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Evaluation of PCR-Based Beef Sexing Methods

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Analysis of the sex of beef meat by fast and reliable molecular methods is an important measure to ensure correct allocation of export refunds, which are considerably higher for male beef meat. Two PCR-based beef sexing methods have been optimized and evaluated. The amelogenin-type method revealed excellent accuracy and robustness, whereas the bovine satellite/Y-chromosome duplex PCR procedure showed more ambiguous results. In addition, an interlaboratory comparison was organized to evaluate currently applied PCR-based sexing methods in European customs laboratories. From a total of 375 samples sent out, only 1 false result was reported (female identified as male). However, differences in the performances of the applied methods became apparent. The collected data contribute to specify technical requirements for a common European beef sexing methodology based on PCR.

KEYWORDS: Beef sexing; PCR; interlaboratory comparison; validation

INTRODUCTION

The volume of beef trade amounts to several billion Euros per year in the European Union (EU). To strengthen the market position of meat producers, measures such as intervention buying and export refunds have been introduced (*1*, *2*). These schemes foresee a considerably higher subsidy for male beef meat, which is regarded to be of higher quality (*3*). To prevent fraud, some control measures have been introduced and are executed by customs offices, such as checking the correct packaging of cuts from randomly sampled consignments and determining the lean meat content of a pooled sample (*4*). However, more specific techniques are required for an unambiguous sex determination of meat samples. Hormone analysis by GC-MS in the single-ion monitoring mode (*5*) reveals specific patterns of steroid hormones, precursors, and metabolites and allows accurate quantification. For instance, the concentration of progesterone and the ratio of progesterone to pregnonelone as well as the levels of testosterone and its metabolites clearly differentiate cow and bull samples. The time-consuming and tedious sample preparation and the need for expensive equipment, however, have limited the application of this methodology for routine testing. ELISA methods detecting epitopes on the male-specific H−Y antigen (*6*) were developee but have meanwhile almost disappeared due to insufficient specificity (*7*) and emerging molecular biology techniques. Polymerase Chain Reaction (PCR) presently is the method of choice. Thanks to the highly specific reaction, it provides accurate results within a short period of time. To date, several different PCR-based methods, most of which have been developed for embryo sexing applications (*8*) are routinely applied, among those are methods amplifying specific sequences within the Y chromosome, the zinc finger protein genes, or the amelogenin gene (*9*). Sex specificity in the amelogenin PCR is accomplished using a common primer pair, which anneals to two transcripts (located on the X and Y chromosomes and named class I and class II, respectively) differing only in a deletion on the latter (*10*). In addition to these methods, duplex PCRs are used that coamplify bovine satellite and Y-chromosome specific sequences (*11*, *12*).

The aim of the present study was to assess the performance of currently applied methods, to detect possible differences in accuracy and robustness as well as critical points in the procedures, with the final goal to set up technical guidelines to be fulfilled for PCR-based sexing methods within the EU. This was accomplished by in-house validation of two of the most widely used procedures (amelogenin PCR and bovine satellite/Y duplex PCR) and by organizing an interlaboratory comparison with customs laboratories of important beef-exporting countries.

MATERIALS AND METHODS

Meat: Procurement, Sample Preparation, and Dispatch. Two pieces of 5 kg each, one originating from a bull, the other one from a cow, were obtained from a slaughterhouse (Heist op den Berg, Belgium), accompanied with a certificate from the Federal Veterinary Office (Brussels) indicating the origin and type of meat. The meat was tested for BSE and found to be negative (results provided from the slaughterhouse). Ostrich meat (filet quality) was obtained from a local farm (Mol, Belgium).

Extensive cleaning of surfaces and instruments was performed before and after handling of each type of meat (cow, bull, and ostrich) using detergent solutions, ethanol, and UV light. The operators wore gloves and protective clothes during the work, which was performed in a laminar flow cabinet. Cutting of meat was done with sterile surgical

