %, the efficiency (proportion of embryos that can be determined) remains at a dissatisfactory level. Furthermore, the technique requires more skill than other methods. In the 1980s, 2) the detection of the HY-antigen on the surface of male mouse embryos led to high expectations. This non-invasive technique is simple to perform and rapid enough to allow field use. Although the accuracy with cattle embryos is at best 80–90 % [1], this method is not shown repeatable [24]. Another approach is to distinguish the sex of embryos based on 3) the difference in metabolism between the sexes. Prior to the inactivation of one of the X-chromosomes in the female, the expression of X-linked genes is expected to be higher than in males. One such gene is the G6PDH (glucose-6-phosphate dehydrogenase), the expression of which can be measured by means of a color reaction. While this method does not require a biopsy it has yet to be proven accurate enough. Moreover, it may not be suitable for embryos that have developed beyond the mid-blastocyst stage as these embryos are already undergoing X-inactivation. Sex diagnosis utilizing 4) deoxyribonucleic acid (DNA) technology has received special attention especially since the development of the polymerase chain reaction (PCR). Sexing by PCR has resulted in acceptable accuracy, efficiency and speed with the major disadvantage being the requirement for biopsy. Embryo sexing methods preceding the PCR era are reviewed by VanVliet et al. [23]. The present review focuses on experiences in PCR sexing of bovine compacted morulae and blastocysts.

2. BASICS OF PCR

PCR is a technique used to amplify specific stretches of DNA and consists of several cycles of three steps: template denaturation, primer annealing and primer extension [19]. Following the release of genetic material from the cells, the complementary DNA strands are separated by heat denaturation (> 90 °C). On cooling to 50–65 °C, two sets of primers (short synthesized DNA sequences) will specifically anneal to the target DNA. The two sets of primers are designed to bind one on each strand of the template usually less than 500 base pairs apart.

The temperature is then raised to 75 °C, the optimal temperature for heat resistant polymerases which, using free single nucleotides, start to synthesize complementary DNA from the primer-template binding sites (primer extension). One primer is oriented so that its extension creates a new binding site for the other primer and vice versa. Thus, in each cycle the amount of

template (PCR product) is doubled. In theory, 30 cycles will result in a billion-fold amplification. Normally, the PCR products are separated and identified by electrophoresis using agarose gels stained with a DNA-specific dye, ethidium bromide.

The high sensitivity of PCR has made it suitable for a preimplantation diagnosis where only a small number of cells can be sacrificed for analysis. On the other hand, the high sensitivity makes this approach susceptible to DNA contamination. In a sexing assay (where the target and product is Y-chromosomal DNA) females would then be diagnosed as males.

3. PCR SEXING OF EMBRYOS

3.1. Biopsy

With compacted morulae the orientation of the embryo is of no relevance. However, from blastocysts the biopsy must be taken from the trophectoderm, leaving the inner cell mass intact. Dead or extruded cells may be included in the biopsy. Since these cells may have degraded DNA (either as a result of or a cause of this abnormality), it is recommended that cells from the embryo itself be included in the biopsy.

The size of the biopsy is a compromise between sexing efficiency and embryo viability. A small biopsy tends to get lost more easily, by possibly sticking to instruments used for biopsy handling. On the other hand, a large biopsy may compromise embryo viability. Lacaze et al. [14] sexed embryos in the field and concluded that the sexing efficiency was significantly lower when biopsy size was two to five cells compared to six to 15 cells, yet they found no influence of biopsy size on pregnancy rate.

Considering the high pregnancy rates achieved following the transfer of demi-embryos [5, 12, 15, 21], a biopsy size representing 10–30 % of the cell mass should be compatible with acceptable pregnancy rates and high efficiency of sexing. However, when ideal conditions cannot be met (due, for example, to prolonged embryo storage, freezing, suboptimal embryo recipient status), a large biopsy size should be avoided.

Many microsurgical procedures require the use of a holding pipette to stabilize the embryo. This is also the case if biopsy by aspiration is preferred. The advantage of aspiration is that a very small sample can be taken with minimal damage to the embryo and zona pellucida. However, an aspirated biopsy may be more difficult to handle (more easily lost) as evidenced by a loss in sexing efficiency [6, 22]. This reason, together with the fact that aspiration may be technically too demanding for widespread implementation by ET practitioners, has made microblade biopsy (microsection) the popular choice. With some experience, about 15 embryos can be microsected in 1 h.

Biopsy of an embryo by means of a vertical movement with a microblade can be done simply by stabilizing the embryo using protein-free medium [9] or by producing scratches on the bottom of the dish, which prevents slippage of the embryo when it is being cut [2]. In both cases only one micromanipulator is used, although a manual biopsy technique has also been developed [3]. The advantage of the scratched-bottom technique is that only one medium, an embryo-holding medium proven free of bovine DNA, can be used for biopsies as well as for other previous and subsequent handlings of the embryo and biopsy. The advantage of the protein-free biopsy medium is that time is saved as the dishes need not be prepared prior to biopsy.

