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Sexing of beef — a survey of possible methods

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Abstract

The beef trade, amounting to several billion Euro per year, is of great importance in the European Union. Several measures have been introduced to support beef producers, such as intervention buying. However, these payments are only effected for male beef, which represents a temptation for fraud. Consequently, reliable methods for sexing of beef are required. This report summarises existing methods in EU countries as well as possible alternatives deduced from the literature. Individual methods are discussed for their advantages and disadvantages as well as their general applicability. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

1.1. Meat sexing

The beef trade, amounting to several billion Euro per year for the import and export of meat, is of great importance in the European Union. To strengthen the position of European meat producers and to stabilise the market, some measures are foreseen in the common agricultural policy (CAP), for example the possibility to sell meat to Intervention or to receive export refunds. When market prices fall below a certain level and other conditions outlined in Commission regulations [(EEC) Nos. 2456/93, 2821/98] are fulfilled, intervention buying is set into force. However, it is clearly stipulated in these regulations that only male beef (further specified as either (a) uncastrated young male animals of less than 2 years of age or (b) castrated male animals) can be taken into Intervention throughout the EC. Male beef is designated to be of higher quality than cow or heifer meat (Branscheid & Lengerken, 1998; Price, 1995) and therefore yields higher prices. Export refunds are another means to support beef producers; again, subventions for male beef are significantly higher than for female meat (Graham Purvis, MAFF, personal communication).

This might pose a temptation to mislabel beef or to offer non-male beef meat, representing considerable fraud to consumers and the EU. This underpins the necessity to have reliable methods for determining the gender of beef meat.

1.2. Requirements for beef sexing methods

Several different methodologies are feasible to determine the gender of meat, for instance, inspection in a slaughterhouse or microscopy of the tissue. The former method requires large beef parts or carcasses to be examined for reliable classification, the latter method is somewhat limited because for frozen cuts, meat cannot be unambiguously classified as male or female. Hence, these techniques could at best serve as preliminary means to assess the gender of beef; however, especially with regard to the huge amount of money to be paid in the frame of Intervention and export refunds, appropriate test methods have to be applied which accurately determine the gender of beef. For these methods, several technical and analytical aspects have to be considered: suitable procedure(s) for isolating and purifying the actual analyte to be determined, appropriate analytical techniques rendering high accuracy (i.e. unambiguous test results; this involves measures to prevent contamination

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throughout (sub)sampling and analysis and appropriate control samples to exclude falsely positive or negative results), sufficient sensitivity, as well as definition of the sample amount needed.

Moreover, these analyses are to be applied routinely in several different laboratories with presumably different infrastructures, such as customs laboratories, universities, and private companies. This implies that the possible method(s) should be robust and simple. The cost per analysis should be moderate, and results should be available in a short period of time.

This report gives an overview about regularly used methods in several laboratories. The methods are described and their advantages and disadvantages highlighted, and considerations about their wider applicability discussed. Furthermore, alternatives are presented as derived from literature.

2. Polymerase chain reaction (PCR)-based techniques

PCR with subsequent gel electrophoresis for analysing the amplicon is by far the most widely used methodology for meat sexing.

Its basic principle is quite simple: to a small amount of DNA to be amplified, the appropriate buffer for PCR, mostly Tris/HCl and magnesium chloride, the dNTPs, suitable primers (oligonucleotides complementary to a part of the target DNA and the starting point of the subsequent amplification reaction) and a thermally stable DNA-polymerase are added. Amplification of the target DNA is accomplished using a thermocycler; normally, about 20–40 cycles are performed, each consisting of the following steps: (1) heat denaturation of double stranded DNA, (2) annealing of the primers to the single stranded DNA, and (3) extension of the primers to new DNA strands performed by the polymerase. One cycle usually takes a few minutes, amounting to a total amplification time of about 1 1/2–2 h.