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Table 1. Sample Preparation and DNA Purification: Procedures Used in the Interlaboratory Comparison

lab	sampling and DNA extraction
A	50 mg subsample, finely minced, hydrolysis and DNA extraction in 50 mM NaOH, 20 min at 95 °C (shaker), supernatant after centrifugation diluted in water and used for PCR
B	removal of outer surface of meat with a sterile scalpel, scratching of a small piece from the frozen part by means of a terile Pasteur capillary pipet, extraction and purification: DNeasy tissue kit (Qiagen)
C	subsample of ∼25 mg of thawed meat taken using a sterile scalpel, further cut to small pieces; extraction and purification: NucleoSpin tissue kit (Macherey & Nagel), including a RNA digestion step
D	meat "cube" of ∼1–10 mm^3 excised from provided meat piece; hydrolysis, proteinase K treatment, chloroform extraction, ethanol precipitation (*17*) for DNA isolation and purification
E	350 mg subsample, DNA extraction and purification: Wizard DNA cleanup kit (Promega); yield and integrity control of extracts by electrophoresis
F	as described under Materials and Methods (DNA Extraction)
G	scratching of a subsample (∼1 mm^3) from the frozen piece as delivered using a sterile Pasteur capillary pipet; extraction and purification: hydrolysis, proteinase K treatment, RNase digestion, Wizard DNA kit (Promega)
H	10–20 mg subsample, extraction and purification: DNeasy tissue kit (Qiagen)

blades. The little meat pieces were then placed in double plastic tubes (biohazard plastic containers, Sarstaedt, Germany).

Samples were stored at −20 °C until shipment. Dispatch to the laboratories was effected in containers with dry ice. For the testing and validation of two PCR methods at IRMM, samples from this production were used.

DNA Extraction. Subsamples of ∼50 mg were excised from the meat sample, further cut down to ∼10 smaller pieces using a sterile scalpel, and transferred into a 1.5 mL Eppendorf tube. The extraction and purification of DNA were accomplished using the High Pure PCR template preparation kit (Roche) comprising the following steps: lysis of the meat in buffer containing urea and proteinase K, application of the solubilized sample to a silica resin, removal of non-DNA compounds by repeated washing steps, and elution of purified DNA from the column. The described procedure for mammalian tissue was followed except for the use of 90 min at 65 °C as incubation conditions for the proteinase K digestion step. The purified DNA was finally eluted from the cartridge in 10 mM Tris/HCl, pH 8.5. The extracts were stored at −20 °C until use. The quality of the extracted DNA was examined by electrophoresis through a 2% agarose (Ultrapure Agarose, Life Technologies) gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) stained with ethidium bromide. As a size reference, a 100−1000 bp mass ruler (Bio-Rad) was used.

Quantification of DNA. DNA concentration in the extracts was determined by applying the PicoGreen assay in the microtiter format (*13*). The working reagent consisted of a 1:200 dilution of the stock solution (Molecular Probes) in TE (10 mM Tris, pH 7.5, and 1 mM EDTA). Calibration was accomplished using λ-DNA diluted with TE to 1, 0.5, 0.25, and 0.1 μg of DNA/mL. All samples were used directly or diluted in TE buffer. On a microtiter plate, 50 μL of sample was mixed with 50 μL of working reagent. After incubation in the dark for 5 min, the plates were read on a BMG Fluorstar Galaxy reader (B&L, Sint-Pieters-Leeuw, Belgium) set at 485 and 530 nm for excitation and emission wavelengths, respectively.

PCR Amplification. The final conditions used for the interlaboratory comparison samples are described here. For starting conditions, refer to the Results.

Amelogenin PCR (14). Amplification reactions were carried out in a total volume of 25 μL in 0.2 mL tubes containing PCR buffer (20 mM Tris/HCl, pH 8.4, and 50 mM KCl), 1 mM $MgCl_2$, 200 μM of each dNTP, 12.5 pmol of each primer (forward 5′-CAGCCAAAC-CTCCCTCTGC-3′, reverse 5′-CCCGCTTGGTCTTGTCTGTTGC-3′), 1 unit of Platinum *Taq* DNA polymerase (Life Technologies), and 5 μL of the 1:10 diluted extract. The PCR reaction was performed on a Mastercycler gradient (Eppendorf). The cycling conditions were as follows: initial denaturation step, 97 °C for 5 min, 30 cycles of amplification (94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min), and final extension at 72 °C for 10 min. The amplicons were analyzed on a 2% agarose gel as described above.

Y/Bovine Satellite Duplex PCR (15). The same composition of the reaction solution as described above applied, except for the primers: 5 pmol of each bovine satellite primer (forward, 5′-TGGAAGCAAA-GAACCCCGCT-3′; reverse, 5′-TCGTGAGAAACCGCACACTG-3′) and 2.5 pmol of each Y chromosome-specific primer (forward, 5′-CCCTTCCAGCTGCAGTGTCA-3′; reverse, 5′-GATCTGTAACT-GCAAACCTGGC-3′). The following temperature program was used: initial denaturation step, 95 °C for 5 min, 30 cycles of amplification (95 °C for 30 s, 63 °C for 30 s, and 72 °C for 1 min), and final extension at 72 °C for 5 min. Analysis of amplicons by agarose gel electrophoresis was done as described above.

