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Application of absolute qPCR as a screening method to detect illicit 17b-oestradiol administration in male cattle

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It has been previously demonstrated that the progesterone receptor gene is up-regulated in the sex accessory glands of pre-pubertal and adult male bovines after 17/S-oestradiol treatment. In the present study, a qualitative screening method was optimised to detect 17/3-oestradiol treatment using absolute quantification by qPCR of the progesterone receptor gene to determine the amount of gene expression in bulbo-urethral glands. An external standard curve was generated and developed with TaqMan® technology. Based on two *in vivo* experiments, the decision limit CCa, sensitivity and specificity of this screening method were established. Trial 1 consisted of 32 Friesian veal calves divided into two groups: group A (w= 12), consisting of animals treated with four doses of 17/S-oestradiol (5mgweek^{-1} per animal); and group B (n = 20), consisting of control animals. Trial 2 was performed on 26 Charolaise beef cattle that either received five doses of 17/3-oestradiol (group C; 20mgweek~^{1} per animal; n = 6) or remained untreated (group D; n = 20). Further, progesterone receptor gene expression was evaluated in beef and veal calves for human consumption. A specific CCa on 20 Piedmontese control beef cattle was calculated to include these animals in a field investigation. Five out of 190 beef cattle and 26 out of 177 calves tested expressed the progesterone receptor gene above their respective CCa and they were classified as being suspected of 17/S-oestradiol treatment. Additionally, 58% of veal calves that tested suspect via qPCR exhibited histological lesions of the bulbo-urethral gland tissue, which are typical of oestrogen administration and are consistent with hyperplasia and metaplasia of the glandular epithelium.

Keywords: absolute qPCR; gene expression; progesterone receptor; 17/3-oestradiol; bovine

Introduction

In cattle fattening, the additional application of oestrogens in anabolic preparations to increase live weight gain is licensed in several countries but not in the European Union. This application leaves a greater amount of residual oestrogens in edible tissues, such as meat, when compared with untreated animals (Daxenberger and Ibarreta 2001). At slaughter, not all the steroids have been metabolised or excreted; measurable levels are, in fact, present in muscle, fat, liver, kidney and other organs in meat products (Henricks et al. 2001).

In humans, the potential effects of oestrogen residues include, but are not limited to, impaired fertility (Skakkebaek 2003), increased incidence of hormone-related cancers (Miller and Sharpe 1998), and congenital malformations (Sharpe and Skakkebaek 1993).

The European Union protects consumer health by banning the use of these anabolic compounds in Directive 1996/22 (European Commission 1996). The Italian National Program for Residue Surveillance (PNR) in meat and meat derivatives showed a marked increase in steroid hormone positivity in cattle (PNR 2010); however, the detection of their use is limited by the increasing number of new growth-promoting compounds, low dosage and cocktail formulations used. These factors limit the efficiency of official analytical methods such as HPLC and LC-MS.

These analytical techniques are based only on the physicochemical characteristics of drugs; several research groups have therefore started to investigate new technologies that use the biological effect of these growth promoters. These screening methods are not meant to replace traditional analytical methods; rather, they may be used as a preliminary screening control to filter the thousands of collected samples and thus improve the overall efficacy of the official controls (Nebbia et al. 2011).

Apart from the desired effects, such as increased muscle development and reduced fat deposits, anabolic agents used in animal production have been shown to produce serious changes in organs that are physiological targets of these agents (Groot et al. 1998; Meyer 2001); in fact, oestrogenic hormones induce characteristic lesions, which are hyperplasia and squamous metaplasia in the prostate and bulbourethral glands of male animals and in Bartholin's glands of females (Grandmontagne 1986; Schilt et al. 1998; Groot et al. 2000; Biolatti et al. 2003). This histological method can identify illicit 17y6-oestradiol (j6E2) treatment up to 15 days after the last drug administration in veal calves (De Maria et al. 2010; Pezzolato et al. 2011) and in adult male animals (Divari et al. 2011). This screening method is officially adopted by the Italian PNR for the detection of illicit hormone treatment (PNR 2010).

However, anabolic steroids are most often used in combination; for example, an androgen and an oestrogen, such as /SE2 and trenbolone acetate, are sometimes used simultaneously (Parr et al. 2011). It was demonstrated that these cocktails induce weak microscopic lesions in sex accessory glands (Divari et al. 2011), and they often elude preliminary screenings; therefore, it is necessary to perform screening tests using other technologies.

For example, Mooney et al. (2008) examined a novel detection strategy based on the profiling of plasma component concentrations in response to growth promoter administration. Specifically, they developed a biosensor that measured the binding capacity of sex hormone-binding globulin that was shown to be significantly reduced within the plasma of growth promoter-treated animals (Mooney et al. 2009).

In addition, the "omics" technologies (i.e. transcriptomics, proteomics and metabolomics) have been proposed as new methods to detect illegally treated animals (Toffolatti et al. 2006; Riedmaier et al. 2009; Davies 2010; Pinel et al. 2010; Lopparelli et al. 2012). In particular, the transcriptomic techniques are based on the concept that once a specific transcriptional marker has been identified, it can be used to develop a novel screening method for the low-cost analysis of anabolic treatment in animal production.

y6E2 affects several oestrogen-regulated genes that contain oestrogen-responsive elements, and its effects are mediated by its binding to two nuclear receptors: oestrogen receptor alfa and oestrogen receptor beta (Cheskis et al. 2007). The progesterone receptor (PR) gene is one of these oestrogen-regulated genes (Risbridger et al. 2001).

In a recent experiment using relative quantification by real-time PCR (qPCR), it was demonstrated that y6E2 alone or in combination with other drugs induces up-regulation of the PR gene in the bulbo-urethral and prostate gland tissues of veal calves (De Maria et al. 2010) and beef cattle (Divari et al. 2011). This result suggests that the PR gene may be used as a biomarker to detect animals illegally treated with oestrogen, particularly when there are no evident histopathological lesions on the sex accessory glands.