Subsequently, the amplified DNA has to be analysed. The most widely used technique is agarose gel electrophoresis. The DNA migration in the gel is dependent on the size of the amplicon which can be determined using appropriate standards. Using ethidium bromide, the DNA is stained and can be visualised under UV light. In the following, some methods are listed which have been submitted from state laboratories, diagnostic companies and universities engaged in this field.

One laboratory has developed a gender determination test based on PCR of a fragment within the SRY gene. This sex-determining region of the Y chromosome (SRY gene) is also known as the testis-determining factor (Sultan, Lobaccaro, Medlej, Poulat, & Berta, 1991) and has been shown to be applicable for sexing of bovine embryos (Utsumi & Iritani, 1993; Zeng et al., 1994). Agarose gel electrophoresis reveals a 300 bp band for males, whereas no band is obtained for female samples. Internal male, female and negative controls are run to ensure accuracy. Three blind trials ($n = 60$) gave an accuracy of 100%. Samples are normally run singly, only samples not found to demonstrate the male-specific band are duplicated.

Another method routinely used in customs laboratories is based upon the work of Ennis and Gallagher (1994): the X-Y homologous gene amelogenin, encoding a protein involved in the development of the tooth enamel matrix, is the target in this assay (Akane, 1998; Mannucci, Sullivan, Ivanov, & Gill, 1994) In cattle, two different transcripts are presented, named class I and class II; these transcripts are located on the bovine X and Y chromosomes, respectively; class II amelogenin exhibits a deletion region and thus renders a shorter fragment than class I. Sample preparation is accomplished by adding water to the small piece of tissue and by a few cycles of heating to 100°C and cooling. An aliquot of the obtained solution is directly used for PCR. Alternatively, the collected sample is boiled in an alkaline buffer, afterwards diluted 1:20 in PCR-grade water and then used for PCR without further clean-up. For both of these isolation procedures, no problems in terms of ambiguous results or PCR inhibitions were reported. Upon PCR amplification, fragments of 280 and 217 bp for class I and class II amelogenin are obtained. No additional internal control is required, as the 280 bp fragment is present in samples of both sexes; an absence of this band on the electrophoresis gel thus indicates failures in the PCR step. The reliability of the assay was validated with 24 randomly chosen animals: in all cases, the PCR results were in agreement with the karyotype determined from each of the animals.

Another frequently applied method is based on the work of Tagliavini, Bolchi, Bracchi, and Ottonello (1993): sample preparation is accomplished by homogenising about 1 g of fresh or frozen meat in extraction buffer containing EDTA, NaCl and Triton. Subsequently, chloroform extraction is used to further purify the DNA. Finally, ethanol precipitation is applied and the DNA content is determined using the fluorescent Hoechst dye 33258. On average, about 1–3 μg DNA can be isolated from 1 g of meat. About 1 ng was found to be sufficient as starting material of DNA for the PCR reaction. Alternatively, two primer pairs for either the bovine-specific repetitive sequence BRY-1 (Matthews & Reed, 1992) or the single copy sequence BOV97M (Miller & Koopman, 1990) are used, and additionally, a fragment of the bovine mitochondrial DNA is co-amplified as an internal control (Kocher et al., 1989). Sometimes, a fragment within the conserved areas of the vertebrate mitochondrial cytochrome b gene (Meyer, Höfelein, Lüthy, & Candrian, 1995) is also used as the internal standard. A double blind study revealed accurate results in all cases ($n = 20$). Furthermore, the

method was shown to be applicable within a range of starting material (template DNA) of 10 pg to 100 ng. About 5 h are required for the whole procedure. Ambiguous results rarely occurred; however, in these cases the analyses are repeated for clarification.

