Comparative Analysis of Bovine Blood and Embryo Sexing

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ABSTRACT

Samples of whole blood were obtained from mature cattle (n=2) to determine the accuracy and consistency of 50 reactions in PCR amplification of the *ZFX/ZFY* loci on the X- and Y-chromosomes. 42% of the samples were correctly sexed, 50% had ambiguous results, and 8% had no reaction occurring. These samples were also used to compare sex-typing results of bovine biopsies (n=50). Sexing of biopsies failed, resulting in 0% success.

BACKGROUND

Pre-implantation embryo sex-typing has had a major role in the livestock industry. Through this technology, animal breeders have developed the ability to control the sex of their animals prior to embryo transfer. This has highly impacted both the beef and dairy industry. Females are more desirable than males in the dairy industry as they are the only ones that can produce milk. Males are sought-after in the beef industry as they have higher feed efficiency and body weight (1).

Many techniques have been developed to perform sex-typing. Some of the methods that have been used are: Fluorescent In-situ Hybridization (FISH) (13), Flow Cytometric Separation (15), and H-Y antibodies (14). Though effective, these techniques are costly and require extended time to complete. One of the newest and most preferred methods for embryo sex-typing is the Polymerase Chain Reaction (PCR) (1). Through PCR, fragments of DNA are amplified exponentially to produce massive amounts of identical genetic material. The replicated DNA products can then be analyzed and/or sequenced. Via PCR, sexing can be completed in a matter of a few hours (3,4)

In this study, the objective was to determine the sex of both sample types via primary and nested PCR amplification of the ZFX/ZFY loci on the X- and Y-chromosomes using a protocol adapted from B.W. Kirkpatrick (2). The accuracy of the sexing will be determined based on the outcome of the PCR results of the blood samples compared to the known sex of the mature cattle from which blood was obtained from. The consistency will also be evaluated by determining whether or not there are any inconsistent results amongst various PCR products from the same blood samples. These samples will then be used as a control to embryo biopsy sexing results.

METHODS AND MATERIALS

DNA Extraction from Bovine Blood

Whole blood samples from male and female cattle (n=2) were obtained, and their DNA extracted and isolated using a DNA extraction kit (Qiagen®, Valencia, CA). Isolated DNA was stored at -20°C.

Embryo biopsy and DNA isolation

Pre-implantation embryos were obtained from pregnant cows. Embryos identified in the 8 – 16 cell stage were biopsied using a micromanipulator. Individual cells were then lysed for strands of DNA by placing them in 5 µl of sterile water and freezing at -20°C.

PCR and Gel Electrophoresis

Using a protocol that was provided by Kirkpatrick, B.W. at the University of Wisconsin – Madison, primary PCR was conducted on thawed DNA samples extracted from both whole blood and embryo biopsies. They were added to a reaction mixture containing sterile Millipore water, 10X *Paq5000* buffer (Stratagene, Cedar Creek, TX), 10mM dNTP mix, and *Paq5000 Polymerase* (5U / µl) (Stratagene). Using primers that were designed by Aasen and Medrano (11), 5 µM Forward Primer with sequence 5'~ ATAATCACATGGAGAGCCACAAGCT ~3', 5 µM Reverse Primer with sequence 5'~ GCACTTCTTTGGTATCTGAGAAAGT ~3' were also added to the reaction mixture for a final volume of 50 µl. They were then placed in a thermal cycler at 95°C, and PCR was run for 30 cycles with denaturation occurring at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds.

After primary PCR was completed, products were added to a secondary, nested PCR reaction mixture containing sterile Millipore water, 10X *Paq5000* buffer, 10 mM dNTP mix, 5 µM XY_P Primer with sequence 5'~ GTGATGACTGCGGGAAGCATTTCTCYCATGCT ~3', 5µM XY_Q Primer with sequence 5'~ GACCTATATTCGCAGTACTGGCATTGGTACGGYTTCTC ~3', 5 µM ZFX Primer with sequence 5'~ GGGGCGGGCGCACAAATGTAAATTCTGTGAA ~3', 5 µM ZFY Primer with sequence 5'~ GGGGCGGGCGGTTCAGCTGTCTCATAC ~3', and *Paq5000* polymerase (5U / µl), for a final reaction volume of 10 µl. Primer sequences were provided by Kirkpatrick (16). Reaction samples were placed in a thermal cycler at 95°C, and PCR was run for 35 cycles with denaturation at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds.

Nested PCR products were combined with 6X Orange Blue loading dye (Promega, Madison, WI) and loaded into a 1.5% agarose gel stained with Ethidium Bromide for UV imaging. A 50 bp ladder was used as a molecular marker to determine the size of PCR products. Electrophoresis was run at 65 volts for 60 minutes.