Capillary Electrophoresis (CE). A Beckman PACE-MDQ system was used, equipped with a laser-induced fluorescence detector (Ar laser, 488 and 520 nm for excitation and emission wavelengths, respectively). Separation was accomplished in a fused silica capillary (Polymicro Technologies) of 75 μm internal diameter and 40 cm total length (30 cm to detector). Separation buffer was 25 mM Tris/MOPS, pH 7.55, containing 0.5% PEO (Aldrich, MW 4 Million), 0.4% PEO (Aldrich, MW 0.9 Million), and 0.4 μL/mL EnhanCE (Beckman-Coulter) as the intercalating dye (*16*). Prior to each run, the capillary was rinsed for 4 min at 20 psi with separation buffer. Samples (PCR products diluted 1:5 in water) were injected hydrodynamically (4 psi, 10 s). Separation was performed in the reversed polarity mode (i.e., anode at the detector side) at 8 kV for 25 min. Capillary temperature was set at 20 °C. A molecular weight standard of 100−1000 bp (Bio-Rad) was used to calibrate the system. Prior to each separation day, the capillary was rinsed with 0.5 M NaOH at 20 psi for 10 min, followed by rinsing with separation buffer (20 psi, 10 min).

Interlaboratory Comparison. *Participants* included the State Laboratory, Abbotstown, Dublin, Ireland; Zolltechnische Prüf- u. Lehranstalt, Hamburg, Germany; LGC, Teddington, U.K.; Douane Laboratorium, Amsterdam, The Netherlands; Laboratoire des Douanes, Paris, France; Biomedisch Onderzoeksinstituut, Dr. L. Willems-Instituut, Diepenbeek, Belgium; Laboratorium der Douane en Accijnzen, Leuven, Belgium; EC-JRC-IRMM, EU. In the following text, the laboratories are coded A−H. This code was assigned in a randomized manner.

The preparation of the samples is described above.

Labels with numbers were printed and assigned to the samples in a randomized way. Two to four samples of the 50 for each laboratory were of ostrich origin. Furthermore, the number of cow (bull) samples for each laboratory was chosen to be not less than 19 and not more than 28. Finally, picking of sample numbers for each laboratory was again done in a randomized manner.

Procedures Used in the Ring Test. The procedures for subsampling, DNA extraction, and PCR are briefly described in **Tables 1** and **2**. As for PCR methods, only deviations from published methods (references listed in **Table 2**) are indicated. Gel electrophoresis was performed using agarose gels (1 or 2%) and TAE or TBE buffers. The gels were stained with ethidium bromide. One laboratory used polyacrylamide gels and silver staining.

RESULTS

Optimization and Validation of the Amelogenin and the Duplex PCR. Genomic DNA was prepared from the meat

Table 2. PCR Conditions Used in the Interlaboratory Comparison

lab	PCR details
A	as published (*14*) except 0.25 mM dNTPs, 1 U of polymerase, and 10 μL of extracted sample (PCR sample = 40 μL)
B	as published (*18*) but Ready-to-go beads (Pharmacia), 20 pmol of each primer, 10 μL of extract, 25 μL total volume of PCR reaction; temperature program: 94 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; final extension, 72 °C for 5 min
C	as published (*19*) but PCR–SuperMix (Life Technologies; contains all ingredients except primers); temperature program: 94 °C for 1 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; final extension, 72 °C for 4 min
D	as published (*20*) but 0.25 pmol of each primer, 0.5 U of polymerase, 0.5 μL of extract, 25 μL total PCR volume; temperature program: 94 °C for 5 min, 35 cycles of 95 °C for 20 s, 57 °C for 20 s, 72 °C for 40 s; final extension, 72 °C for 5 min
E	as published (*14*) but 5 pmol of each primer, 1 μL of extract used, 50 μL total volume; temperature program: as published, except 58 °C annealing temperature
	as published (*21*) but 5 pmol of each primer, 1 μL of extract used (50 μL total volume); temperature program: as published, but 53 °C annealing temperature in all cycles
F	as described under Materials and Methods (PCR Amplification)
G	as published (*22*) but 1.5 mM $MgCl_2$, 10 pmol of each bovine satellite primer, 0.75 U of polymerase, 5 μL of extract, 25 μL total volume; temperature program: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; final extension, 72 °C for 5 min
	as published (*22*), as described in B except Y-specific BRY4a primers
	as published (*18*) but 5 pmol each of primers ZFX and ZFY, 10 pmol of common reverse primer ZFA, 5 μL of extract, 100 μL total volume of PCR reaction; temperature program: 95 °C for 5 min; touchdown program: 2 cycles of 95 °C for 1 min, 64 °C for 1 min, 72 °C for 3 min and then 2 cycles each as described but annealing temperatures of 62, 60, 58, 56, and 54 °C; final conditions, 30 cycles of 95 °C for 1 min, 52 °C for 1 min, 72 °C for 3 min; final extension 72 °C for 5 min
H	as published (*14*) but 20 pmol of each primer; 2.5 U of polymerase, 5 μL of extract, 100 μL total volume; temperature program: 95 °C for 15 min, 15 cycles of 94 °C for 1 min, 72 °C for 1 min, 72 °C for 1 min, with decreasing annealing temperature of 1 °C/cycle and then 20 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min; final extension, 72 °C for 10 min