Another method, frequently used for embryo sexing (Schmoll, Brem, & Schellander, 1996; Wilson & Erlandsson, 1998) and based on determination of a X-Y homologous gene encoding a zinc finger protein (ZFX/ZFY), is also applied on a regular basis for meat sexing (Aasen & Medrano, 1990). DNA is isolated using phenol/chloroform extraction and ethanol precipitation. Conserved primers for both ZFX and ZFY are used, yielding bands of 445 and 447 bp, respectively on agarose electrophoresis gels. Distinction between male and female is accomplished by restriction fragment length polymorphism (RFLP) analysis (Schellander, Mayr, Ertl, & Peli, 1993); aliquots for the PCR amplificates are subject to PstI restriction endonuclease digestion, which for male samples generates two new fragments of 103 and 344 bp, whereas the female fragment remains undigested as demonstrated by agarose gel electrophoresis. Additionally, a PCR generated digoxigenin (DIG)-labelled probe is used to check the identity of the ZFX/ZFY bands observed by electrophoresis.

Another laboratory which performs beef sexing has developed three different PCR-based methods which are applied alternatively. For sample preparation, one of the following procedures is used: firstly, extraction of DNA using Chelex-resins; a small cube of meat is dissected from the inside of the dispatched sample and incubated in water. The remaining pellet after centrifugation is then dissolved in a solution of 5% Chelex-100 in water. After incubations at 56 and 95°C, respectively, the supernatant is used for PCR. Secondly, extraction of DNA is accomplished using a haemolysis buffer which contains SDS and Proteinase K followed by precipitation of DNA in ethanol (requires 2 days). Finally, DNA extraction kits especially dedicated to animal tissue and blood samples are used. As for PCR, the following three protocols are used alternatively: first, a duplex PCR is performed using primers for BOV97M, a bovine Y-specific DNA fragment, and for actin, the internal control (Agrawala, Wagner, & Geldermann, 1992; Miller et al., 1990; Schröder et al., 1991). The respective bands obtained on the gel exhibit sizes of 97 and 220 bp, respectively. The second method also represents a duplex PCR using primers for OY11.1, a bovine Y-specific repeated sequence (Genebank U303307), and the bovine-specific satellite DNA sequence 1.715 (Genebank V00125) serving as internal control. Subsequent agarose gel electrophoresis exhibits bands of 173 and 216 bp, respectively (Machaty et al., 1993). The third protocol describes a nested PCR procedure using the ZFX and ZFY genes as targets (Aasen et al., 1990; Kirkpatrick & Monson, 1993): the first PCR step with universal primers for both ZFX and ZFY yields fragments of 445 and 447 bp, respectively; in a subsequent nested PCR step, X and Y specific primers are used resulting in amplicons of 167 and 247 bp, respectively. The 247 bp band is obtained in all samples (female and internal control at the same time); male samples exhibit the additional band of 167 bp. An internal laboratory comparison revealed that the three reported methods are equally applicable for accurate beef sexing.

Other applied PCR-based methods differ from those mentioned above in the detection of the amplicon by using either capillary electrophoresis, real-time PCR, ELISA techniques or hybridisation to specific probes instead of slab gel electrophoresis. Additionally, variations are also found in sample preparation steps. For instance, grinding of the meat sample in the presence of liquid nitrogen prior to incubation in lysis buffer and organic extraction was reported (Appa Rao, Kesava Rao, Kowale, & Totey, 1995). Zeng et al. (1994) published a method using SRY as the template DNA and β-globin as the internal control. A SRY-specific hybridisation probe was used to confirm the identity of the electrophoretic band upon blotting onto a membrane.