The aim of the present study was to develop this transcriptomic technique for potential use in food safety monitoring. For the first time it was applied to unknown samples that were collected at slaughterhouses. In addition, an absolute quantitative qPCR approach was developed to improve the detection specificity and sensitivity of PR gene expression levels in the bulbo-urethral glands of beef cattle and veal calves treated with /?E2. Potential physiological differences in PR gene expression between two breeds of adult male bovines, Charolaise and Piedmontese, were also investigated. A further in-field investigation of veal calves and beef cattle in the Piedmont region (north-west Italy) was conducted to evaluate the possible illicit use of oestrogens in these animals.

Materials and methods

Animal treatment and sample collection

In trial 1, 32 male Friesian veal calves, 6-7 months old, were randomly divided into two groups of animals: group A (n = 12) received four doses of /SE2 valerate (Sigma-Aldrich, St. Louis, MO, USA) diluted in 10 ml of benzyl alcohol and 1 ml of ethyl oleate (5mgweek^{-1} per animal; i.m.); and group B (n = 20) was maintained as a control group. The animals were sacrificed 15 days after the last treatment (trial 1, partially reported in Pezzolato et al. 2011). In trial 2, 26 male Charolaise beef cattle, 17-22 months old, were randomly divided into two groups of animals: group C (n = 6) received five doses of betaE2 (20 mg week^{-1} per animal; i.m.), and group D (n = 20) was kept as a control. The animals were sacrificed 7 days after the last treatment (trial 2, partially reported in Divari et al. 2011).

The animals were housed as reported previously (Pezzolato et al. 2011; Divari et al. 2011), and a placebo was administered to the animals used as controls. In addition, a group (n = 20) of non-treated male Piedmontese beef cattle (group E), 14-24 months old, was used to determine the decision limit CCa for PR gene expression in this breed as a control for the in-field investigation. These animals were bred as in Divari et al. (2011).

The bulbo-urethral glands were collected at slaughter and preserved for molecular analyses. The experiment was authorised by the Italian Ministry of Health and the Ethics Committee of the University of Turin. The carcasses of the treated animals were destroyed (2003/74/CE - DL 16 March 2006, 2n. 158).
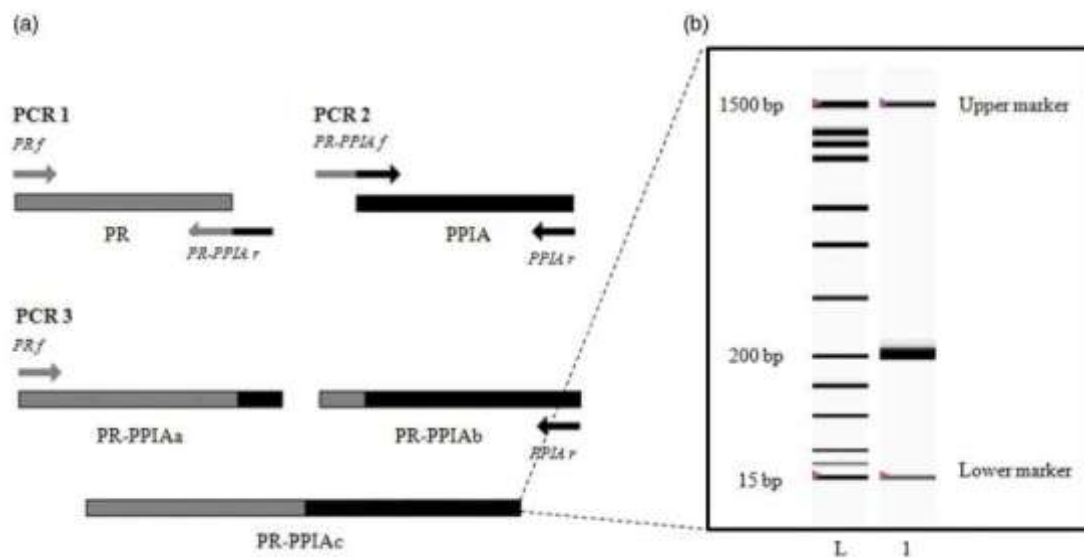

Figure 1. (a) PCR strategy used to join the PR and PPIA gene fragments and to obtain PR-PPIAc. The *PRf/PR-PPIAr* and *PR-PPIAf/PPIAr* primer sets were used in PCRs 1 and 2, respectively, to produce two overlapping fragments (PR-PPIAa and PR-PPIAb, respectively). Purified amplicons were then joined together in the third step using the external primers, *PRf* and *PPIAr*. (b) A virtual gel of the automated capillary electrophoresis of the final PR-PPIAc gene fragment. Lane L: molecular weight marker; and lane 1: PR-PPIAc gene fragment (199 bp). Two DNA internal markers (lower, 15bp; higher, 1500bp) were added to indicate peak alignments.

Animals infield

A group of 190 male beef cattle, 14-24 months old, including Piedmontese (n = 91) and Charolaise *(n* = 99) breeds, was selected for the beef cattle studies. In addition, Friesian pre-pubertal veal calves (w=177), 5-8 months old, were selected for analysis. This sampling method is already used for official analytical controls in PNR and was performed as previously described (Cannon and Roe 1982). The expected prevalence was 50% with a 95% confidence interval (CI). All the animals originated from the Piedmont region (north-west Italy).

Sample collection, RNA extraction and reverse transcriptase PCR

Bulbo-urethral gland samples were collected from each animal after slaughter. A portion of the gland (100 mg) was immediately fixed in RNAlater (Ambion, LifeTechnologies, Carlsbad, CA, USA) and stored at —80°C for the molecular studies; the remaining tissue was fixed in 10% neutral-buffered formalin for histological analyses.