RESULTS

Accuracy of PCR from blood DNA

12 out of the 25 female blood samples displayed a single band at 190 bp, indicating the amplification of *ZFX* (Figure 1) (16). 10 of the 25 female samples had ambiguous results with a band of another size other than our target gene. 3 of the 25 samples displayed no bands (image not shown), suggesting that no reaction occurred. 25 male blood samples were also used for PCR. 9 were correctly sexed with two bands at 119 bp and 190 bp indicating amplification of both *ZFY* and *ZFX*, respectively (Figure 2) (16). There were no samples that were incorrectly sexed, 15 with ambiguous results, and 1 that displayed no reaction. A total of 21 male/female samples were correctly sexed, 25 were ambiguous, and 4 that had no reaction. See Table 1 for results in tabular form.

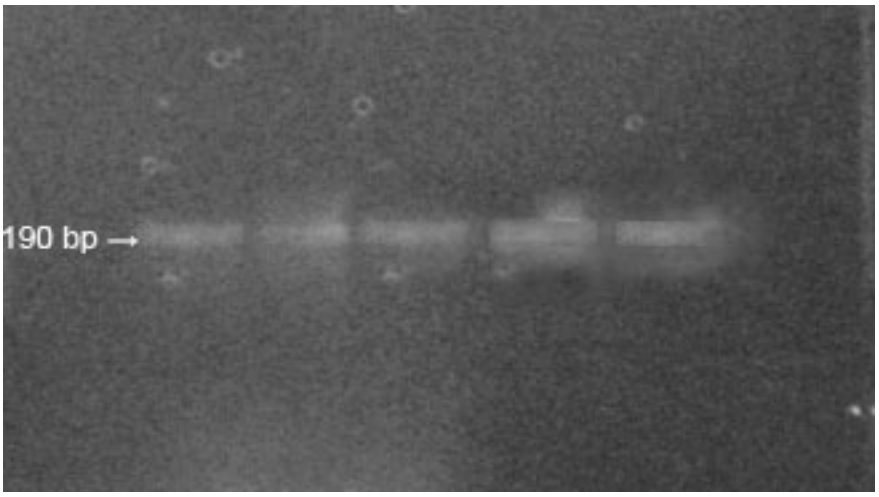


Figure 1. Gel electrophoresis of amplified PCR product from bovine female blood. Single bands at 190 bp in lanes 1-5 indicate amplification of *ZFX*. No reaction occurred in lane 6. A 50 bp ladder was used as a molecular marker.

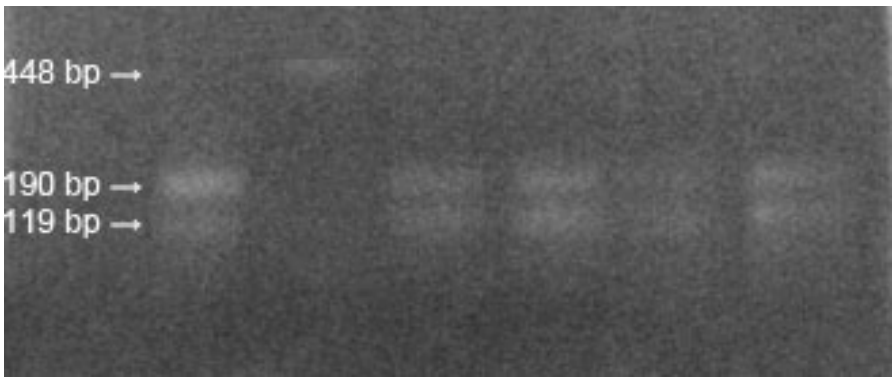


Figure 2. Gel electrophoresis of amplified PCR product from bovine male blood. Two bands in lanes 1, 3-6 at 190 bp and 119 indicate amplification of *ZFX/ZFY*, respectively. A single band in lane 2 at 448 bp provides ambiguous results, thus sex cannot be determined. A 50 bp ladder was used as a molecular marker.

Sex of Cattle	Number of blood samples	Number correctly sexed / %	Number incorrectly sexed / %	Number of ambiguous results / %	No reactions (%)
Male	25	12 (48%)	0 (0%)	10 (40%)	3 (12%)
Female	25	9 (36%)	0 (0%)	15 (60%)	1 (4%)
Total	50	21 (42%)	0 (0%)	25 (50%)	4 (8%)

Table 1. Results from gel electrophoresis of bovine blood DNA PCR products.