A valuable alternative to gel electrophoresis for the detection of PCR amplicons is ELISA (Hahn, Dörsam, Friedhoff, Fritz, & Pingoud, 1995). For the selective detection of ZFY-sequences in mixtures of male and female cells, two procedures were successfully applied. They allow sensitive quantification of DNA in the the low femto molar level and are briefly described here (Miele, Peri, Cordella-Miele, & Mukherjee, 1994): in the first assay (dual labelling direct incorporation assay), PCR is performed in the presence of biotinylated and DIG-labelled dNTPs; the amplified DNA binds to an avidin-coated microtiter plate. Subsequently, the bound DNA is detected by means of anti-DIG antibody labelled with alkaline phosphatase in the presence of the *p*-nitrophenyl phosphate. The extinction is measured at 405 nm. Alternatively, a so-called dual-labelled oligonucleotide capture assay was applied. PCR is performed in the presence of biotinylated dUTPs and the resulting amplicons are incubated with a 3′-11-DIG-labelled oligonucleotide probe. The hybridised DNA is then detected by ELISA as described above.

Capillary electrophoresis (Geldart, Brown, & Armstrong, 1998) offers several advantages to slab gel electrophoresis such as better resolution, automatisation and more accurate size determination of the amplicons. In forensic applications, capillary electrophoresis with laser induced fluorescence detection has been successfully applied to the sexing of human tissue (LaFountain, Schwartz, Cormier, & Buel, 1998; Liu, Rampal, Evangelista, Lee, & Chen, 1995; Pouchkarev, Shved, & Novikov, 1998) where nanogram to high picogram quantities of DNA could be detected. Separation is

accomplished in coated capillaries using basic buffers and intercalating dyes such as Yo-Pro-1 (Molecular Probes, Eugene). X- and Y-specific fragments, which exhibit a size difference of only 6 bp for human amelogenin, could be separated; moreover, the threshold to identify mixtures of male and female amelogenin was as low as about 2% male in female. This represents a means which could also help to identify contaminants during PCR.

Another possibility which avoids the application of slab gels with hazardous chemicals such as ethidium bromide is real-time PCR. This technique is quickly emerging and is already applied for example, in the detection of bacterial DNA in human and animal tissues (Pahl, Kuhlbrandt, Brune, Rollinghoff, & Gessner, 1999). The big advantage is in obtaining rapid results as the PCR and analysis occur in one step (2 h are normally sufficient). The amplicons can be either continuously monitored during PCR amplification ("on-line" monitoring, using the *LightCycler*™ technology, Roche) or after completion of PCR (*TaqMan*®, PE Applied Biosystems). However, only a few labs are yet equipped with these sophisticated and costly instruments.

Finally, it should be mentioned that gel electrophoresis is not restricted to agarose gels, although these are most widely used. Polyacrylamide gels represent a suitable alternative. Silver staining is usually applied for the detection of DNA. Advantages of this post-PCR analysis technique are a better resolution of bands and a higher sensitivity (about 5–10 fold) compared to agarose gels stained with ethidium bromide (Santos, Pandya, & Tyler-Schmidt, 1998).

All the currently used procedures represent PCR-based methods with subsequent slab gel electrophoresis. They differ mainly in sample preparation procedure, the type of template DNA chosen and consequently in the respective primers used for PCR, the PCR protocol itself, which is still is largely lab dependent, and minor differences in the electrophoresis step (e.g. % agarose gel). As far as sample preparation procedures are concerned, various extraction and purification possibilities are available. The more sophisticated procedures yield DNA of higher quality which reduces the probability of inhibitory effects in the subsequent PCR reaction; however, simple preparation methods as described by Ennis and Gallagher (1994) do not seem to represent a potential drawback.

Sampling as well as sample handling can be regarded as being the most critical steps for PCR analysis with respect to contamination; however, if this work is performed according to good handling practices (properly cleaned instrumentation, extensive washing of the meat piece, excision of a small subsample from inside of the meat, a different location for sample preparation and PCR analysis, good laboratory practices such as using dedicated pipettes for PCR only etc.), no major problems are expected.

