Development and Application of a Screening Method of Absolute Quantitative PCR to Detect the Abuse of Sex Steroid Hormone Administration in Male Bovines

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Introduction

The use of growth promoters (GPs), namely, sex steroid hormones and glucocorticoids, is tempting in bovine husbandry to gain greater muscle mass and reduce fat content. Drug residues represent a potential risk for beef and beef products consumers. Consequently, the administration of such molecules as GPs in livestock is illegal in the European Union (EU). Nevertheless, 10% of animals has been estimated to be illicitly treated in the EU, whereas the official analytical methods find as noncompliant <0.5% of tested samples. Although highly sensitive, these methods (mainly GC-MS and LC-MS/MS) are time-consuming and costly. Additionally, the official assays are not able to identify unknown molecules, which are often present in seized black market preparations (1) or added in amounts well below the minimum quantification threshold. Therefore, alternative screening methods have been developed. They are focused on the identification of the biological effects of illicit compounds in the target organs, independent of applied substance and the manner of application (2-7). For example, in the bulbo-urethral glands and prostate of veal calves and beef cattle, typical lesions, namely, hyperplasia and squamous metaplasia, are caused by estrogenic hormones. (3, 8-11)

The advantage of these alternative methods is that alterations in cell or tissue persist a long time after the treatment withdrawal, even if drugs and their metabolites are no longer measurable. Indeed, the illicit 17β-estradiol treatment can be identified by histological methods in veal calves (12, 13) and beef cattle (3) up to 15 days after the last treatment was discontinued. Since 2008 the Italian PNR officially adopted this method to detect illegal hormone administration.

Nevertheless, anabolic compounds are frequently administered in low-dose cocktails (1) inducing weak microscopic lesions in sex accessory glands, (3) and then they can elude preliminary screenings. Therefore, screening tests using more sensitive and specific technologies are desirable.

The transcriptomic techniques are based on the use of certain transcriptional markers for the low-cost screening analysis of GP administration in livestock production. (14) A number of novel gene expression profiles have been examined in different bovine tissues, and some of these findings could be applicable in the field as routine screening methods. (2, 14-17) For instance, the expression of progesterone receptor (PR) gene in the prostate and bulbo-urethral glands has been identified as a successful biomarker for the detection of beef cattle and veal calves illegally treated with estrogens. (3, 12, 18) In the same way, gene expression change of oxytocin (OXT) in beef cattle muscle may suggest an illicit

treatment with glucocorticoids and estrogens. (4) The University of Turin (Italy) patented PR- and OXT-based screening tests, due to their ability to detect illegally treated animals long after GP withdrawal. (4, 18)

Recently, it was demonstrated that sex steroid hormones alone or in association with other drugs induce the decrease of RGN gene and protein expression in bovine testis. (19, 20) These results suggest that the RGN gene may be used as a biomarker to detect animals illegally treated with sex steroid hormones.

A technique for the absolute quantification of RGN gene via quantitative PCR (qPCR) was developed to prove that the decrease of this gene expression in the testis is an effective biomarker to detect sex steroid hormone abuse in veal calves and beef cattle. A further in-field investigation of veal calves and beef cattle in the Piedmont region (northwestern Italy) was conducted to evaluate possible sex steroid hormone abuse in these animals.

Materials and Methods

Animals, Experimental Designs, and Sample Collection

In trial 1, 18 4-month-old Friesian male veal calves were randomly divided as follows: group A (n = 6) was intramuscularly administered once a week 17β-estradiol for 6 times (for a total of 190 mg/calf); group B (n = 6) was intramuscularly administered once a week testosterone propionate for 6 times (for a total of 1050 mg/calf); group K1 (n = 6) represented the control. The first treatment was administered at nearly 140 days of age, and the challenge was carried out for 44 days. Six days after the last treatment, animals were slaughtered.

In trial 2, 16 4-month-old Friesian male veal calves were randomly divided as follows: group C (n = 8) was treated with 150 mg/calf of Nandrosol (17β,19-nortestosterone phenylpropionate) every 2 weeks for 4 times in association with 80 mg/calf/day of ractopamine per os for the last 31 days of treatment; group K2 (n = 8) represented the control. Three days after the last treatment, animals were slaughtered.

The calves of trials 1 and 2 were housed as previously reported. (19)

In trial 3, 12 male Charolaise beef cattle 17–22 month old were randomly divided as follows: group D (n = 6) was intramuscularly administered for 5 weeks 25 mg/beef/week of 17β-estradiol; group K3 (n = 6) was the control. Six days after the last treatment the animals were slaughtered.

In trial 4, 32 10–18-month-old Friesian male beef cattle were randomly divided as follows: group E (n = 8) was treated with Revalor-200 (Intervet, USA) slow-release subcutaneous pellets, containing 200 mg of trenbolone acetate and 20 mg of 17β-estradiol for 89 days; group F (n = 8) was treated with Revalor-200 slow-release subcutaneous pellets for 89 days in association with Desashock 0.7 mg/beef/day per os for the last 40 days of treatment; group G (n = 8) was treated with Finaplix-H (Intervet, USA) slow-release subcutaneous pellets, containing 200 mg of trenbolone

acetate for 89 days; group K4 (*n* = 8) represented the control. The pellets remained in place until slaughter.

The beef cattle of trials 3 and 4 were housed as previously reported (19).

The Ethical Committee of the University of Turin and the Italian Ministry of Health authorized all of the trials. According to national legislation (2003/74/CE-DL 16 March 2006, No. 158), the carcasses of the treated animals were properly destroyed.

At slaughtering, samples of testes were collected from all animals and stored for molecular investigations.

Animals in Field and Sample Collection

A group of 54 male veal calves (5–8 months old) and a group of 70 male beef cattle (13–24 months old) were selected for the study. The animals originated from the Piedmont region (northwestern Italy).

Samples from testes were collected from each animal at the slaughterhouse, immediately fixed in RNAlater (Sigma, St. Louis, MO, USA) for molecular studies, and preserved at 4 °C. The following day the supernatant was discarded, and the samples were stored at −80 °C.

Some of the remaining testes were fixed in Bouin–Hollande solution for 24 h for immunohistochemistry (IHC). After the fixation, the samples were repeatedly washed in water and then paraffin-embedded overnight at room temperature.