The microblade should be cleaned between each biopsy because debris attached to the blade may contain DNA. This can be done by washing the blade sequentially in distilled water, 70 % alcohol and phosphate buffered saline. An alternative is to clean the blade with a cotton swab moistened in alcohol.

The transfer of the biopsy into the PCR reaction tube is a critical step in PCR sexing. After performing the biopsy it is important to aspirate the biopsy in a medium with a good surfactant to avoid sticking of the cells to the tip. In addition to synthetic alternatives (e.g. polyvinyl alcohol) proteins can be used. One should, however, be careful in using proteins of bovine origin because these may contain trace amounts of male bovine DNA [10]. The choice of pipette tips may be important and siliconizing is recommended at least if glass capillaries are used.

3.2. Lysis

DNA can be released from the blastomeres in various ways. A rapid method is to snap-freeze the biopsies in liquid nitrogen followed by heating at 95 °C. One disadvantage of this approach is that intact nucleases may also be released from the cells. Nucleases can destroy the DNA unless appropriate measures are taken. Lysis by proteinase K will circumvent this problem as this enzyme inactivates nucleases in addition to releasing DNA. Furthermore, proteinase K alone will not release DNA from frozen-thawed spermatozoa, which may occur with chemical or physical methods. Hence, spermatozoa are unlikely candidates for contamination when proteinase K is used for DNA lysis. With this enzyme a 5-min digestion at 55 °C followed by a 5-min inactivation at 98 °C is sufficient to release DNA from blastomeres.

3.3. PCR and electrophoresis

The traditional PCR approach involves the co-amplification of a Y-chromosomal sequence and an autosomal sequence which acts as a control for the presence of biopsy and appropriate conditions for amplification [17]. The PCR is designed to yield different fragment sizes for the Y-chromosomal and the autosomal product.

In a PCR cycle the primer annealing temperature is optimized for each protocol. A too low annealing temperature will allow the primers to bind to non-specific sites, which can lead to amplification of other fragments rather than the intended ones. For the same reason starting a PCR at room temperature will not work in some protocols. Therefore, PCR is often performed with a 'hot start', i.e. either one or a few necessary ingredients are added to the reaction tubes during the first, prolonged denaturation step.

Typically, a preimplantation embryo PCR assay consists of 30 to 50 PCR cycles. With the most rapid conventional-type thermocyclers sufficient amplification can be completed within 1 h, and with air thermal cyclers using glass capillaries as reaction vessels, in 30 to 45 min (P. Bredbacka, unpublished data). With the air thermal cyclers, the advantage in speed is at least partially offset by other factors, such as the need to heat-seal the glass capillaries.

The common way to detect products generated by PCR is to perform electrophoresis on agarose gels stained with ethidium bromide. The DNA fragments can be seen as pink/orange bands under ultraviolet illumination. The lengths of the DNA fragments are determined by comparing the migration rate to that of known standards in adjacent lanes. Excluding the time for applying samples to the gel, electrophoresis usually takes at about 15 to 45 min, depending on the resolution required for reliable separation and identification of products.

Unfortunately, electrophoresis increases the risk of contamination. Although other sources of contamination are possible (e.g. carry-over of cellular material via the biopsy instrument), PCR products from previous assays represent the greatest risk of contamination [9]. It is almost impossible to ensure that no amplified DNA fragments are released into the environment between the time of opening the reaction tubes and the end of electrophoresis. Even single copies of Y-chromosomal DNA will be

amplified if they end up in a sample tube used in a subsequent assay. Hence, contamination is associated with an increased incidence of females diagnosed as males.

To minimize the contamination risk, diagnostic laboratories have taken measures such as separating the laboratory into pre- and post-amplification areas. This is, however, sometimes difficult to apply in small laboratories and especially when sexing is performed on the farm.

To decrease the risk of contamination under farm conditions, it is important to 1) separate the equipment for PCR and electrophoresis, 2) remove (and pack) all PCR equipment, including microscopes, micromanipulators, pipettes, thermal cycler, etc., before the PCR tubes are opened for electrophoresis, and 3) avoid overloading the wells when applying the samples to minimize buffer contamination. It is equally essential to 4) dispose of reaction tubes, pipette tips, gel and buffer immediately after use, and 5) carefully clean all equipment that is to be reused in electrophoresis, preferably using chemicals designed for DNA decontamination.

A sexing assay based on PCR and electrophoresis can be completed in about 1.5 h (60 min for PCR, 30 min for electrophoresis) plus the time needed for biopsy.