Regarding the applied PCR methods, different approaches are found: either single copy genes, such as BOV97M, or repetitive sequences (e.g. BRY-1), are chosen as target DNA. For internal controls, normally a second, autosomal locus is co-amplified with the bovine Y-specific fragment (e.g. actin). However, in case of homologous gene fragments as template DNA (e.g. amelogenin) the need for an additional internal control is omitted as the X-derived fragment must be present in all samples and thus serves as an internal control validating DNA extraction, PCR amplification and electrophoresis at the same time. Nested PCR as applied by one laboratory significantly improves the specificity of the reaction. For routine analysis however, PCR with subsequent electrophoresis is sufficient provided that respective control samples are co-analysed. Nevertheless, a second, complementary method in laboratories performing meat sexing is preferable. Alternative detection techniques for PCR amplicons reveal advantages to conventional slab gel electrophoresis in terms of higher specificity (hybridisation probes), less labour expenditure (CE, ELISA), avoidance of handling hazardous chemicals (CE), better resolution of amplicons (CE), and numeric data which are preferable to quantitative or semi-quantitative results.

3. Hormone analysis

The analysis of sex-specific hormones represents a more conventional approach to determine the gender of a tissue such as meat. As steroid hormone levels vary, not only between individuals, but also within a given individual, often quite dramatically due to several physiological factors such as age, puberty, seasonal changes, and administering of steroid hormones in animal breeding, this poses a challenge to establish methods which allow an unambiguous gender determination. Two different methodologies are feasible for steroid hormone analyses: immunochemical determination using ELISA techniques, and highly sophisticated chromatographic techniques with mass-spectrometric detection such as GC–MS and HPLC–MS/(MS). The methods applied in some laboratories are as follows. Hartwig (Hartwig, Hartmann, & Steinhart, 1995, 1997) developed a GC–MS method for the determination of naturally occurring steroid hormones, precursors and metabolites. Selective detection is accomplished by electron impact mass spectrometry operating in the single ion-monitoring mode. It was demonstrated that the observed pattern can be used to accurately distinguish between male and female samples, based on the following parameters (indicated figures are median values obtained by Hartwig et al., 1997): the concentration of progesterone is significantly higher in female animals (18.1 μg/kg) than in male cattle (0.4 μg/kg). The ratio of progesterone to

pregnenolone gave values of 6.7 and 0.4 for male and female samples, respectively. Finally, the levels of typical male hormones or respective metabolites, such as testosterone, epitestosterone and 5α-dihydro-testosterone in females are at or below the detection limit of the method. The sample preparation procedure comprises the following main steps: hydrolysis of the meat sample with proteolytic enzymes, defatting of the hydrolysate with hexane, extraction with dichloromethane, removal of phenolic compounds using KOH, purification by solid-phase extraction using a C_{18}-column followed by a Al_2O_3 column and finally derivatisation with trimethylsilyl-reagents.

Other hyphenated techniques such as HPLC–MS/MS can be applied, allowing quantification of progesterone, testosterone and their metabolites from bovine serum (Draisci, Palleschi, Ferretti, Lucentini, & Cammarata, 2000). This technique has proven to be highly specific and sensitive, but requires sophisticated instrumentation.

Enzyme-linked immunosorbent assays (ELISA) for the determination of anabolic steroids, commercially available as kits, represent an alternative, for screening purposes (Simontacchi, Marinelli, Gabai, Bono, & Angeletti, 1999). For example, progesterone which occurs in female animals in significantly higher concentrations than in males can be determined (limit of detection about 0.02 pg/ml). However, only one analyte can be determined with a given ELISA test, which limits the applicability of this methodology.

The fact that hormone analysis is not performed on a regular basis for meat sexing can be explained by the technical limitations of the ELISA tests, the tedious sample preparation procedure for gas chromatographic analysis (3 working days, which extends the total analysis time), and the expensive equipment required for GC–MS and LC–MS analysis. From an analytical point of view, hormone analyses by GC–MS nevertheless proved to be a valuable alternative for sexing of beef meat (Hartwig et al., 1997), as qualitative and quantitative data is obtained.