RNA Extraction and Reverse Transcription

RNA was extracted from 50 mg for each sample through a TissueLyser II (Qiagen, Hilden, Germany) disruption in 1 mL of TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA) by means of stainless steel beads, according to the manufacturer's instructions. Using a spectrophotometer, the RNA concentration was quantified and the RNA integrity was verified by means of Experion Instrument (Bio-Rad, Hercules, CA, USA). cDNA was obtained from 1 µg of total RNA by means of the QuantiTect Reverse Transcription Kit (Qiagen), including an optimized blend of random primers and oligo-dT.

Selection of Reference Genes

Four genes commonly utilized as references in qPCR analysis were selected: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), ribosomal protein S5 (RPS5), and succinate dehydrogenase complex, subunit A (SDHA). Genes from different functional classes were considered to minimize a potential co-regulation of genes. The primer sequences of GAPDH and RPS5 were designed using Primer3 software (vers. 4.0.0) on the basis of reference sequence NM_001034034 and NM_001015531, respectively. Primer sequences of PPIA and SDHA were previously reported. (4, 21) Primer information is summarized in Table 1.

Table 1. Primer Sequences for qPCR

gene	GenBank accession no.	sense	exon	amplicon length (bp)	melting temperature (°C)
GAPDH	NM_001034034	F	5	102	63.1
		R	6		62.4
PPIA	NM_178320	F	4	95	59.5
		R	5		61.2
RPS5	NM_001015531	F	4	71	62.1
		R	5		64.1
SDHA	NM_174178	F	14	185	60.4
		R	15		62.5
RGN	NM_173957	F	3	115	60.7
		R	4		58.8

Analysis of Reference Genes Expression Stability

The reference genes expression stability was analyzed though geNorm version 3.5. (22) The software generates an internal control stability measure (M value) for all examined genes which is arbitrarily set less than 1.5. A lower M value indicates a greater gene stability across the samples.

Primer Design and PCR Strategy

The PCR strategy used to amplify the synthetic gene fragment (RGN-PPIAc), containing the RGN target gene and PPIA housekeeping gene, is schematically presented in Figure 1A. The primer and probe sequences of RGN were designed using Primer3 software (vers. 4.0.0) on the basis of reference sequence NM_173957. Primer and probe sequences of PPIA were previously reported. (4) Primer information is summarized in Table 1.

Figure 1

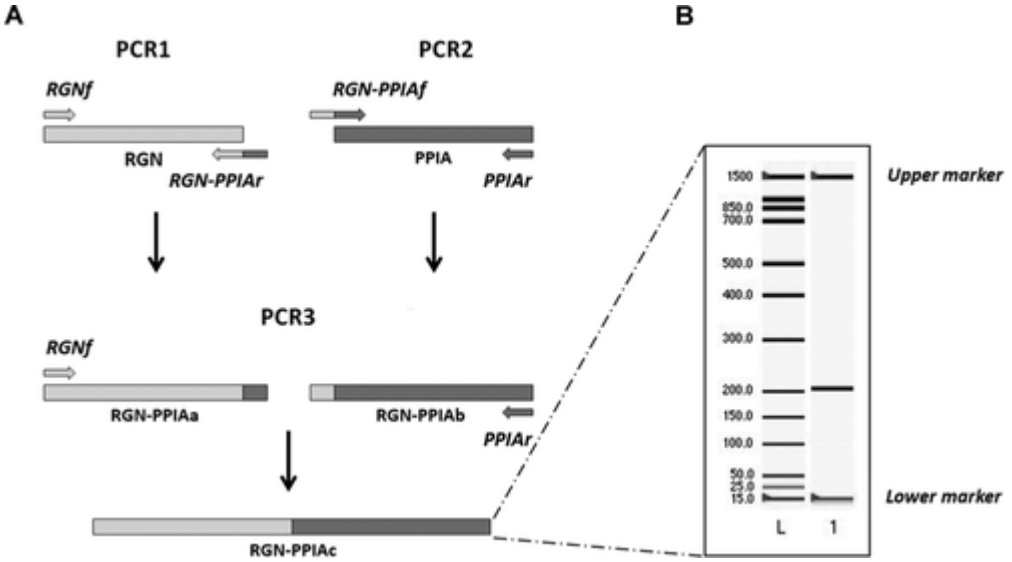

Figure 1. (A) PCR strategy used to join the RGN and PPIA gene fragments and to obtain RGN-PPIAc. The RGNf/RGN-PPIAr and RGN-PPIAf/PPIAr primer sets were used in PCRs 1 and 2, respectively, to produce two overlapping fragments (RGN-PPIAa and RGN-PPIAb, respectively). Purified amplicons were then joined together in PCR3 using the external primers, RGNf and PPIAr. (B) Virtual gel of the automated capillary electrophoresis of the final RGN-PPIAc gene fragment. Lanes: L, molecular weight marker; 1, RGN-PPIAc gene fragment (210 bp). Two DNA internal markers (lower, 15 bp; higher, 1500 bp) were added to indicate peak alignments.

Briefly, two separate runs of amplification were performed: the first PCR reaction (PCR1) used the RGN forward (RGNf) and RGN-PPIA reverse (RGN-PPIAr) primers; the second PCR reaction (PCR2) used the RGN-PPIA forward (RGN-PPIAf) and PPIA reverse (PPIAr) primers. These PCRs generated the overlapping RGN-PPIAa and -b subfragments that were then joined together (RGN-PPIAc) in a final run (PCR3) using external RGNf and PPIAr primers. Each PCR protocol was performed through the Taq DNA Polymerase (Qiagen) and the following cycling program: a denaturation (94 °C, 3 min), an amplification program repeated 35 times (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min), and a final elongation (72 °C, 10 min). The size of RGN-PPIAc (approximately 210 bp) was verified through the Experion DNA 1K Analysis Kit (Bio-Rad) by Experion Instrument (Figure 1B).