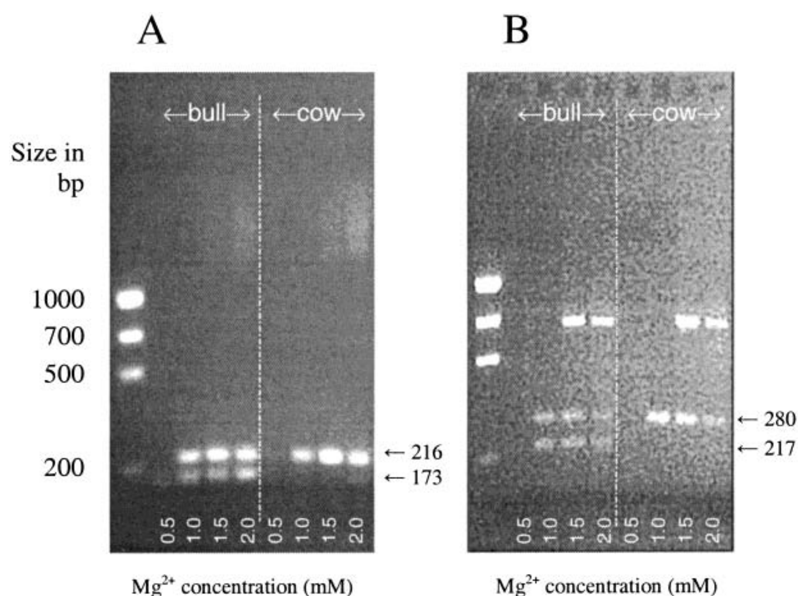

Figure 1. Influence of Mg^{2+} concentration on PCR amplicons: agarose gel electrophoresis (2%) of (A) Y/bovine satellite duplex PCR and (B) amelogenin PCR. Molecular weight standards (Bio-Rad). Arrows indicate the positions of the specific bands: in both PCRs, males exhibit two bands, whereas females reveal only one.

samples using an extraction kit as described above. Agarose gel electrophoresis yielded DNA bands of ~4500 bp. The amount of DNA in the extracts, as determined by PicoGreen quantification, varied between 0.7 and 1.5 μg/mL.

The procedures for the amelogenin and duplex PCR as published (*14*, *15*) were applied as starting conditions besides reduced holding times in the PCR temperature program: 30 s for denaturation, 30 s for annealing, and 1 min for extension. The first results revealed necessary optimization as unspecific bands appeared on the gel in addition to the expected fragments: in the case of the amelogenin method, a band at ~700 bp appeared, which was even more intense than the product bands at 217 and 280 bp, respectively. The duplex PCR showed a smear between 1500 and 500 bp.

As a first step, the magnesium chloride concentration was varied between 0.5 and 2 mM for both methods (**Figure 1**). For both methods, a Mg^{2+} concentration of 1 mM yielded best results in terms of a compromise between sensitivity and exclusion of unspecific amplification.