Total RNA of bulbo-urethral gland samples was extracted using TRIzol reagent (Invitrogen, LifeTechnologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity and integrity were verified by an automated gel electrophoresis system (Experion Instrument, BioRad, Hercules, CA, USA), and cDNA was synthesized from 1 mg of total RNA using the QuantiTect Reverse Transcription Kit

(Qiagen, Hilden, Germany), which included a DNase reaction.

Primer design and PCR strategy

The PCR strategy used to amplify the synthetic gene fragment (PR-PPIAc), make up PR target gene and cyclophilin A (PPIA) housekeeping gene is schematically presented in Figure 1(a), while the primer sequences are listed in Table 1. Briefly, two separate runs of amplification were performed: one PCR reaction (PCR1) utilised the PR forward *(PRf)* and PR-PPIA reverse *(PR-PPIAr)* primers; the other PCR reaction (PCR2) used the PR-PPIA forward *(PR-PPIAf)* and PPIA reverse *(PPIAr)* primers. These two PCRs generated overlapping PR-PPIAa and -b sub-fragments. These sub-fragments were then joined together (PR-PPIAc) in a final run (PCR3) using external *PRf* and *PPIAr* primers. Each PCR protocol was performed using Taq DNA Polymerase (Qiagen) and the following PCR cycling programme: initial denaturation (94°C for 3min), an amplification programme repeated 35 times (94°C for 1 min, 60° C for 1 min, 72°C for 1 min), and a final extension (72°C for 10 min). The size of PR-PPIAc (approximately 200 bp) was verified by an automated electrophoresis station (Experion Instrument) (Figure 1b); DNA analysis was performed using the Experion DNA 1K analysis kit (BioRad) (Delibato et al. 2009).

Table 1. Primer sequences and TaqMan® probes for the PCRs and qPCR.

Gene (RefSeq ID)	Primer	Sequence (5'–3')	Amplicon size (bp)
PR (NM_001205356)	PRf[a]	CCAGAGCCCACAGTACAGCTT	105
	PRr[a]	CAGCTTCCACAGGTGAGGACA	
	PR Probe	FAM-ACAGCCTGATGCTTCATCCCCA CAG-BHQ1	
PPIA (NM_178320)	PPIAf	GCCCCAACACAAATGGTT	95
	PPIAr	CCCTCTTTCACCTTGCCAAAG	
	PPIA Probe	HEX-TGCTTGCCATCCAACCACTCAG TC-BHQ1	
	PR-PPIAr	AACCATTTGTGTTGGGGCCAGCTTCC ACAGGTGAGGACA	123
	PR-PPIAf	TGTCCTCACCTGTGGAAGCTGGCCCC AACACAAATGGTT	116

Note: [a]f and r at the end of the primer name indicate forward and reverse orientations, respectively.

Cloning of PR-PPIAc into the pDRIVE plasmid vector and generation of the recDNA external calibration curve

The PR-PPIAc gene fragment was mixed with the pDRIVE vector in a ligation reaction mixture for 30min at 16°C using a Qiagen PCR Cloning Kit, and the ligation product was transformed into QIAGEN EZ Competent Cells. The cell-vector mixture was incubated on ice for 5min, heat-shocked for 30 s at 42° C in a water bath and immediately transferred to ice for 2min. The cells were then plated onto LB agar containing kanamycin, IPTG and X-gal and incubated at 37°C. White recombinant colonies were picked and subjected to colony PCR to confirm the presence of the PR-PPIAc gene fragment. Positive colonies were grown in LB overnight. The bacteria were harvested, and the plasmid was purified with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

After purification, the recombinant plasmid DNA (recDNA) was sequenced by BMR Genomics (Padova, Italy) using M13R and M13F primers to confirm its identity as PR-PPIAc. The recDNA obtained from the cloning reaction was stored at — 80°C and it was utilised for hundreds of external standard curves. The concentration of the purified recDNA was calculated by multiple optical measurements at 260 nm using an Ultrospec 3100 Pro UV/vis spectrophotometer (Amersham, GE Healthcare, Buckinghamshire, UK). The total number of recDNA bases, 4051 (pDRIVE vector, 3851 bp; PR-PPIAc, 200 bp), was multiplied by 650 Da (the average molecular weight of a DNA base pair) to determine its single-copy molecular weight as 2.63×10^6 Da. One Dalton is 1.67×10^{-24} g, and thus the recDNA weighs 4.39×10^{-18} g; this number was then used to calculate the recDNA copy number μl_{-1} of product purified. The recDNA was used as a starting template in the 20 ml qPCR reaction mix, and a standard curve was generated using serial dilutions of the recDNA from 1.82×10^7 to 1.82×10^2 molecules.

The absolute qPCR reactions were performed in a final volume of 20 ml containing 10 ml of 2x IQ Multiplex Powermix (BioRad), 1 ml of each 6 mM primer (PRf and PPIAr), 1ml of each 2mM TaqMan® probe (Table 1), 1 ml of template DNA and 5ml of nuclease-free water. The PCR cycling conditions consisted of 3min incubation at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 30 s.

Absolute quantification by qPCR was performed using the iQ5 Detection System (BioRad). Each sample was amplified in triplicate.

Primer and TaqMan® probe specificity was documented by an automated electrophoresis station (Experion Instrument). The amplification efficiency and the sensitivity and linearity of the absolute qPCR were evaluated using different starting amounts of the recDNA; these parameters were calculated for PR and PPIA fragments separately given the different TaqMan® probes. PCR amplification efficiencies (E) were calculated from the slopes of the standard curves using the following formula (Rasmussen 2001) (Table 2):

$$E (\%) = [10(-1/slope)] - 1 \times 100$$

Assuming that the relationship between the log of the number of molecules of the PR and PPIA gene fragments present in the qPCR reaction mix and the quantification cycle (Cq) value is linear, the equation of the external standard curve was used to determine the detection limit of the qPCR. The total number of PCR amplification cycles was set to 40.