Cloning of RGN-PPIAc and Development of the External Standard Curve

The RGN-PPIAc gene fragment and the pDRIVE vector were mixed in a ligation reaction mixture (16 °C, 30 min) by means of a Qiagen PCR Cloning Kit. After ligation, the obtained product was transformed into Qiagen EZ Competent Cells, and the mixture was incubated on ice for 5 min, heat-shocked at 42 °C for 30 s, and then carried on ice for 2 min. The cells were subsequently plated onto LB agar with ampicillin, X-gal, and IPTG and incubated at 37 °C. Recombinant colonies were

grown in LB overnight, and the presence of RGN-PPIAc gene fragment was confirmed via PCR. The plasmid was purified from harvested bacteria through the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. After purification, the identity of the recombinant DNA (recDNA) as RGN-PPIAc was confirmed by sequencing at BMR Genomics (Padova, Italy). The recDNA achieved from the cloning reaction was preserved at −80 °C, and it was used to generate the external standard curves. Repeated optical measurements at 260 nm by means of a BioPhotometer Plus (Eppendorf, Hamburg, Germany) were performed to quantify the purified recDNA. The single-copy molecular weight of the plasmid is 2.64×10^6 Da, and it was calculated by multiplying the total number of recDNA bases, 4061 (pDRIVE vector, 3851 bp; RGN-PPIAc, 210 bp) by 650 Da (the DNA base pair mean molecular weight). One dalton is 1.67×10^{-24} g, and thus the recDNA weighs 4.41×10^{-18} g; this number was then used to calculate the recDNA copy number per microliter of product purified. The recDNA represented the starting template in the 20 μL qPCR reaction mix, and a standard curve was created through serial dilutions of the recDNA in the range of 2.54×10^9–4.06×10^6 molecules. The absolute qPCR reactions were performed with 10 μL of 2× IQ Multiplex Powermix (Bio-Rad), 5 μL of nuclease-free water, 1 μL of each 6 μM primer (RGNf and PPIAr), 1 μL of each 2 μM TaqMan probe, and 1 μL of DNA template. The PCR cycling program was the following: incubation at 95 °C, 3 min; 40 cycles of 95 °C, 10 s, and 60 °C, 30 s.

The iQ5 Detection System (Bio-Rad) was used for the absolute quantification by qPCR. The amplification of each sample was performed in triplicate. The linearity, amplification efficiency, and sensitivity of the absolute qPCR were assessed by means of different recDNA starting amounts. Given the different TaqMan probes used, the previous parameters were considered separately for RGN and PPIA fragments. On the basis of the slopes of the standard curves, PCR amplification efficiencies (*E*) were calculated from the following formula: (23)

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

A covariance analysis (ANCOVA) was performed to verify the precision (intra-assay) and reproducibility (interassay) of the standard curves' amplification. A possible significant difference between slopes and intercepts of two standard curves (A and B) amplified in the same qPCR run (intra-assay) and among slopes and intercepts of six standard curves amplified in three independent qPCR runs (qPCR assays 1, 2, and 3) (interassay) was tested by the calculation of the *P* and *F* values. (4, 24)

Absolute Quantification of RGN Gene Expression in the Testes of Animals from Trials 1–4

The RGN/PPIA ratio was calculated by the normalization of the RGN cDNA copy number to the PPIA cDNA copy number, and it was used to absolutely quantify the RGN gene expression in the testes of the examined animals.

The variability in the RGN and PPIA absolute quantification in a single qPCR run (intra-assay) and among different qPCR runs (interassay) was determined by qPCR experiments. Subsequently, three independent qPCR assays (qPCR assays 1, 2,

and 3) were performed as described in the previous paragraph. Each qPCR assay included three serial dilutions of an unknown testis sample (1, 1:2, and 1:4), replicated four times. The presence of a standard curve in all qPCR runs allowed the RGN/PPIA ratio to be quantified for all sample dilutions. The mean, standard deviation, and coefficient of variation (CV%) were determined on the basis of the change of RGN/PPIA gene ratio.

For each gene, the copy number was obtained from the recDNA standard curves. The qPCR for RGN and PPIA genes were performed in a 20 µL reaction, using 10 µL of 2× IQ Multiplex Powermix (Bio-Rad), 3 µL of nuclease-free water, 1 µL of each 6 µM PPIA primer (RGNf/RGNr, PPIAf/PPIAr), 1 µL of each 2 µM TaqMan probe, and 1 µL of DNA template. The same qPCR cycling conditions used for the standard curve construction experiments was then applied. All of the samples of the testes were amplified in duplicate.

Parameters of the RGN Screening Test

A receiver operating characteristic (ROC) analysis was performed to investigate the efficacy of the decrease of RGN gene expression as a potential biomarker to detect veal calves and beef cattle illegally treated with sex steroid hormones. The values of the RGN/PPIA ratio achieved from the experimental groups were used to generate the ROC curves, which allowed the criterion value (cutoff), specificity, and sensitivity to be calculated (with a 95% confidence interval, CI).

Sensitivity is the probability that the test rightly identifies a sex steroid hormone-treated animal (true positive), and 100 – specificity % indicates the probability that the test incorrectly identified a negative subject as positive (false positive). The calculation of the likelihood ratios of positive (+LR) versus negative (−LR) outcomes, with a 95% CI, allowed the cutoff to be selected. Moreover, Youden's index (*J*) was considered. It maximizes the difference between true positives and false positives and was calculated using the following formula:

$$J = \text{sensitivity}\,\% - (100 - \text{specificity}\,\%)$$

Then, the maximum value of the Youden's index may be used as a criterion for selecting the optimum cutoff point. Graphically, the index is represented as the highest vertical distance between the diagonal line and the ROC curve.

Immunohistochemistry

The IHC was performed on the testis samples by means of an anti-RGN rabbit polyclonal antibody (Sigma). The 3 µm deparaffinized sections were rehydrated, and then the blocking of the endogenous peroxidases was performed by incubating the slides in 3% hydrogen peroxide for 15 min. Following multiple washings in PBS, the antigen retrieval was performed by immersing the sections in citrate buffer (10 mM, pH 6.0) at 98 °C for 40 min. The slides were incubated for 1 h at room temperature with the primary antibody (1:150). An EnVision Kit (Dako, Glostrup, Denmark) was used to visualize the RGN protein, using an HRP-labeled secondary antibody. As chromogen, a diaminobenzidine–hydrogen peroxide solution (Dako)

was applied on the slides for 5 min. The slides were rinsed in distilled water, hematoxylin counterstained, dehydrated, and finally mounted with a coverslip.