For the duplex PCR, ostrich meat rendered bands of similar size to cow samples (bovine satellite fragment) as deduced from slab gel electrophoresis. Further investigation by CE (co-injection of cow and ostrich amplicons) proved that these bands were of identical size. However, the intensity of this band for

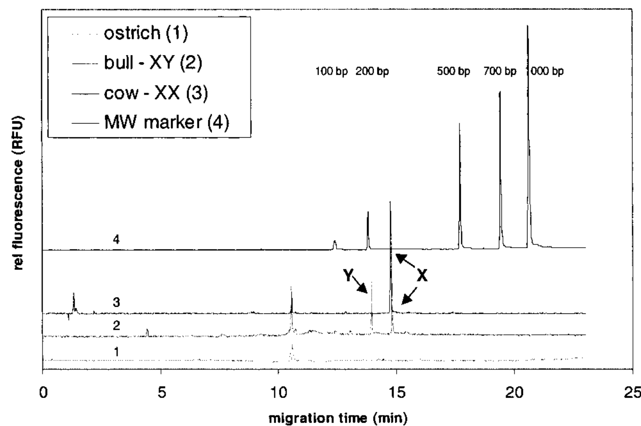

Figure 2. Analysis of amelogenin PCR products by CE. Electrophoretic conditions: fused silica capillary, 75 μm i.d., 40 cm total length (30 cm to detector); buffer, Tris/MOPS, pH 7.55, 0.5% PEO (MW 4 Mio), 0.4% PEO (MW 0.9 Mio); voltage, 8 kV (reverse polarity). Samples: PCR amplicons 1:5 diluted in water, injection by pressure (4 psi, 10 s). Peaks at 10.5 min are primer dimers.

ostrich samples was always considerably lower than that of cow samples. Contamination as the possible explanation could be excluded due to the thorough sample preparation procedure, separation of DNA isolation and PCR work, and appropriate DNA extraction and PCR control. Furthermore, the same sample extracts were used for both the amelogenin and the duplex PCR. In the former, no bands were observed with any ostrich sample. To overcome the unspecific amplification, different annealing temperatures (two levels) and different primer concentrations (two levels) were tested as follows: for the amelogenin PCR, annealing temperatures were 57 and 63 °C and primer concentrations were 0.5 and 0.2 μM, respectively; for the duplex PCR, annealing temperatures were 57 and 63 °C and primer concentrations were 0.6 μM (Y) and 0.3 μM (bovine satellite) ("higher level") and 0.2 μM (Y) and 0.1 μM (bovine satellite ("lower level"), respectively.

All four possible combinations were tested using two samples each of ostrich, cow, and bull. As for the amelogenin method, all combinations showed the same, unambiguous result: no bands for the ostrich, the 217 bp band for the cow, and two bands of 217 and 280 bp, respectively, for the bull. **Figure 2** depicts CE elution profiles of ostrich, cow, and bull samples. Generally, the lower primer concentrations resulted in fewer byproducts such as primer dimers as deduced from CE electropherograms (data not shown). The duplex PCR revealed a different result (**Figure 3**). Only an annealing temperature of 63 °C and primer concentrations of 0.2 μM and 0.1 μM for Y and bovine satellite primers, respectively, led to a disappearance of the band for the ostrich sample while showing the expected bands of 173 and 216 bp for the bovine- and Y-specific fragments, respectively. Another observation made was that the relative intensities of the two bands varied quite substantially not only with different PCR conditions applied but also within replicates of given conditions.

For the ring test, the initial primer concentration and an annealing temperature of 57 °C were used for the amelogenin method, whereas the optimized conditions were employed for the duplex PCR (63 °C annealing temperature, primer concentrations for Y and bovine satellite of 0.2 and 0.1 μM, respectively).

Proficiency Testing. Customs laboratories from the main beef-exporting countries as well as the IRMM as the organizing institution participated in this study. Each laboratory received