To confirm the precision and reproducibility of the qPCR, the intra-assay precision was determined using four repeats within one qPCR experiment.

Table 2. Characterisation of the absolute qPCR using the recDNA as a starting template for the calibration curve. The quantitative amplification parameters were calculated as mean values of four different experimental runs.

	PR TaqMan® probe	PPIA TaqMan® probe
Amplification efficiency, % (± SD)	98.5 (5.6)	99.3 (4.0)
Intercept (± SD)	45.0 (1.4)	44.9 (0.9)
Slope (± SD)	−3.36 (0.14)	−3.33 (0.11)
Detection limit (number of molecules)	38	33
Quantification range (number of molecules)	$182–1.82 \times 10^7$	$182–1.82 \times 10^7$
Test linearity, r^2 (± SD)	0.993 (0.004)	0.989 (0.007)

Table 3. Intra-assay (test precision) and inter-assay variation (test variability) of two calibration curves using the recDNA as a starting template.

	Intra-assay variation ($n=4$)		Inter-assay variation ($n=4$)	
	Mean C_q	CV%	Mean C_q	CV%
PR TaqMan® probe				
1.82×10^7	15.4	0.47	16.41	4.55
1.82×10^6	18.23	1.69	19.39	4.38
1.82×10^5	22.96	0.21	23.33	1.98
1.82×10^4	25.34	2.68	26.15	2.57
1.82×10^3	29.47	1.12	29.90	2.30
1.82×10^2	31.61	0.89	33.09	3.84
Average		1.18		3.27
PPIA TaqMan® probe				
1.82×10^7	16.1	0.62	16.58	2.90
1.82×10^6	18.74	4.71	19.47	4.46
1.82×10^5	23.89	0.15	23.42	3.49
1.82×10^4	25.85	3.89	25.93	2.14
1.82×10^3	29.76	2.21	29.89	2.05
1.82×10^2	32.07	0.24	33.13	2.44
Average		1.97		2.91

Inter-assay variation was investigated using four repeats in different experimental qPCR runs over 4 days (Table 3) (Pfaffl and Hageleit 2001; Dionisi et al. 2003). Calculation of variation is based on the variation of C_q (mean and coefficient of variation, CV%).

Absolute quantification by qPCR of PR gene expression on experimental and in-field samples

Amplification of an endogenous control was performed to standardise the amount of DNA added to the qPCR reaction. For the absolute quantification of PR gene expression in each sample, the molecule number of PR cDNA was normalised to the molecule number of PPIA cDNA (PR PPIA"1). PPIA is a housekeeping gene used as a loading control in De Maria et al. (2010). The absolute qPCR reactions were performed in a final volume of 20 ml containing 10 ml of 2x IQ Multiplex Powermix (BioRad), 1 ml of each 6 mM primer (PRf and PRr, PPIAf and PPIAr), 1 ml of each 2mM TaqMan® probe (Table 1), 1 ml of template DNA and 3 ml of nuclease-free water. The qPCR cycling conditions were the same as for the standard curve construction. Each sample was amplified in duplicate.

Decision limit, sensitivity and specificity of the bE2 screening method

In the context of European Commission (2002) Decision 2002/657, the CCa (a = 1%), was defined as the mean signal of 20 blank samples plus three times the corresponding standard deviation (SD). Samples with a signal below this CCa were classified as compliant, while samples with a signal above this CCa were classified as suspect.

In this case, the CCa was calculated from the corrected PR PPIA^{-1} cDNA copies obtained from the bulbo-urethral glands of 20 control Friesian veal calves, 20 control Charolaise beef cattle and 20 control Piedmontese beef cattle (Figure 2).

The sensitivity and specificity of this screening method were calculated using a 2 x 2 contingency table (Table 4) from the qPCR results obtained from the experimental Friesian veal calves and Charolaise beef cattle.

Histopathology

The formalin-fixed samples were embedded in paraffin, cut in 4-mm-thick sections and stained with haematoxylin and eosin. The histopathological investigation was performed on tissue sections from the bulbo-urethral glands of all experimental animals from groups A-D. The bulbo-urethral gland sections of the group E animals were also analysed for the typical histological alterations that are easily visible after betaE2 treatment (Divari et al. 2011). The in-field animals that were suspected of betaE2 treatment via absolute qPCR were also submitted for histological diagnosis to investigate for lesions typical of oestrogen treatment.
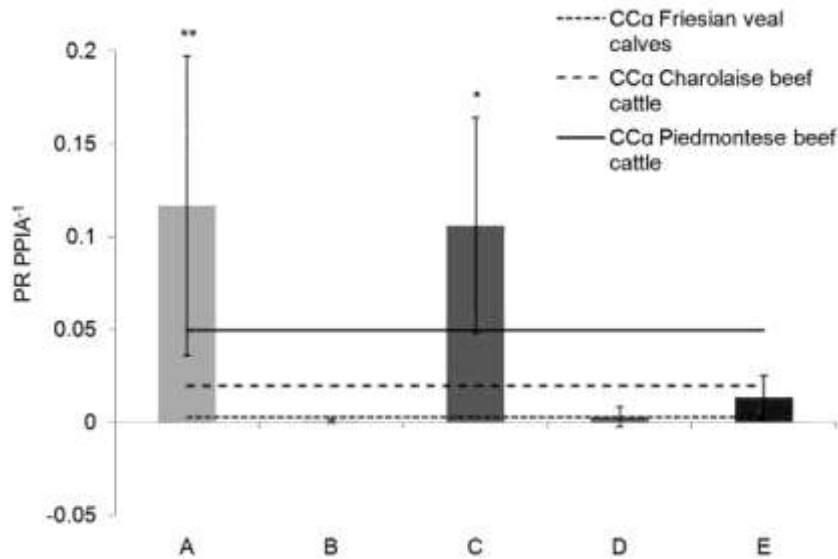
Figure 2. Mean number of PR PPIA ^{1} gene copies calculated from bulbo-urethral glands of control male Friesian veal calves (group B), Charolaise beef cattle (group D), Piedmontese beef cattle (group E) and the PR PPIA^{-1} mean values obtained from bulbo-urethral glands of treated animals of group A (Friesian veal calves) and group C (Charolaise beef cattle). The horizontal lines correspond to CCa values determined from the control groups *(**p 5 0.001, *p 5 0.01)*.