Immunolabeling of RGN was scored by use of a semiquantitative scale incorporating both the proportion of positively stained target cells (scored on a scale of 0–5) and the intensity of the staining (scored on a scale of 0–3), according to the modified Allred score (25) used in breast cancer. The proportion score (PS) was given as 0 (none), 1 (1%), 2 (1–10%), 3 (10–33%), 4 (33–66%), or 5 (>66%). The intensity score (IS) was given as 0 (no staining), 1 (weak staining), 2 (intermediate), or 3 (strong). A sum of PS and IS scores of ≤3 was considered positive, and all other scores were considered negative.

Statistical Analyses

GraphPad Prism (vers. 3.05) software (GraphPad Inc., San Diego, CA, USA) was used to perform all of the statistical analyses. Each examined group was tested for deviations from Gaussian distribution using the Kolmogorov–Smirnov (KS) test. The opportune test was consequently applied. A one-way analysis of variance (ANOVA), followed by Dunnett's post-test, was used to analyze the absolute quantification of the RGN/PPIA ratio in trials 1 and 4. The unpaired *t* test and the Mann–Whitney test were respectively applied in trials 2 and 3. Grubbs' test was performed to identify and exclude potential outliers.

The slopes and intercepts of the six standard curves were compared using ANCOVA, and *P* and *F* values were calculated.

The mean, standard deviation, and coefficient of variation (CV%) were determined on the basis of the change of RGN/PPIA gene ratio. The Kruskal–Wallis test, followed by Dunn's multiple-comparison post test, was performed to analyze the absolute quantification variability of the unknown sample. A $P < 0.05$ was considered statistically significant.

The best relationship between sensitivity and specificity was achieved through a ROC curve analysis.

Results

GeNorm Analysis

The PPIA gene with an *M* value of 0.865 was indicated as the most stable reference gene by GeNorm analysis (Table 2). Then, the PPIA gene was designated as reference for the development of the test.

Table 2. Gene Expression Stability of Candidate Reference Genes in Bovine Testis Analyzed by GeNorm Software

gene	*M* value
PPIA	0.865
SDHA	0.875
GAPDH	0.925
RPS5	1.015

RGN-PPIAc Cloning

After the PCR1 and PCR2 runs, two single bands of the correct length (RGN-PPIAa, 132 bp; RGN-PPIAb, 116 bp) were detected. In the final PCR, a 210 bp gene fragment was obtained, which was similar to the expected length of PR-PPIAc (Figure 1B). The RGN-PPIAc fragment was effectively cloned into the vector and transformed into a bacterial host. The sequencing of the purified recDNA confirmed its identity as RGN-PPIAc (a 99% match).

qPCR Amplification Efficiency and Linearity

The amplification efficiency percent, based on the slope of the standard curves, the test linearity (or r^2 value), and the intercepts were calculated for each standard curve (Table 3). The qPCR amplification efficiency for the RGN-PPIAc fragment was very close to 100% and was similar for both the RGN and PPIA TaqMan probes. A great linearity was pointed out in the range of 10^6–10^9 DNA copies.

Table 3. Parameters of the qPCR for Six Standard Curves Concerning RGN and PPIA Amplified in Three Independent qPCR Runs[a]

	qPCR assay 1		qPCR assay 2		qPCR assay 3	
RGN recDNA standard curve	**A**	**B**	**A**	**B**	**A**	**B**
amplification efficiency %	106.7	112.0	108.5	110.0	96.1	100.6
slope	–3.170 ± 0.060	–3.065 ± 0.066	–3.133 ± 0.065	–3.103 ± 0.040	–3.420 ± 0.054	–3.307 ± 0.037
intercept	47.28 ± 0.48	46.55 ± 0.53	46.52 ± 0.52	46.39 ± 0.32	49.02 ± 0.44	48.24 ± 0.30
S*y.x*	0.2305	0.2517	0.248	0.1543	0.2085	0.1427
quantification range	2.54×10^9–4.06×10^6					
test linearity, r^2	0.9953	0.9941	0.9945	0.9978	0.99675	0.9984
	intra-assay 1 (*P*; *F*)		intra-assay 2 (*P*; *F*)		intra-assay 3 (*P*; *F*)	
slope	0.2458; 1.41016		0.6976; 0.154398		0.09868; 2.93315	
intercept	0.1947; 1.76826		0.1646; 2.04065		0.06598; 3.67198	
	interassay (*P*; *F*)					
slope	0.3287; 1.28275					
intercept	**0.017***; 6.29462					
	qPCR assay 1		**qPCR assay 2**		**qPCR assay 3**	
PPIA recDNA standard curve	**A**	**B**	**A**	**B**	**A**	**B**
amplification efficiency %	108.3	112.9	111.1	109.9	96.3	97.7

	qPCR assay 1		qPCR assay 2		qPCR assay 3	
PPIA recDNA standard curve	**A**	**B**	**A**	**B**	**A**	**B**
slope	–3.139 ± 0.060	–3.047 ± 0.059	–3.082 ± 0.091	–3.105 ± 0.062	–3.413 ± 0.066	–3.378 ± 0.073
intercept	47.53 ± 0.48	46.92 ± 0.47	46.58 ± 0.73	46.91 ± 0.50	49.39 ± 0.53	49.24 ± 0.59
S*y.x*	0.2296	0.2251	0.3472	0.238	0.2529	0.2801
quantification range	2.54×10^9–4.06×10^6					
test linearity, r^2	0.9953	0.9952	0.9889	0.9948	0.9952	0.9939
	intra-assay 1 (*P*; *F*)		intra-assay 2 (*P*; *F*)		intra-assay 3 (*P*; *F*)	
slope	0.2857; 1.1883		0.8375; 0.0429249		0.72441; 0.127294	
intercept	0.146; 2.24051		0.1845; 1.85446		0.1713; 1.97531	
	interassay (*P*; *F*)					
slope	0.07121; 4.9281					
intercept	**0.02964** *****; 4.9281					

[a]

ANCOVA tested the variation of intra- and inter-run standard curves.
The *P* and *F* values were calculated to determine a potential significant difference between the slopes and the intercepts in the same qPCR run (intra-assay) and among the slopes and the intercepts in the three independent qPCR runs (interassay).