3.4. Non-electrophoretic PCR sexing

Non-electrophoretic PCR sexing [3] is a simplification of the standard PCR-sexing protocol. In this assay the electrophoretic step is omitted. The reaction mixture includes ethidium bromide as an amplification marker. The sex can thus be diagnosed immediately after PCR using an ultraviolet illuminator. Pink fluorescence in the reaction tube indicates a male sample and no fluorescence indicates a female sample or the absence of a sample. Apart from less labor and less equipment needed, the main advantage is that the risk of contamination by previously amplified products is virtually negligible since the tubes do not need to be opened. In about 300 assays performed at EmTran, Inc., Elizabethtown, PA, USA blank controls never showed fluorescence while male controls always did [Hasler et al., in prep.]. The disadvantage of the assay in its present form is that the reaction mixture does not contain control primers. Consequently, the assay cannot distinguish between a female and a blank sample. For instance, a 5 % failure in inserting the biopsy into the tube would lead to 2.5 % misdiagnosed males. With an assay using control primers, the interpretation would be 5 % unambiguous determinations. With the original protocol the PCR amplification took about 2 h 15 min to be completed. Following a number of improvements the time required for amplification was reduced to 1.5 h (for use at test sites). Shorter amplification programs are currently being tested.

4. FIELD APPLICATIONS

Considering that PCR sexing has been performed commercially for nearly 10 years, the amount of published data is disappointingly limited. In many cases, however, accurate records may rely on whether the client has a need to supply calving data. Furthermore, as the results improve with experience, the companies that provide sexing services may be reluctant to release results until they are considered excellent. For these reasons an estimate of success is difficult to obtain.

4.1. Efficiency and accuracy

4.1.1. Sexing with electrophoresis

With product detection based on electrophoresis both efficiency and accuracy appears to be between 90 and 100 %. Field trials in France have resulted in 95 % efficiency and 98 % accuracy for microblade-biopsied embryos [13, 22]. Using an aspi-

ration technique for biopsy, Roschlau et al. [18] determined the sex in 91 % of the samples with a 96 % accuracy.

4.1.2. Non-electrophoretic sexing

Due to the nature of the non-electrophoretic assay, efficiency is 100 % with the only exception being the rare intermediate signals. With the latest version of the assay no intermediate signals have been detected in the last 1 000 assays [J.F. Hasler, pers. comm.]. The accuracy of the method depends strongly on successful transfer of the biopsy into the tube.

The first field trials of non-electrophoretic sexing were performed on farms in Finland and Estonia using manual biopsies [4]. Of the calves born, 18 were diagnosed as females and one as a male by PCR. All except one (a male diagnosed as a female) were correctly sexed (95 %).

At other test sites micromanipulators were used for biopsy. In one of the test sites (EmTran, Inc.) 145 pregnancies from transferred embryos sexed as females, a 92 % sexing accuracy was confirmed by ultrasound [Hasler et al., unpublished data]. The false-negatives were probably due to failure in successfully inserting the biopsies into the tube. These biopsies were handled using a positive displacement glass capillary, to which the biopsy may stick more easily than with a plastic tip. There is some indication that results improve when capillaries are siliconized.

The assumption that the choice of the type of pipette is critical in avoiding false-negatives is supported by the data at Premier Embryos in Ireland: in 1997 the proportion of embryos diagnosed as males was 47 % for in vivo embryos and 49 % for in vitro embryos. After changing from glass tips to plastic tips in 1998, the proportion of male-diagnosed embryos increased to 50 % (392 assays) for in vivo embryos and 55 % (605 assays) for in vitro embryos [P. Ryan, pers. comm.]. Although this change could be attributed to increased experience in biopsy handling, the sudden turnover to expected values suggests that plastic tips may be the better choice. At another test site (Holland Genetics, Beers) non-siliconized glass capillaries were used, but with a different pipetting technique than used at EmTran, Inc. All of the 22 first pregnancies (five males, 17 females) were correctly sexed as verified by ultrasound scanning. It is evident that the choice of the pipette and pipetting technique are the critical issues, especially in non-electrophoretic sexing. The different types of pipettes and techniques are currently under investigation and calving results should ultimately provide more precise guidelines for transferring biopsies into tubes.

4.2. Pregnancy rates

4.2.1. In vivo embryos

Thibier and Nibart [22] reported a 53 % pregnancy rate ($n = 301$) with microsected Grade 1 embryos. These embryos had been maintained about 6 h before transfer. By using Grade 1 microsected embryos for transfer, a pregnancy rate of 61 % ($n = 178$) was recorded at EmTran, Inc. [Hasler et al., unpublished data] and 68 % ($n = 197$) at Premier Embryos, Ireland (P. Ryan, pers. comm.). The higher pregnancy rates at the latter two sites may partly be attributed to a shorter holding time between biopsy and embryo transfer due to the more rapid sexing assay. The present speed of sexing allows biopsied embryos to be held at ambient temperature until sexing except in situations when a large number of embryo biopsies considerably prolongs the holding time. Storage at +4 °C may be preferable in situations when the holding time exceeds 6 h [22].