Table 4. The 2 x 2 contingency table for the evaluation of sensitivity and specificity of this qualitative /JE2 screening method in veal calves (trial 1, groups A and B) and beef cattle (trial 2, groups C and D).

PR absolute qPCR	Experimental animal groups	
(Trial 1)	A	B
+	12[a]	0[b]
−	0[c]	20[d]
(Trial 2)	C	D
+	6[a]	1[b]
−	0[c]	19[d]

Notes: [a]Number of true positives.
[b]Number of false positives.
[c]Number of false negatives.
[d]Number of true negatives.

Statistical analysis

Statistical tests were performed using GraphPad InStat software (v.3.05; GraphPad Inc., San Diego, CA, USA). The gene expression change of PR PPIA^{-1} between the experimental animal groups was analysed using an unpaired t-test that compared the treated animal group with each control group. Grubbs's test was used to reveal potential outliers. *p 5* 0.05 was considered to be statistically significant.

Fisher's Exact test was used to evaluate the differences in prevalence of illicit y6E2 treatment among veal calves and beef cattle for the in-field investigation.

Results *PR-PPIAc cloning*

After the first and the second PCR runs, two single bands of the correct length (PR-PPIAa, 123bp; PR-PPIAb, 116bp) were detected. In the final PCR, a 199-bp gene fragment was obtained, which was similar to the expected length of PR-PPIAc (Figure 1b). The PR-PPIAc fragment was successfully cloned into the pDRIVE vector and transformed into a bacterial host. After purification, the recDNA was sequenced by BMR Genomics to confirm its identity as PR-PPIAc (a 99% match) (see Supplementary Material Figure S1).

qPCR amplification efficiency, linearity and limit of detection

The amplification efficiency of the qPCR for the PR-PPIAc fragment was very close to 100%, and was similar for both the PR and PPIA TaqMan® probes. A high linearity was detected in the range of 10^7-10^2 DNA molecules. The theoretical detection limits for the PR and PPIA TaqMan® probes were calculated from the calibration curve and were found to be similar (38 and 33, respectively) (Table 2).

qPCR precision and reproducibility

qPCR precision and reproducibility were calculated by comparing results within a plate (intra-assay experiment) and between plates (inter-assay experiment). The calculations for test precision and test variability were based on the variation of C_q from the C_q mean value

$$(CV\% \quad PR = 1.18-3.27; \quad CV\% \quad PPIA = 1.97-2.91)$$

(Table 3).

Table 5. Total number of male bovines analysed in the infield investigation and the PR positivity distribution. Animals were classified as positive for /JE2 treatment when the PR PPIA^{-1} gene copy total was found to be greater than the respective CCa value.

Breed	Age (months)	PR positive/ number tested	Percentage PR positive
Charolaise	14–21	3/99	3.0
Piedmontese	14–24	2/91	2.2
Friesian	5–8	26/177	14.7

Decision limit of the bE2 screening method

CCa values were calculated for the Friesian veal calves and the Charolaise beef cattle. The CCa of the beef cattle (0.0194) was found to be higher than that of the veal calves (0.0029), indicating that physiological PR gene expression was increased in adult male animals. To include the Piedmontese beef cattle in the in-field investigation, the CCa on 20 control animals was calculated and found to be higher than the value found for the other beef cattle (0.0496) (Figure 2).

Absolute quantification of PR gene expression in experimental animals

The PR PPIA-1 gene expression levels of both the treated veal calves (group A) and the treated beef cattle (group C) were higher than their respective CCa values. The PR gene expression difference between groups A and B (veal calves control group) was statistically significant (p 5 0.001), as was the PR gene expression difference between groups C and D (beef cattle control group) (p 5 0.01). These results confirm previous studies on the fold change of PR gene expression (2_ΔΔCq method) (Divari et al. 2011) (Figure 2).

Sensitivity and specificity of bE2 screening method

Table 4 shows the 2x2 contingency table that evaluates the validity of this method as a qualitative screening test. The method had 100% sensitivity for both the veal calves (95% CI = 73.5-100%) and the beef cattle (95% CI = 54.1-100%). The specificity was 100% for veal calves (95% CI = 83.2-100%) but 95% for adult animals (95% CI = 75.1-99.9%) (Table 4).

In-field investigation

A PR PPIA-1 gene expression value above the respective CCa was common in all three breeds examined, but the highest prevalence of suspected individuals was in the Friesian veal calves (14.7%). In particular, 19 of the veal calves that exhibited a PR PPIA"1 value higher than the CCa 0.0029 came from the same three farms (Table 5). Furthermore, the difference in the treatment prevalence between the beef cattle and veal calf categories was statistically significant (p 5 0.001).

Histopathology

The bulbo-urethral glands of experimental animal groups A and C showed typical histological lesions (Figure 3), which have been widely described in previous studies (Schilt et al. 1998; De Maria et al. 2010; Divari et al. 2011; Pezzolato et al. 2011).

Only one of the five in-field beef cattle that were classified as suspect by qPCR analysis exhibited histological changes in its bulbo-urethral gland tissue; however, 58% of the suspect veal calves showed diffuse hyper-secretion, cysts, epithelial hyperplasia and metaplasia in their bulbo-urethral glands, as shown in previous studies (De Maria et al. 2010; Pezzolato et al. 2011).