For each recDNA, the similarity of the standard curve parameters was determined to verify the reproducibility of absolute quantification through qPCR. The two intra-assay standard curves in each assay were highly reproducible. No significant differences among the slopes of the interassay standard curves were revealed by

ANCOVA, whereas a statistical difference was pointed out for the intercepts ($P < 0.05$) (Table 3).

Variability in the Absolute Quantification of the Copy Number of the RGN and PPIA Genes in Testis

Using three independent qPCR assays, the number of copies of RGN/PPIA in three different dilutions (1, 1:2, and 1:4) of the same unknown sample was determined by means of a recDNA standard curve. The intra-assay showed a CV% range of 13.1447–34.1993. The interassay showed a CV% range of 20.3631–21.7588 (Table 4).

Table 4. Intra-assay (Test Precision) and Interassay Variation (Test Variability) for RGN/PPIA Values Calculated through Three Independent qPCR Runs[a]

	interassay (n = 36)			intra-assay (n = 12)								
				qPCR assay 1			qPCR assay 2			qPCR assay 3		
dilution	RGN/PPIA	SD	CV%	RGN/PPIA	SD	CV%	RGN/PPIA	SD	CV%	RGN/PPIA	SD	CV%
1	0.0150	0.00030	20.3631	0.0161	0.00039	23.9433	0.0155	0.30031	19.7484	0.0133	0.00020	15.3139
1:2	0.0126	0.00027	21.7588	0.0141	0.00019	13.1447	0.0127	0.00018	14.3964	0.0109	0.00037	34.1993
1:4	0.0141	0.00029	20.5968	0.0162	0.00033	20.0928	0.0131	0.00024	18.4068	0.0129	0.00023	17.7415

[a] RGN/PPIA mean, standard deviation (SD), and coefficient of variation (CV%) were determined on the basis of three different dilutions of an unknown sample.

qPCR Absolute Quantification of RGN Gene Expression in the Testes of Experimental and In-Field Animals

All of the examined samples showed a RNA quality indicator (RQI) >7.

The absolute quantification of RGN gene expression (RGN/PPIA ratio) is reported in Figure 2. The RGN/PPIA ratio significantly decreased in group A (mean ± SEM, $3.10 \times 10^{-3} \pm 3.43 \times 10^{-4}$) ($P < 0.01$) and group B ($8.26 \times 10^{-3} \pm 1.44 \times 10^{-3}$) ($P < 0.01$) compared to group K1 ($3.82 \times 10^{-2} \pm 7.50 \times 10^{-3}$) (Figure 2A). The RGN/PPIA ratio significantly decreased in group C ($7.34 \times 10^{-3} \pm 1.23 \times 10^{-3}$) compared to group K2 ($1.62 \times 10^{-2} \pm 2.43 \times 10^{-3}$) ($P < 0.01$) (Figure 2B). Only two treated animals (no. 9 and 20) showed a RGN/PPIA ratio higher than the optimal criterion value, whereas one calf belonging to the control group K2 (no. 31) was under the threshold.

Figure 2

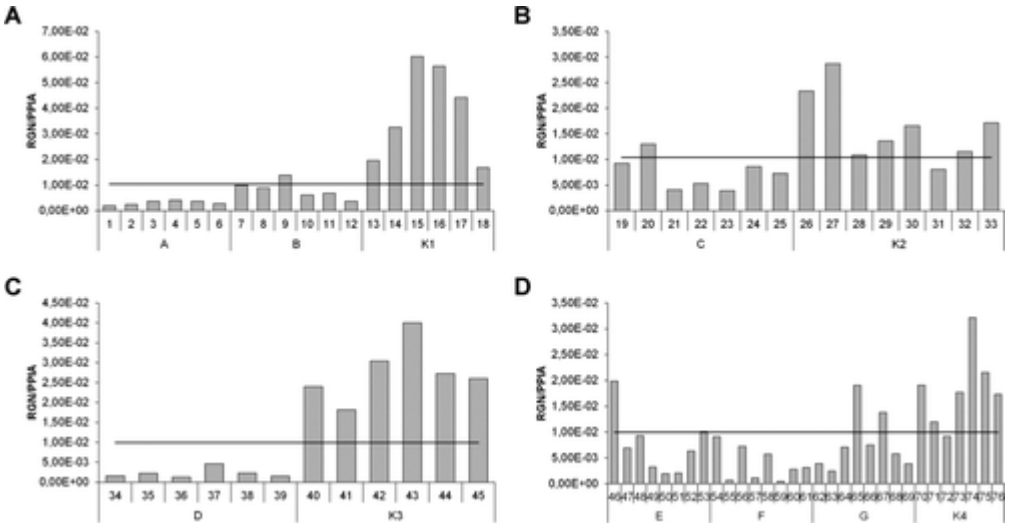

Figure 2. Absolute qPCR data (RGN/PPIA ratios) from experimental groups of trial 1 (A), trial 2 (B), trial 3 (C), and trial 4 (D). The black lines indicate the optimal criterion value calculated by ROC curve analysis for veal calves (A, B) and beef cattle (C, D). A, 17β-estradiol; B, testosterone propionate; K1, control of trial 1; C, Nandrosol (17β,19-nortestosterone phenylpropionate); K2, control of trial 2; D, 17β-estradiol; K3, control of trial 3; E, trenbolone acetate and 17β-estradiol (Revalor-200); F, trenbolone acetate and 17β-estradiol (Revalor-200) in combination with Desashock; G, trenbolone acetate (Finaplix-H); K4, control of trial 4.

The RGN/PPIA ratio significantly decreased in group D ($2.25 \times 10^{-3} \pm 4.97 \times 10^{-4}$) compared to group K3 ($2.76 \times 10^{-2} \pm 2.98 \times 10^{-3}$) ($P < 0.01$) (Figure 2C). The RGN/PPIA ratio decreased in group E ($3.79 \times 10^{-3} \pm 1.15 \times 10^{-3}$), group F ($7.49 \times 10^{-3} \pm 2.08 \times 10^{-3}$) ($P < 0.01$), and group G ($7.93 \times 10^{-3} \pm 2.01 \times 10^{-3}$) ($P < 0.01$) compared to group K4 ($1.84 \times 10^{-2} \pm 2.01 \times 10^{-3}$) (Figure 2D). Only four treated animals (no. 46, 53, 65, and 67) showed a RGN/PPIA ratio higher than the optimal criterion value, whereas one beef cattle belonging to the control group K4 (no. 72) was under the threshold.