4. Other methods for sexing of beef

4.1. ELISA determination of the H-Y antigen

In the 1980s, a male-specific histocompatibility antigen (H-Y antigen) was discovered on male mouse embryonic cells (Ostrand-Rosenberg, 1980). This led to high expectations that this protein could also serve as the target analyte for sexing tests, mainly using ELISA (Muller, 1996). Several tests for various species have been developed, including one for beef embryo sexing (Avery & Schmidt, 1989).

Veerhuis, Hendriksen, Hengst, Kruijt, Tieman, and Booman (1994) applied an ELISA method using monoclonal antibodies to H-Y to evaluate the usefulness of this test compared with PCR-based methods. In the ELISA, the tissue is probed with each of the four monoclonal antibodies alternatively. Detection of binding is accomplished by a fluorescently labelled goat anti-mouse antibody. Two PCR-based procedures served as reference methods: firstly, PCR with subsequent analysis using a male-specific hybridisation probe (Cotinot, Kirszenbaum, Leonard, Gianquinto, & Vaiman, 1991); secondly, a duplex PCR employing primers for a Y-chromosome specific repeated DNA and an autosomal sequence of bovine satellite DNA was applied (Herr, Holt, Matthaei, & Reed, 1990). Of the tested bovine embryos of known karyotype (sex), 58–71% could be assessed correctly by the ELISA method.

Furthermore, another study (Utsumi & Iritani, 1993) using an indirect immunofluorescent assay applying anti-H-Y antibodies and a PCR-based method amplifying a Y-specific target sequence revealed similar results; for example, from the tested embryos, 15 showed binding to H-Y and were thus typed male, whereas PCR results clearly revealed 14 male and one female samples. On the other hand, 20 samples were typed as females according to the ELISA test, whereas PCR indicated only 16 female embryos. The increasing evidence that PCR-based sex typing is highly accurate and superior to H-Y antigen-based determinations (several studies including the PCR methods presented above which are applied already in several laboratories for beef sexing imply that this holds true) led to an almost complete disappearence of the latter technique in routine meat sexing (Bredbacka, 1998).

4.2. Hybridisation assay

Hybridisation techniques which lack a PCR step to amplify template DNA, but use PCR-generated probes for specifically detecting the isolated template DNA, were originally developed for species identification in meat (Buntjer, Lenstra, & Haagsma, 1995; Janssen, Hägele, Buntjer, & Lenstra, 1998), but can easily be adapted to meat sexing using the respective primers. Briefly, DNA is either isolated and purified by proteinase K treatment followed by binding and elution from a DNA-binding resin or simply by extracting DNA in 0.5 M NaOH, — an aliquot of DNA is spotted on a positively charged nylon membrane. Hybridisation is performed by means of PCR-generated probes (DIG-labelled), specific for either a bovine satellite DNA and/or bovine male-specific BRY-1 repeat (Appa Rao et al., 1995; Schwerin, Gallagher, Miller, & Thomsen, 1992). Detection of the probe is accomplished by means of an anti-DIG-IgG conjugate and Fast-Violett B as the colouring agent. Down to about 10 ng of template DNA can be detected using this approach.

5. Conclusions

PCR-based methods with subsequent gel electrophoresis proved to be reliable tools for gender determination of meat samples. Accurate results can be obtained in a short time, usually a few hours. Furthermore, additional means are available to underpin the obtained results, such as sequencing, hybridisation, RFLP analysis, and a second PCR method with other targets and primers. These techniques are not required for routine analyses, but can serve as complementary methods in ambiguous cases. The actual cost per analyses (some 30–50 Euro/sample) is moderate compared to hormone analysis (about 300–500 Euro/sample).

Methods such as hybridisation assays, PCR with subsequent CE analysis or real-time PCR are also applicable for beef sexing, but are more costly than the above technique.

Hormone analysis by GC–MS represents a valuable tool for meat sexing, but is not competitive with PCR-based methods in terms of low cost and rapid availability of results.

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