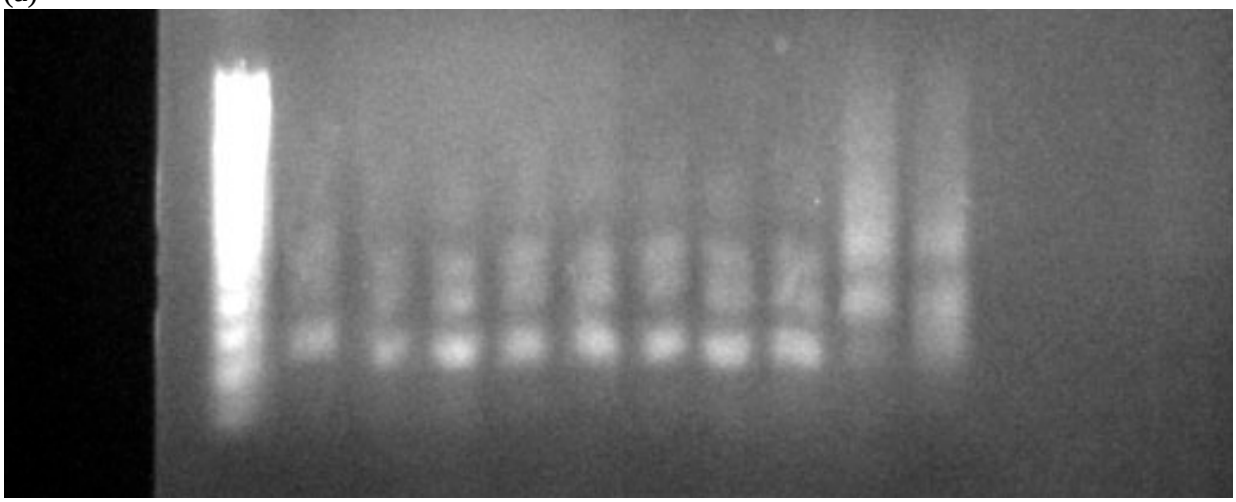
Consistency of PCR from blood DNA

Though sexing was not completely successful with DNA obtained from whole blood samples, the results were relatively consistent among each correctly sexed, incorrectly sexed, and ambiguous groups. Each ambiguous sample had a band that appeared at 448 bp. From this, we were able to determine that there is some DNA fragment that is being amplified, but it is not of our desired target *ZFX/ZFY*.