In 11 of 54 veal calves (20.4%) and in 5 of 70 beef cattle (7.1%) the RGN gene was expressed under their respective cutoffs; therefore, they were identified as suspected of sex steroid hormone administration (Table 5).

Table 5. Total Number of Male Bovines Analyzed in the In-Field Investigation and the RGN Positivity Distribution[a]

	age (months)	RGN positive/no. tested	% RGN positive
veal calves	5–8	11/54	20.4
beef cattle	13–24	5/70	7.1

[a]

Animals were classified as positive for sex steroid hormone treatment when the RGN/PPIA gene copy was found to be lower than the respective cutoff.

Parameters of the ROC Curves for RGN Screening Test

A good diagnostic value was obtained by a ROC curve analysis for the RGN/PPIA ratio in sex steroid hormone-treated veal calves and beef cattle, in comparison to the respective control groups (Figure 3). For the veal calves the following results were pointed out: an area under the curve (AUC) of 0.9586, with a 95% confidence interval (0.8988–1.000; $P < 0.0001$) (Figure 3A); a diagnostic sensitivity of 89.47%, a specificity of 92.86%, a positive likelihood ratio (+LR) of 12.53, and a negative likelihood ratio (−LR) of 0.11 for the optimal criterion value of 0.01041 ($J = 82.33$) (Table 6). The experimental groups A, B, and C revealed RGN/PPIA values lower than the cutoff calculated by the ROC curve (Figure 2A,B).

Figure 3

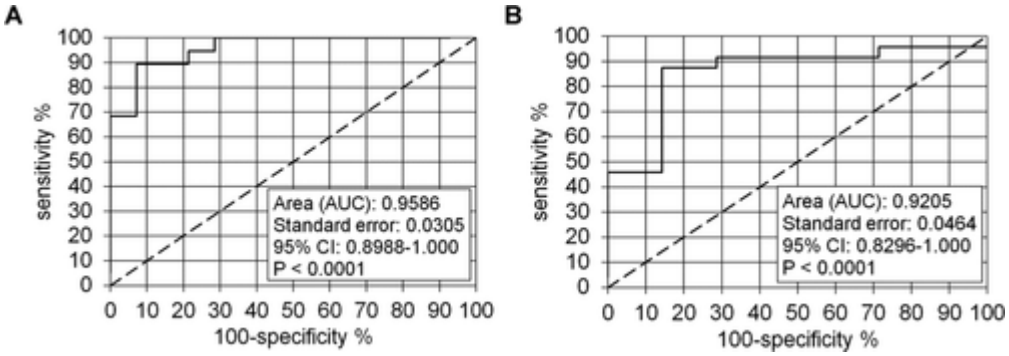

Figure 3. ROC curve for RGN decrease in sex steroid hormone-treated animals versus untreated control animals for veal calves (A) and beef cattle (B). (A) The area under the ROC curve (AUC) for veal calves was 0.9586 with a 95% CI (0.8988–1.000; $P < 0.0001$). The optimal criterion value selected was 0.01041 with 89.47% sensitivity and 92.86% specificity. (B) The AUC for beef cattle was 0.9205 with a 95% CI (0.8296–1.000; $P < 0.0001$). The optimal criterion value selected was 0.009947 with 90% sensitivity and 92.31% specificity.

Table 6. Cutoff (Reported as RGN/PPIA Values) and ROC Curve Parameters for the Decreasing RGN Expression in Veal Calves as Screening Test[a]

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.00218	52.63	0.1332–26.03	100	76.84–100.0		0.95	5.263
<0.002615	10.53	1.301–33.14	100	76.84–100.0		0.89	10.53
<0.003245	15.79	3.383–39.58	100	76.84–100.0		0.84	15.79
<0.00368	21.05	6.052–45.57	100	76.84–100.0		0.79	21.05
<0.003705	26.32	9.147–51.20	100	76.84–100.0		0.74	26.32
<0.003815	31.58	12.58–56.55	100	76.84–100.0		0.68	31.58
<0.00399	36.84	16.29–61.64	100	76.84–100.0		0.63	36.84
<0.004085	42.11	20.25–66.50	100	76.84–100.0		0.58	42.11
<0.004705	47.37	24.45–71.14	100	76.84–100.0		0.53	47.37
<0.00576	52.63	28.86–75.55	100	76.84–100.0		0.47	52.63
<0.0065	57.89	33.50–79.75	100	76.84–100.0		0.42	57.89
<0.007045	63.16	38.36–83.71	100	76.84–100.0		0.37	63.16
<0.007675	68.42	43.45–87.42	100	76.84–100.0		0.32	68.42
<0.008330	68.42	43.45–87.42	92.86	66.13–99.82	9.58	0.34	61.28
<0.008785	73.68	48.80–90.85	92.86	66.13–99.82	10.32	0.28	66.54

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.009095	78.95	54.43–93.95	92.86	66.13–99.82	11.06	0.23	71.81
<0.009575	84.21	60.42–96.62	92.86	66.13–99.82	11.79	0.17	77.07
<0.01041	**89.47**	**66.86–98.70**	**92.86**	**66.13–99.82**	**12.53**	**0.11**	**82.33**
<0.0112	89.47	66.86–98.70	85.71	57.19–8.22	6.26	0.12	75.18
<0.01225	89.47	66.86–98.70	78.57	49.20–5.34	4.17	0.13	68.04
<0.0133	94.74	73.97–99.87	78.57	49.20–5.34	4.42	0.07	73.31
<0.01375	94.74	73.97–99.87	71.43	41.90–1.61	3.32	0.07	66.17
<0.01525	100	82.35–100.0	71.43	41.90–91.61	3.50	0	71.43
<0.0167	100	82.35–100.0	64.29	35.14–87.24	2.80	0	64.29
<0.0170	100	82.35–100.0	57.14	28.86–82.34	2.33	0	57.14
<0.0184	100	82.35–100.0	50	23.04–76.96	2.00	0	50
<0.0215	100	82.35–100.0	42.86	17.66–71.14	1.75	0	42.86
<0.02605	100	82.35–100.0	35.71	12.76–64.86	1.56	0	35.71
<0.0306	100	82.35–100.0	28.57	8.389–58.10	1.40	0	28.57
<0.03835	100	82.35–100.0	21.43	4.658–50.80	1.27	0	21.43

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.0503	100	82.35–100.0	14.29	1.779–42.81	1.17	0	14.29
<0.0583	100	82.35–100.0	7.143	0.1807–33.87	1.08	0	7.143

[a]

CI, confidence interval; +LR positive likelihood ratio; −LR, negative likelihood ratio; *J*, Youden's index. The optimal cutoff is printed in bold.