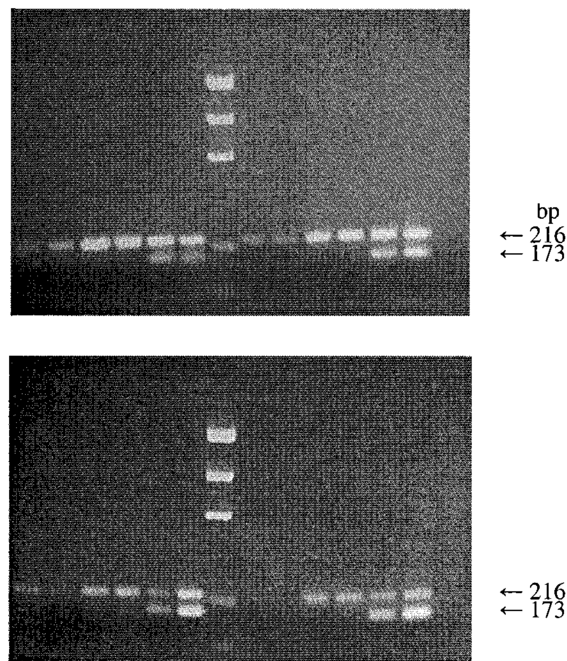

Figure 3. Optimization of the Y/bovine satellite duplex PCR: variation of annealing temperature and primer concentration. M, molecular weight standard (Bio-Rad); O, ostrich; C, cow; B, bull sample; Ct, negative PCR control. Annealing temperatures were 57 °C (upper gel) and 63 °C (lower gel). Higher primer concentrations (0.6 and 0.3 μM for Y and bovine satellite, respectively) are shown on the left, whereas lower primer concentrations (0.2 and 0.1 μM, respectively) are shown on the right. Arrows indicate positions of bovine satellite (216 bp) and Y fragment (173 bp).

Table 3. Performance of Beef Sexing Methods in the Interlaboratory Comparison

lab	principle of PCR method	correctly determined male/female of beef samples received	detection of nonbeef or ostrich samples received
A	amelogenin	47/47	3/3
B	ZFX/ZFY	48/48	0/2
C	Y	46/46	0/4
D	ZFX/ZFY	46/46	4/4
E	amelogenin	45/46[a]	4/4
	amelogenin	45/46[a]	4/4
F	amelogenin	47/47	3/3
	Y/bovine satellite	47/47	3/3[b]
G	bovine satellite[c]	–[d]	3/3
	ZFX/ZFY	46/47 (47/47)[e]	3/3
	Y	45/47 (47/47)[f]	3/3
H	amelogenin	48/48	2/2

[a] Amelogenin methods mainly differ in primer sets and annealing temperature used. [b] Distinction of ostrich/cow possible after PCR optimization. [c] Bovine satellite PCR performed first to check amplifiability of DNA; ZFX/Y and Y PCR to distinguish between cow and bull samples. [d] One beef sample failed to amplify. [e] One was sample not classified; upon request this sample was reanalyzed and then typed correctly. [f] Two samples failed to amplify but were typed and submitted as the ZFX/Y PCR run in parallel rendered clear results.

50 samples in frozen form; 46−48 of these were beef samples and 2−4 consisted of ostrich meat, which served as a negative control. The gender of each sample had to be determined with the respective PCR method(s) of the laboratories.

Table 3 gives an overview of the laboratories' performances. The third column indicates how many of the sent beef samples

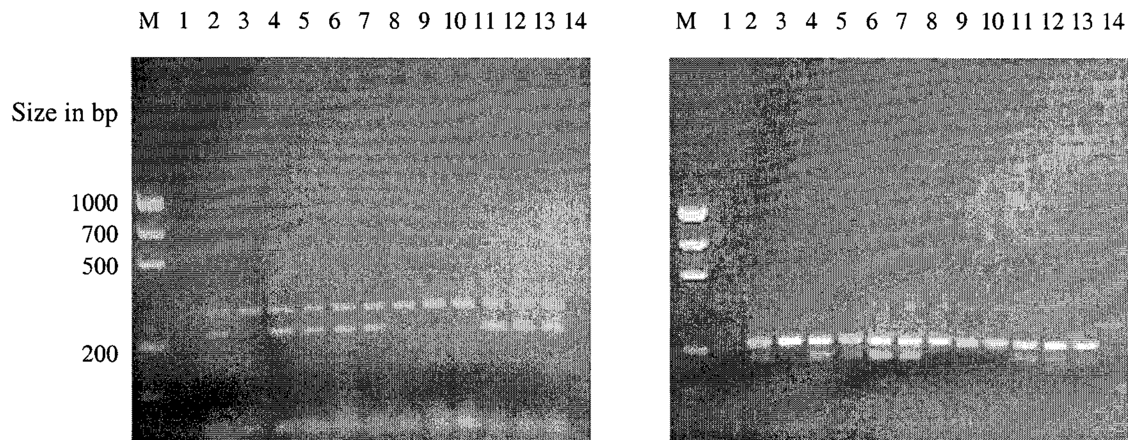

Figure 4. Electrophoretic analysis of ring test samples on 2% agarose gels: (A) amelogenin PCR; (B) Y/bovine satellite duplex PCR. M, molecular weight marker; lane 1, ostrich; lanes 2, 4–7, and 11–13, bull; lanes 3 and 8–10, cow; lane 14, negative PCR control.

were classified correctly; the fourth column then depicts how many of the nonbeef samples provided were recognized as such.