Discussion

The European Community established criteria for the analytical control of residues in animals and their by products with European Commission (2002) Decision 2002/657/EC, which recognises direct chemical analysis as the only legal and valid method. This method, although specific and sensitive, is limited because it is very expensive and time-consuming. Moreover, the constant refinement of illicit treatments often prohibits the recognition of residues in food. Therefore, the development of innovative and reliable

methods for the identification and quantification of illicit molecules and their metabolites is important to prevent consumer exposure to potentially dangerous and illegal substances.

This work is an extension of previous studies by De Maria et al. (2010) and Divari et al. (2011) which demonstrated that PR gene expression is up-regulated by betaE2 in the bulbo-urethral glands of veal calves and beef cattle. The up-regulation of the PR gene was calculated using qPCR and the comparative Cq method, and this up-regulation was expressed as a normalised fold change versus control animals.

The first aim of the present study was to verify whether the absolute quantification of PR gene expression by qPCR in conjunction with the standard curve method could be used as an   appropriate
screening method for the detection of betaE2 treatment in pre-pubertal and adult male bovines. This method allowed for the quantification of unknown levels of PR gene expression in a given sample by comparing the unknown level with a known quantity of the recDNA standard.

First, a standard curve was created. Then, unknown samples were compared with the standard curve and their values extrapolated. Thus, it was possible to correlate the number of copies of a gene target to an anabolic treatment of the animal. A calibration model was optimised for two channel qPCR using TaqMan® technology applied to a recDNA that expressed a chimera of the PR and PPIA genes (PR-PPIAc). As demonstrated, the amplification of both calibration curves (PR and PPIA) was efficient and linear over a wide range of starting template copies. These results allowed the use of the recDNA for the generation of a calibration curve, which could then be used to quantify PR PPIA^{-1} gene expression in an unknown sample.

The test had high reproducibility and low variability (maximum of 4.71%), and these parameters were calculated over the whole range of the calibration curve reflecting the real PCR variation (Pfaffl and Hageleit 2001).

The linearity was approximately 0.99 for both calibration curves, and it was stable over a wide range of gene copies (102-107 molecules). This is necessary to quantify the number of PR gene copies in an unknown sample where the number of molecules ranges from 106 to 102 . The copy number of a housekeeping gene in all unknown samples was stable at approximately 106, which is a value that is included in the qPCR quantification range.

The second aim of this study was to determine CCa, sensitivity and specificity values for the qualitative betaE2 screening method of both Friesian veal calves and Charolaise beef cattle using the absolute PR PPIA-1 gene expression values found in 20 control animals. It was evident that the control beef cattle expressed higher levels of the PR gene than control veal calves as previously described (Divari et al. 2011). This is likely due to physiological levels of hormone synthesis in the control adult male animal; in fact, the endogenous betaE2 increases the PR gene expression in accessory sex glands of adult beef cattle and the relative CCa value is higher than in pre-pubertal animals. Based on trials 1 and 2, it was possible to validate the parameters of this screening method as both sensitive and specific. The sensitivity was very high (100%) in veal calves and in beef cattle, which resulted in the identification of betaE2-treated animals using the test. The specificity was 100% in veal calves but only 95% in beef cattle, which reduced the fraction of animals correctly identified as non-treated by the test. This result is probably due to high PR gene expression variability in experimental animal groups C and D.

To extend the scope of the in-field investigation to the Piedmontese breed of beef cattle, a CCa value was calculated using 20 Piedmontese adult male animals. This was necessary because the meat of this breed is particularly consumed in the north-west of Italy, which is the area under consideration in this in-field investigation. The CCa value of Piedmontese animals was found to be higher than the value found for the Charolaise beef cattle. Piedmontese is a breed culled for meat consumption; thus, it is probable that this breed produces a physiologically higher quantity of sex hormone, which results in increased muscle mass. It was shown that an inactivated myostatin gene is responsible for the double-muscling phenotype found in Piedmontese beef (Kambadur et al. 1997); however, Hanset (1982) concluded that other genes independent of the inactive myostatin gene also contributed to the muscling.

Both veal calves and beef cattle from two trials showed a significant increase in PR gene expression after betaE2 treatment, and this up-regulation was detectable 7-15 days after drug administration. The anabolic treatment was confirmed by histopathological analysis as in previous studies (De Maria et al. 2010; Divari et al. 2011; Pezzolato et al. 2011).

These results allow for the application of qPCR analysis as a screening method for betaE2 treatment, and the in-field investigation found that 14.7% *(p 5* 0.001) of the veal calves analysed exhibited a PR PPIA^{-1} value above the relative CCa. In this case, the animals were classified as suspected of *fiE2* treatment, and 58% of these animals showed histological lesions typical of betaE2 treatment (Figure 3). Furthermore, 73% of the suspected animals came from the same three livestocks. With respect to the beef cattle, neither the Charolaise breed nor the Piedmontese breed showed a significant prevalence of animals suspect for betaE2 treatment. It is evident that not all suspect animals exhibit histological alterations of the glandular epithelium; however, this does not exclude the possibility of /SE2 treatment. In fact, when this hormone is administered at a low dosage and in association with other growth promoters that could gloss over its effects on the tissue (e.g. the implant technique), there is no induction of obvious histological alterations in the bulbo-urethral glands (see the results of trial 2 in Divari et al. 2011). For this reason, in these particular conditions the histological test is not a good reference method and, after 7 days from the point at which the low dosage hormones administration was discontinued, the official analytical methods cannot confirm the treatment (Divari et al. 2010). Therefore, at the moment a PR PPIA"1 value higher than CCa value allows one to consider that the animal is suspect of betaE2 treatment although it is not possible to confirm this statement with the other techniques. This method is a screening test that is sensitive and specific to betaE2 treatment in male bovines, and in vitro tissue culture studies

are underway to test the level of PR gene expression after treatment with different oestrogen molecules. Obviously, this method has some limitations such as the need to establish the CCa value for different cattle breeds and cattle age groups. The decision limit in fact depends on the breed and age of animals, the basal PR expression being different in each of these categories. This test is an indirect method that evaluates the biological effect of the drug and does not detect the residue itself. At present, it is necessary to confirm the illicit treatment of animals by analytical methods which are able to detect the presence of residues in the suspected sample. On the other hand, the application of this kind of screening test could reduce the number of samples that need to be analysed by official chemical analysis.