For the beef cattle the following results were pointed out: an AUC of 0.9205, with a 95% confidence interval (0.8296–1.000; $P < 0.0001$) (Figure 3B); a diagnostic sensitivity of 90.00%, a specificity of 92.31%, a +LR of 11.70, and a −LR of 0.11 for the optimal criterion value of 0.009947 ($J = 82.31$) (Table 7). Experimental groups D–G revealed the RGN/PPIA values lower than the cutoff calculated by the ROC curve analysis (Figure 2C,D).

Table 7. Cutoff (Reported as RGN/PPIA Values) and ROC Curve Parameters for the Decreasing of RGN Expression in Beef Cattle as Screening Test[a]

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.001001	3.333	0.08436–17.22	100	75.29–100.0		0.97	3.333
<0.001111	6.667	0.8178–22.07	100	75.29–100.0		0.93	6.667
<0.001193	10	2.112–26.53	100	75.29–100.0		0.90	10
<0.001245	13.33	3.755–30.72	100	75.29–100.0		0.87	13.33
<0.001283	16.67	5.642–34.72	100	75.29–100.0		0.83	16.67
<0.001403	20	7.713–38.57	100	75.29–100.0		0.80	20
<0.001554	23.33	9.934–42.28	100	75.29–100.0		0.77	23.33
<0.001797	26.67	12.28–45.89	100	75.29–100.0		0.73	26.67
<0.00207	30	14.73–49.40	100	75.29–100.0		0.70	30
<0.002175	33.33	17.29–52.81	100	75.29–100.0		0.67	33.33
<0.002222	36.67	19.93–56.14	100	75.29–100.0		0.63	36.67
<0.002255	40	22.66–59.40	100	75.29–100.0		0.60	40
<0.002493	43.33	25.46–62.57	100	75.29–100.0		0.57	43.33
<0.002853	46.67	28.34–65.67	100	75.29–100.0		0.53	46.67

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.0030	50	31.30–68.70	100	75.29–100.0		0.50	50
<0.003026	53.33	34.33–71.66	100	75.29–100.0		0.47	53.33
<0.003054	53.33	34.33–71.66	92.31	63.97–99.81	6.93	0.51	45.64
<0.003402	56.67	37.43–74.54	92.31	63.97–99.81	7.37	0.47	48.98
<0.00378	60	40.60–77.34	92.31	63.97–99.81	7.80	0.43	52.31
<0.004205	63.33	43.86–80.07	92.31	63.97–99.81	8.24	0.40	55.64
<0.005618	66.67	47.19–82.71	92.31	63.97–99.81	8.67	0.36	58.98
<0.006665	70	50.60–85.27	92.31	63.97–99.81	9.10	0.32	62.31
<0.006871	73.33	54.11–87.72	92.31	63.97–99.81	9.54	0.29	65.64
<0.007276	76.67	57.72–90.07	92.31	63.97–99.81	9.97	0.25	68.98
<0.007706	80	61.43–92.29	92.31	63.97–99.81	10.40	0.22	72.31
<0.007974	83.33	65.28–94.36	92.31	63.97–99.81	10.84	0.18	75.64
<0.008805	86.67	69.28–96.24	92.31	63.97–99.81	11.27	0.14	78.98
<0.009947	**90**	**73.47–97.89**	**92.31**	**63.97–99.81**	**11.70**	**0.11**	**82.31**
<0.01150	90	73.47–97.89	84.62	54.55–98.08	5.85	0.12	74.62

cutoff	sensitivity (%)	95% CI	specificity (%)	95% CI	+LR	–LR	*J*
<0.01308	93.33	77.93–99.18	84.62	54.55–98.08	6.07	0.08	77.95
<0.01582	93.33	77.93–99.18	76.92	46.19–94.96	4.04	0.09	70.25
<0.01838	93.33	77.93–99.18	69.23	38.57–90.91	3.03	0.10	62.56
<0.01867	93.33	77.93–99.18	61.54	31.58–86.14	2.43	0.11	54.87
<0.01925	93.33	77.93–99.18	53.85	25.13–80.78	2.02	0.12	47.18
<0.02187	96.67	82.78–99.92	53.85	25.13–80.78	2.09	0.06	50.52
<0.02502	96.67	82.78–99.92	46.15	19.22–74.87	1.80	0.07	42.82
<0.02631	96.67	82.78–99.92	38.46	13.86–68.42	1.57	0.09	35.13
<0.02689	96.67	82.78–99.92	30.77	9.092–61.43	1.40	0.11	27.44
<0.02881	96.67	82.78–99.92	23.08	5.038–53.81	1.26	0.14	19.75
<0.03170	96.67	82.78–99.92	15.38	1.921–45.45	1.14	0.22	12.05
<0.03326	96.67	82.78–99.92	7.692	0.1946–36.03	1.05	0.43	4.362
<0.03678	100	88.43–100.0	7.692	0.1946–36.03	1.08	0	7.692

[a]

CI, confidence interval; +LR, positive likelihood ratio; −LR, negative likelihood ratio; *J*, Youden’s index. The optimal cutoff is printed in bold.

Immunohistochemistry

The testes of subjects tested positive via qPCR showed a total score of <3 (Figure 4A,D). All of testes of negative animals (Figure 4B,E) and control subjects (Figure 4C,F) showed a total score >3.