The overall results revealed that from 375 beef samples sent out to 8 laboratories employing 4 different approaches (amelogenin, ZFX/Y, Y, and duplex PCR), 374 were typed correctly, indicating 99.7% accuracy. However, one laboratory reported four ambiguous results in total; the submitted results were correct, but typing was done on the basis of taking the data from all three PCR methods into account. The procedures used in six of the eight laboratories allowed the detection of the ostrich samples as nonbeef meat (19 of 25 ostrich samples). Looking at the data sets, 550 single results originating from 11 methods were submitted. From the 515 beef samples tested, 513 (99.6%) were correctly typed as male or female. From the 35 ostriches, 29 were recognized as being of nonbeef origin.

Figure 4 shows typical results for some ring test samples at IRMM using the amelogenin-type method (A) and the duplex PCR (B). The amelogenin-type PCR rendered clear results, and a further examination of the samples by CE revealed that the relative band intensities of the X and Y fragments are equal, which held true for all samples analyzed. The duplex PCR gave broader bands, and the intensity ratio of the Y to the bovine satellite fragment differed from sample to sample.

In the following, special remarks are given for each laboratory.

Laboratory A. The sample preparation procedure used was the simplest one applied in the study and apparently works well as in all cases clear and accurate results were obtained. For the ostrich samples no bands were obtained on the gel; repeated extraction and a different extraction procedure confirmed the result (no signals), which led to the conclusion that these samples were of nonbeef origin.

Laboratory B. All beef samples were analyzed correctly; for the ostriches, bands of similar size to female-specific fragments appeared on the gel. This problem might be overcome by altering the annealing temperature of the PCR program or other optimization measures such as using purified primers.

Laboratory C. The applied method detects only a Y-specific fragment and therefore does not allow a distinction between cow and nonbeef samples.

Laboratory D. The conventional DNA extraction procedure followed by a ZFX/Y PCR delivered clear and accurate results in all cases. As for the ostriches, the missing bands on the gel led to additional analysis, which confirmed the nature of these samples being of ostrich origin (cytochrome *b* sequencing).

Laboratory E. In both methods, all but one beef sample were typed correctly (one false bull reported). The same sample (piece of meat) was mistyped with both methods. As for the ostrich samples, no bands were obtained in the first method, whereas an unrecognized pattern was seen in the second method. Repeated extractions and further work such as RFLP suggested clearly a nonbeef nature of these samples.

Laboratory F. In the case of the amelogenin PCR, all results found were correct, and ostrich samples gave no bands and could thus be clearly distinguished from cow samples. The duplex PCR also rendered correct results in all cases; an unspecific band for ostrich samples could be eliminated after optimization of the method (higher annealing temperature and lower primer concentrations).

Laboratory G. First, a PCR using a bovine-specific primer pair was employed to check the amplifiability of the extracted DNA. Second, a Y-specific PCR and a ZFX/Y PCR were performed in parallel to distinguish between male and female samples. One of the beef samples failed to show a bovine-specific band with the first PCR but gave clear results with both the Y and ZFX/Y methods. The Y-PCR failed for two of the male samples, but as the ZFX/Y PCR rendered clear results, the samples were typed as males. The ZFX/Y PCR performed best, although one of the 50 samples had to be reanalyzed as the first attempt did not give not a clear result. As for the ostriches, in neither method were bands obtained, and a repetition of the extraction and amplification confirmed these results; therefore, it was suggested that these samples were of different origin.

Laboratory H. All samples were typed correctly. As for the ostrich samples, no bands were obtained, and these samples thus were suspected to be of nonbeef origin. This laboratory was the only one using polyacrylamide gels and silver staining to analyze PCR products.

The one sample incorrectly analyzed by laboratory E was sent back to IRMM and further analyzed using the amelogenin and the duplex PCR methods. Furthermore, the PCR products were characterized by capillary electrophoresis. All of these analyses revealed the clear result that this sample was of female origin.

DISCUSSION

The implementation of two PCR procedures led to some necessary optimization in terms of Mg^{2+} concentration for the amelogenin PCR, Mg^{2+}, and primer concentration as well as

elevated annealing temperature for the Y/bovine satellite duplex PCR. Clear and correct results were obtained for all beef samples. However, the amelogenin method shows to be more robust to changes in the reaction conditions such as annealing temperature and primer concentration. The duplex PCR was revealed to be more susceptible to variations in the master mix. Ambiguous results obtained in the beginning for the ostrich samples could be solved by increasing the annealing temperature and reducing the primer concentrations. The relative intensities of Y and bovine satellite amplicons varied quite substantially.