Conclusions

An absolute qPCR method was developed to improve the specificity and sensitivity of the detection of PR gene expression in the bulbo-urethral glands of beef cattle and veal calves treated with betaE2. The detection of PR gene up-regulation could be a valid qualitative screening method to identify animals illegally treated with betaE2 alone (De Maria et al. 2010) or in a cocktail with other growth promoters (Divari et al. 2011). This is the first time that the detection of gene expression changes has been extended to an in-field investigation of Friesian veal calves and Charolaise and Piedmontese beef cattle.

This method identifies a substance, such as betaE2, based on its biological properties, and it gives a binary positive/negative response to betaE2 treatment. This transcriptomics test could become a rapid screening method that is easily incorporated into routine practice and for this reason it has been patented by the University of Turin, Italy (Biolatti et al. 2012).
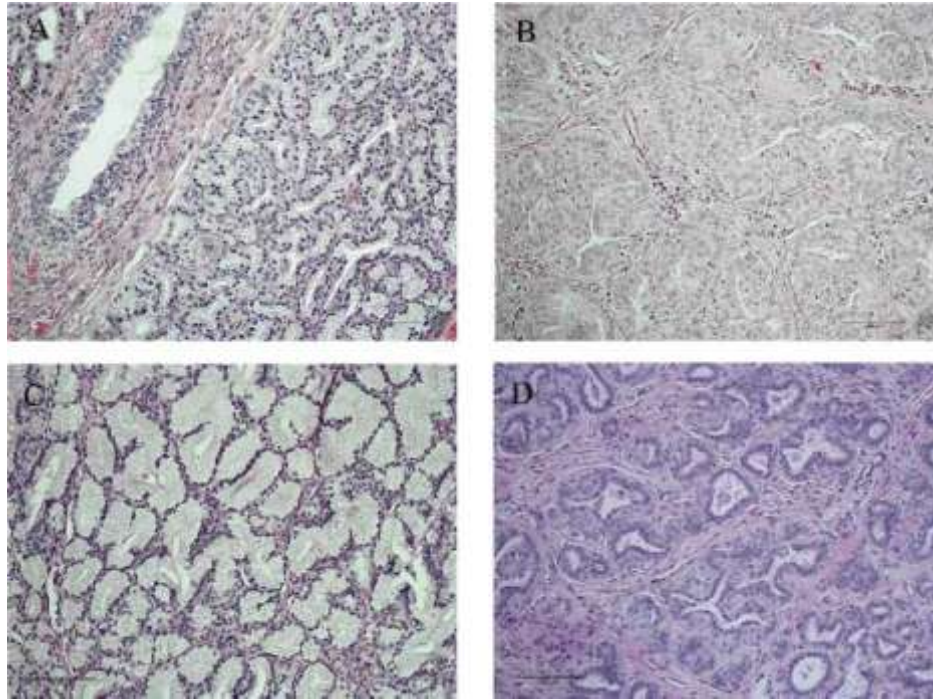
Figure 3. Examples of the histopathological analysis (haematoxylin-eosin (HE) staining) of the bulbo-urethral glands of untreated (A, veal calves; C, beef cattle) and /3E2-treated animals (B, veal calves; D, beef cattle); the bulbo-urethral glands of treated animals exhibited typical epithelial hyperplasia and metaplasia in the secreting portions of the gland (HE, 20x).

References

Biolatti B, Cabassi E, Rosmini R, Groot M, Castagnaro M, Benevelli R, Gilioli G, Ghizzinardi A, Giorgi P, Mazzini C, et al. 2003. Lo screening istologico nella prevenzione dell'uso di anabolizzanti nel bovino. Large Anim Rev. 9:9-19.

Biolatti B, De Maria R, Divari S. 2012. Procedimento e Kit per la rilevazione della somministrazione di sostanze estrogeniche esogene nei bovini. Patent Italy 0001393755. Registered on 8 July 2008 and granted on 30 May 2012.

Cannon RM, Roe RT. 1982. Livestock disease surveys: a field manual for veterinarians. Canberra, ACT, Australia: Australian Bureau of Animal Health.

Cheskis BJ, Greger JG, Nagpal S, Freedman LP. 2007. Signalling by estrogens. J Cell Physiol. 213:610-617.

Davies H. 2010. A role for 'omics' technologies in food safety assessment. Food Contr. 21:1601-1610.

Daxenberger A, Ibarreta DH. 2001. Possible health impact of animal oestrogens in food. Hum Reprod Update. 7:340-355.

De Maria R, Divari S, Spada F, Oggero C, Mulasso C, Maniscalco L, Cannizzo FT, Bianchi M, Barbarino G, Brina N, et al. 2010. Progesterone receptor gene expression in the accessory sex glands of veal calves. Vet Rec. 167:291-296.

Delibato E, Gattuso A, Minucci A, Auricchio B, De Medici D, Toti L, Castagnola M, Capoluongo E, Gianfranceschi MV. 2009. PCR Experion automated electrophoresis system to detect Listeria monocytogenes in foods. J Sep Sci. 32:3817-3821.

Dionisi HM, Harmas G, Layton AC, Gregory IR, Parker J, Hawkins SA, Robinson KG, Saylor GS. 2003. Power analysis for real time PCR quantification of genes in activated sludge and analysis of the variability introduced by DNA extraction. Appl Environ Microbiol. 69:6597-6604.

Divari S, De Maria R, Cannizzo FT, Spada F, Mulasso C, Bovee TFH, Capra P, Leporati M, Biolatti B. 2010. A RIKILT yeast estrogen bioassay (REA) for estrogen residue detection in urine of calves experimentally treated with 17/S-estradiol. Food Addit Contam A Chem Anal Control Expo Risk Assess. 27:19-28.