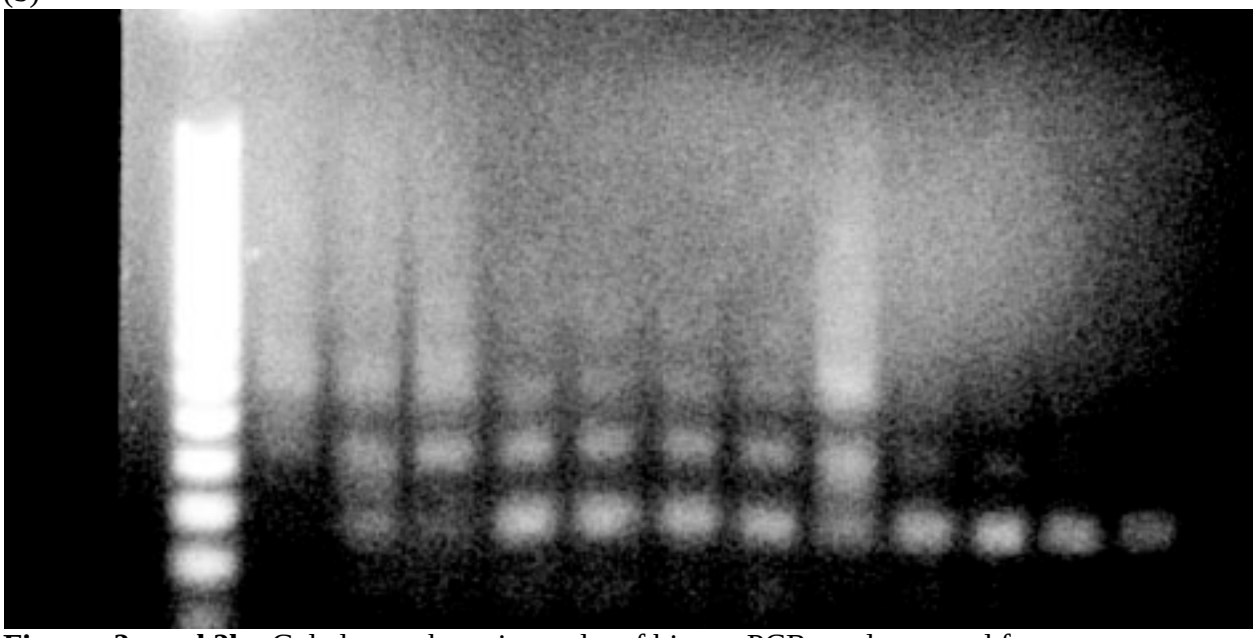
Embryo Sexing

All embryo biopsies used for sexing (n=50) returned ambiguous results that did not allow for the determination of sex. Multiple bands appeared on gel electrophoresis of PCR products. Though most of the bands appeared in roughly the same base pair size of *ZFX*/*ZFY*, we were unable to come to a conclusion about the sex or exact size of each sample. In Figures 3a and 3b PCR products created a wave-like alignment of bands.

(a)



(b)



Figures 3a and 3b. Gel electrophoresis results of biopsy PCR products used for sex determination.

DISCUSSION

PCR is a powerful method that is frequently used for sex-typing. Not only is it applicable to the livestock industry, but it is also used in human diagnostic testing to determine the possibility of sex-linked illnesses being passed on to offspring (17). Many studies have reported

successful results of sexing in many different species (1,2,3,5,6); however, in this particular one, sex-typing using DNA from known sex bovine blood samples was only 48%. Sexing of bovine embryo biopsies failed and prohibited us from determining the sex, resulting in 0% success. Previous studies that were able to accomplish sex-typing reported 98% success (1,2,3,5,6). Several reasons for failure may include: improper storage and handling of samples, primer design (18), and sample contamination.

Due to the high sensitivity of PCR, sample contamination may have affected our results. Contamination could have caused amplification of other fragments other than *ZFX/ZFY*. This may explain why bands other than those at 119 bp and 190 bp appeared. False results may have occurred due to the amplification of contaminates and not of the actual samples. Contamination could have occurred by using non-sterile materials such as pipette tips and micro centrifuge tubes. Though every effort was put into using aseptic technique, it may not have been entirely possible due to the sharing of materials within a classroom research laboratory environment. To decrease the chances of contamination, all samples should be handled with sterile material dedicated to this study under a flow hood. Contamination also may have occurred during the biopsying process. Cross contamination from various embryos sitting in the same petri-dish could have contributed to false results; however, it is not possible to verify this without embryo transfer, pregnancy, and the birth of a calf. In addition to contamination, inconsistencies of many biopsy results in which no reaction occurred were likely due to the absence of biopsy material for PCR. We were unable to verify the presence of any genetic material in our samples prior to PCR.

Another problem that may have contributed to poor results was the handling and storage of samples. Because access to fresh biopsies was not always feasible, frozen embryo biopsies were used in this study. Freezing and thawing of samples may have caused some degradation of DNA resulting in 0% success (19). Similarly, the freezing and thawing of reagents for PCR reaction may have also decreased their effectiveness in completing PCR reactions (20). To avoid this, all reaction mixtures should be aliquoted into individual vials to reduce the number of freezing/thawing cycles. Furthermore, the bands that resulted from electrophoresis were often times indistinct. Delayed time between sample loading into gel wells could have allowed for the dispersion of DNA, causing large fuzzy bands similar to those seen in lanes 2 and 3 of Figure 3b (20).

Primer design plays a large role in the success of PCR reactions. It is imperative that one creates primers that are specific to the target gene to avoid amplification of other sequences. Primer length, melting temperature, and GC content also must be taken into consideration when designing primers. Ideally, a primer should be 18 – 22 bp long to allow for specificity and ease of binding to template during the annealing process (18). In this study, Primer XY_Q was 38 bp long, violating the 18 – 22 bp rule. Furthermore, the formation of secondary structures such as hairpins and primer-dimers may adversely affect annealing and amplification (21). Primer-dimers are by far the most common secondary structures that form. With the large amount of primers that are present in our reaction mixture, primers may have the ability to anneal to another primer and amplify itself (21). This phenomenon may cause the formation of excess bands seen during UV imaging resulting in ambiguous results. Many programs have been developed by reputable companies to assist in primer design such as *Net Primer* (Premier Biosoft International,

Palo Alto, CA, USA), Primer *3*, *Gene Fisher* (Bielefeld University Bioinformatics Server, Bielefeld, Germany) and *OligoAnalyzer* (Integrated DNA Technologies, Inc., USA). These programs are easily accessible through the World Wide Web.

In conclusion, the success of sex-typing of embryo biopsies and whole blood was poor. Many of the results were inconclusive. Further troubleshooting and development of protocol needs to be completed. In addition, samples that provided ambiguous results should be extracted from the agarose gel and sent to a sequencing laboratory to determine what is exactly being sequenced.

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