This can be explained by different amplification efficiencies for different primer pairs, as well as by different sensitivities to inhibition from impurities in the template DNA. Additionally, unequal copy numbers of these two repeats in the genome (*23*) give a reasonable explanation. Suggestions of how to overcome the latter problem were reported, such as to carry out a two-step PCR, thereby using Y primers for the first 10 cycles before the bovine satellite specific primers were added. This measure, however, increases the risk of contamination and does not present an optimal solution for routine testing. Another alternative to compensate for the unequal copy numbers of Y and bovine satellite repeats was presented by Peura et al. (*22*). The master mix contained Y-specific and bovine satellite specific primers (*11, 24*) in a concentration ratio of 4:1. Nevertheless, in 2 of 29 male samples analyzed, the duplex PCR failed to reveal the respective bands. A second PCR using only Y-specific primers with specificity (*12*) different from those in the duplex PCR was therefore run in parallel to ensure high accuracy of results (*22*).

The analysis of amplicons by capillary electrophoresis proved to be a valuable alternative to slab gel electrophoresis. Thanks to the superior resolution and high sensitivity using LIF detection, CE allows a better assessment of the quality of PCR products and therefore represents an interesting tool for PCR optimization (*25*). Moreover, size determination of amplicons can be effected more accurately, and quantitative results are obtained in contrast to slab gels with ethidium bromide staining.

The interlaboratory comparison revealed that several different procedures as for subsampling, DNA extraction, PCR protocol, and electrophoresis are applied in the participating laboratories. A high overall accuracy of results was obtained nevertheless.

Sampling is a crucial step as contamination has to be avoided—it should be therefore performed in a dedicated environment using appropriate equipment. Good working practices, such as having a spatial separation of sample preparation, PCR, and electrophoresis, as well as dedicated equipment (pipets) and working place (clean bench) for preparing the master mix, have been reported by the participants. As for the different extraction and purification procedures used, any of them showed to be suitable.

PCR is another key step in the procedure; therefore, parameters such as quality (purity) of reagents (primers, dNTPs, and polymerase), relative concentrations of these compounds in the master mix, quality and amount of template DNA, and Mg^{2+} concentration, as well as the cycling conditions (annealing temperature, holding times, and number of cycles), can substantially influence the result.

With regard to the beef samples sent out, only one sample was typed incorrectly. In addition, four more samples in one laboratory could be typed correctly only by taking into account the results from all PCR methods performed in parallel.

The intention of incorporating ostrich samples in the proficiency testing was to obtain some data concerning the specificity of the PCR reactions, although it has to be said that the presented methods are not designed for the purposes of species identification, which would require a different approach, for instance, cytochrome *b* sequencing by RFLP (*26*). In most of the applied methods, no bands or unrecognizable patterns were obtained, indicating the nonbeef origin of these samples. For two methods a band of similar size to female beef samples but of less intensity was obtained for the ostrich samples. In one case, an elevated annealing temperature and reduced primer concentrations eliminated this problem. One laboratory used only a Y-specific primer pair, which limits the information of the result; the reported procedure in case no band is obtained on the gel is to retest the sample.

An important measure to achieve reliable results and to verify the integrity of the procedure is to incorporate controls within the series of samples to be analyzed; these should comprise cow and bull reference samples (meat or extracted DNA) as well as a blank extraction and a PCR negative control. Another important feature in method validation is to ensure an appropriate in-house confirmation method such as a second PCR procedure, PCR-RFLP, or sequencing (*26*, *27*). Other methodologies serving as confirmation analyses that employ hybridization probes are real-time PCR and PCR-ELISA (*28*, *29*).

In conclusion, most of the beef sexing methods tested revealed accurate results. However, PCRs of homologous genes, which exhibit a length polymorphism of the X and Y chromosomes such as amelogenin, and allele-specific PCRs, as employed in the case of the zinc finger protein, are to be preferred, as they deliver accurate results, include the internal standard in themselves, and tend to be more robust than the tested Y/bovine satellite duplex PCR.

ABBREVIATIONS USED

CE, capillary electrophoresis; PCR, Polymerase Chain Reaction; PEO, polyethylene oxide; RFLP, restriction fragment length polymorphism, PT, proficiency testing; MW, molecular weight; TAE, Tris acetate EDTA; TBE, Tris borate EDTA.

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