Divari S, Mulasso C, Uslenghi F, Cannizzo FT, Spada F, De Maria R, Brina N, Biolatti B. 2011. Progesterone receptor up-regulation: a diagnostic tool for the illicit use of oestrogens in adult beef cattle. Food Addit Contam A Chem Anal Control Expo Risk Assess. 28:1677-1686.

European Commission. 1996. EC Council Decision 1996/22 of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repeating Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Off J Eur Comm. L125:3-9.

European Commission. 2002. EC Council Decision 2002/ 657, of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical meth-ods and the interpretation of results. Off J Eur Comm. L221:8-36.

Grandmontagne C. 1986. Modifications histologiques de la prostate du veau traité par les différents anabolisants utilisés en élevage. Rev Mèd Véterinaire. 137:37-47.

Groot MJ, Ossenkoppele JS, Bakker R. 2000. Changes in cytokeratin expression in accessory sex glands of growth promoter treated veal calves: influences of treatment schedule and combination of drugs. Eur J Vet Pathol. 6:19-24.

Groot MJ, Schilt R, Ossenkoppele JS, Berende PLM, Haasnoot W. 1998. Combinations of growth promoters in veal calves: consequences for screening and confirmation methods. Zentralbl Veterinarmed A. 45:425^40.

Hanset R. 1982. Muscular hypertrophy as a racial characteristic: the case of the Belgian Blue breed. In: King JWB, Menissier F, editors. Muscle hypertrophy of genetic origin and its use to improve beef production: current topics in veterinary medicine and animal science. Vol. 16. The Hague, the Netherlands: Martinus Nijhoff Publishers, p. 437-449.

Henricks DM, Gray SL, Owenby JJ, Lackey BR. 2001. Residues from anabolic preparations after good veterinary practice. APMIS. 109:273-283.

Italian National Program for Residue Surveillance (PNR). 2010. Relazione sui risultati dei controlli ufficiali svolti in attuazione del piano nazionale residui 2010 [cited 2012 Oct 31]. Available from: http://www.salute.gov.it/imgs/ C_1 7_pubblicazioni_1 637_allegato.pdf

Kambadur R, Sharma M, Smith TPL, Bass JJ. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese Cattle. Genome Res. 7:910-915.

Lopparelli RM, Giantin M, Pozza G, Stefani AL, Ravarotto L, Montesissa C, Dacasto M. 2012. Target gene expression signatures in neutrophils and lymphocytes from cattle administered with dexamethasone at growth promoting purposes. Res Vet Sci. 93:226-233.

Meyer HHD. 2001. Biochemistry and physiology of anabolic hormones used for improvement of meat production. APMIS. 109:1-8.

Miller WR, Sharpe RM. 1998. Environmental oestrogens and human reproductive cancers. Endocr-Relat Cancer. 5:69-96.

Mooney MH, Bergwerff AA, van Meeuwen JA, Luppa PB, Elliott TC. 2009. Biosensor-based detection of reduced sex hormone binding globulin in response to growth-promoter administration. Anal Chim Acta. 637:235-240.

Mooney MH, Situ C, Cacciatore G, Hutchinson T, Elliott C, Bergwerff AA. 2008. Plasma biomarker profiling in the detection of growth promoter use in calves. Biomarkers. 13:246-256.

Nebbia C, Urbani A, Carletti M, Gardini G, Balbo A, Bertarelli D, Girolami F. 2011. Novel strategies for tracing the exposure of meat cattle to illegal growth-promoters. Vet J. 189:34-42.

Parr SL, Chung KY, Hutcheson JP, Nichols WT, Yates DA, Streeter MN, Swingle RS, Galyean ML, Johnson BJ. 2011. Dose and release pattern of anabolic implants affects growth of finishing beef steers across days on feed. J Anim Sci. 89:863-873.

Pezzolato M, Maurella C, Varello K, Meloni D, Bellino C, Borlatto L, Di Corcia D, Capra P, Caramelli M, Bozzetta E. 2011. High sensitivity of a histological method in the detection of low-dosage illicit treatment with 17/toestradiol in male calves. Food Contr. 22:1668-1673.

Pfaffl MW, Hageleit M. 2001. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett. 23:275-282.

Pinel G, Weigel S, Antignac J-P, Mooney MH, Elliott C, Nielen MW, Le Bizec B. 2010. Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle. Trends Anal Chem. 29:1269-1280.

Rasmussen RP. 2001. Quantification on the LightCycler. In Meuer S, Wittwer C, Nakagawara K, editors. Rapid cycle real-time PCR, methods and applications. Heidelberg: Springer Press. p. 21-34.

Riedmaier I, Becker C, Pfaffl MW, Meyer HH. 2009. The use of omic technologies for biomarker development to trace functions of anabolic agents. J Chromatogr A. 1216:8192-8199.

Risbridger GP, Wang H, Frydenberg M, Cunha G. 2001. The metaplastic effects of estrogen on mouse prostate epithelium: proliferation of cells with basal cell phenotype. Endocrinol. 142:2443-2450.

Schilt R, Groot MJ, Berende PL, Ramazza V, Ossenkoppele JS, Haasnoot W, van Bennekom EO, Brouwer L, Hooijerink H. 1998. Pour on application of growth promoters in veal calves: analytical and histological results. Analyst. 123:2665-2670.

Sharpe RM, Skakkebaek NE. 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet. 341:1392-1395.

Skakkebaek NE. 2003. Testicular dysgenesis syndrome. Horm Res. 60(Suppl. 3):49.

Toffolatti L, Rosa Gastaldo L, Patarnello T, Romualdi C, Merlanti R, Montesissa C, Poppi L, Castagnaro M, Bargelloni L. 2006. Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. Domest Anim Endocrinol. 30:38-55.