Figure 4

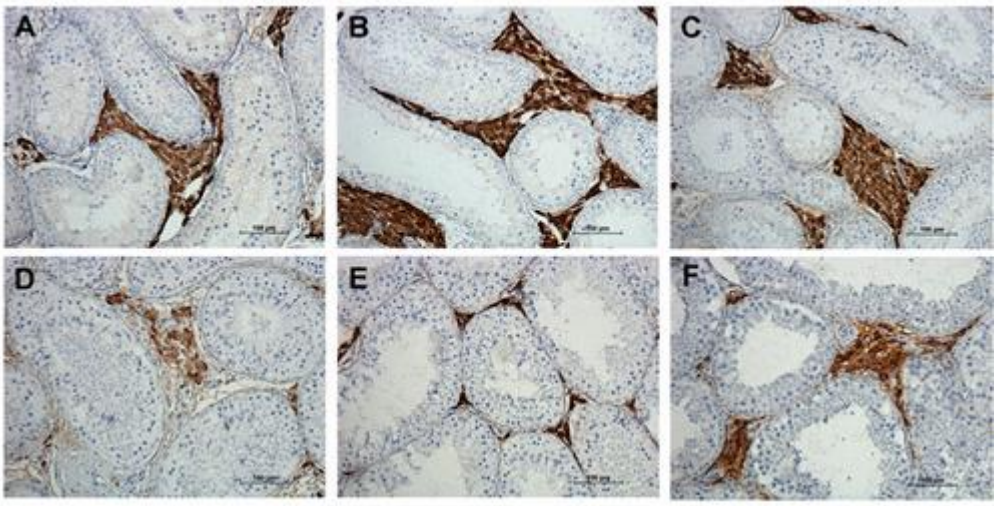

Figure 4. Representative image of the immunohistochemical localization of RGN protein in paraffin sections of the testis of veal calves (A–C) and beef cattle (D–F). Immunolabeling of RGN was scored by use of a semiquantitative scale incorporating both the proportion of positively stained target cells (scored on a scale of 0–5) and the staining intensity (scored on a scale of 0–3), according to the modified Allred score used in breast cancer. The proportion score (PS) was given as 0 (none), 1 (1%), 2 (1–10%), 3 (10–33%), 4 (33–66%), and 5 (>66%). The intensity score (IS) was given as 0 (no staining), 1 (weak staining), 2 (intermediate), and 3 (strong). A sum of PS and IS score of ≤3 was considered positive, and all other scores were considered negative. The testes of subjects testing positive via qPCR showed a total score <3 (A, D). All of the testes of negative animals (B, E) and control subjects (C, F) showed a total score >3. Bar = 100 μm.

Discussion

European Commission Decision 2002/657/EC establishes direct chemical analysis as the only legal and valid technique for the analytical control of residues in bovine meat and byproducts. (26) Nevertheless, the results of the current official approach are unsatisfactory. Therefore, the discovery and validation of new methods to track the GP abuse in bovine husbandry raise an increasing interest.

The first aim of the present work was to verify whether the qPCR absolute quantification of RGN gene expression associated with the standard curve method could be used as a suitable screening test to identify the sex steroid hormone administration in beef cattle and veal calves.

This method allowed for the quantification of RGN gene expression in a given sample by comparing the unknown level with a standard curve and then extrapolating the value. Then, an association between the RGN gene copy number and a sex steroid hormone treatment has been pointed out.

The amplification of both standard curves (RGN 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 standard curve, which could then be used to quantify RGN/PPIA gene expression in an unknown sample.

The intra-assay analysis revealed a high reproducibility of qPCR recDNA standard curves (Table 1), whereas the significant difference among the intercept values observed through the interassay would imply changes in the calculation of gene copy number. Then, the use of an internal standard curve in each qPCR run is recommended. The sample dilution, rather than the standard curve, mainly seemed to affect the results, as indicated by the precision and variability test. Then, a higher error may occur in the quantification of low concentrations of cDNA. However, no significant differences were detected in the unknown sample quantification (P = 0.1000).

ROC analysis was used to estimate the accuracy of the diagnostic test to discriminate between untreated and treated animals. The curve is generated by plotting the true-positive rate (sensitivity) against the false-positive rate (100% – specificity). AUC values of 0.9586 for veal calves and 0.9205 for beef cattle indicated a clear distinction between sex steroid hormone-treated and control animals. For the criterion values selected for veal calves and beef cattle, the ROC curve data showed that only about 7% of the untreated subjects were incorrectly identified as positive, whereas approximately 90% of treated animals were properly identified.

A significant decrease of RGN gene expression following sex steroid hormone treatment was pointed out in both beef cattle and veal calves, and this reduction was detectable 3–6 days after drug administration was discontinued.

Considering the possibility of getting false-positive outcomes, the obtained results allow for the application of qPCR analysis as a screening test to detect the sex steroid hormone administration in bovine husbandry. The in-field investigation found that 20.4% of the veal calves and 7.1% of the beef cattle analyzed exhibited a RGN/PPIA value under the respective cutoff. In this case, the animals were classified as suspected of sex steroid hormone treatment, and the molecular results were confirmed by staining reduction in the IHC assay.

A RGN/PPIA value lower than cutoff value allows one to consider that the animal is suspect of sex steroid hormone treatment despite the possibility to confirm this result with other techniques. Indeed, the administration of low dosages of sex steroid hormones and/or the association with other GPs could not lead to a marked effect on the target tissue, for example, at histological level. (3) Additionally, a few days after the treatment was discontinued, the official analytical methods cannot confirm the treatment. (27) Therefore, in these particular cases the reference methods fail to identify an animal illegally treated with GPs.

This study could provide a valid qualitative screening method to circumvent the current limitations in the control of GP abuse in bovine husbandry. Indeed, the veal calves and beef cattle illicitly treated with sex steroid hormones alone or in combination with other GPs can be identified by this test. Moreover, to the authors’

best knowledge, the detection of RGN gene decreasing in testis is the first molecular biomarker available for the detection of androgen abuse. The application of this test could further improve the safety of bovine meat and meat products because the changes of RGN expression may last for some days after the administration is discontinued.

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- ## Notes

  The authors declare no competing financial interest.

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