By using manual biopsies under farm conditions, a 66 % pregnancy rate ($n = 29$) was recorded [5]. In that trial, the embryos were of Grade 1 or Grade 2 quality with the

exception of one Grade 3 quality embryo. The embryo quality appears to have a significant effect on viability, at least when the biopsy is performed using a microblade. Thibier and Nibart [22] reported a pregnancy rate of 53 % with Grade 1 embryos, but only 34 % with Grade 2 embryos. A similar difference has been observed at EmTran: a 61 % pregnancy rate with Grade 1 embryos, but only a 45 % pregnancy rate with Grade 2 embryos [Hasler et al., unpublished data].

4.2.2. In vitro produced embryos

In vitro produced (IVP) embryos appear to survive biopsy reasonably well. At Premier Embryos, Ireland, a 46 % pregnancy rate was recorded following the transfer of 93 Grade 1 IVP blastocysts (P. Ryan, pers. comm.). Carbonneau et al. [6] reported a pregnancy rate as high as 66 % following the transfer of 65 IVP blastocysts. Nevertheless, embryo quality and, in contrast to in vivo embryos, the embryonic stage appears very critical when biopsying IVP embryos. Whereas the pregnancy rate was 51 % for Grade 1 blastocysts (n = 267), it was 29 % for Grade 2 blastocysts (n = 55) and only 5 % for Grade 1 morulae (n = 22) [Hasler et al., unpublished data]. In the in vitro culture system used, the morula/blastocyst ratio on the day of biopsy was only about 1:10. Therefore, the morulae may be considered retarded and the poor survival of morulae is possibly a consequence of this. Carbonneau et al. [6] compared the aspiration biopsy of day-5 IVP embryos with the microsection of day-7 IVP embryos and concluded that viability was better maintained in the latter case. This result also may imply that the developmental stage, rather than retardation alone, decreases the resistance of IVP embryos to microsurgery.

4.2.3. Cryopreservation and sexing

Pregnancy rates of about 45 % have been reported following the transfer of thawed embryos biopsied by aspiration prior to freezing [7, 17]. Despite some earlier concern about the freezability of microsected embryos [8], encouraging results have recently been obtained. The transfer of IVP embryos frozen after microblade biopsy resulted in a 53 % pregnancy rate (n = 141) [Hasler et al., unpublished data]. Furthermore, Nibart et al. [16] reported encouraging results on direct transfer of frozen-thawed microsected embryos. The choice of cryoprotectant (ethylene glycol) and the removal of a small biopsy (less than 10 % of embryo mass) may be factors particularly crucial in freezing embryos biopsied by microsection. Encouraging results have also been obtained with the transfer of embryos sexed after freezing and thawing: 31 pregnancies were established from 63 transfers [Hasler et al., unpublished data].

5. CONCLUDING REMARKS AND FUTURE PROSPECTS

The reports reviewed here demonstrate that PCR is a highly efficient and accurate approach for sexing bovine embryos. The success is, however, highly dependent on the performer. Before taking the technique to the commercial level, it must be noted that the time required for training should not be underestimated. Problems related to biopsy should be particularly considered during the training period. These include sticking of the biopsies to the bottom of the dish, the microblade and the aspiration pipette. A good way to evaluate success is to run assays on parallel samples from the same embryos. Skill in transferring the biopsy into the tube is essential.

With the electrophoretic approach a working routine must be established in order to avoid problems with contamination. Contamination is avoided with the non-electrophoretic assay, but the lack of control primers in the reaction tube stresses the importance of successful handling of the biopsy. The inclusion of a control primer in

a non-electrophoretic assay is, however, under investigation. Other issues that need to be further investigated include freezability of biopsied embryos and biopsy sensitivity of IVP morulae.

It is possible to determine other genetic traits of the embryo in combination with sex. In the future genetic markers associated with economic trait loci may be used in embryo assays. Designs to perform multiple locus assays have been devised [11, 20], but at present the assays involve a number of additional steps in comparison with a standard sexing assay, thus restricting their use to a laboratory environment. There are, however, traits such as genetic diseases that can be determined in combination with sexing under farm conditions. For instance, simultaneous determination of sex and bovine leukocyte adhesion deficiency (BLAD) can be performed in less than 4 h [P. Bredbacka, unpublished data]. It should also be possible to detect polyploidy (e.g. by polyspermy) and perhaps other abnormal traits in combination with sexing.

With major practical application of sperm sorting still a few years away, PCR sexing of embryos remains the principal method for selective predetermination of sex. Simplifications of the protocol during the last few years has increased the interest in PCR sexing and work currently in progress should contribute to this interest.

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