# UNIVERSITÀ DEGLI STUDI DI TORINO

# High sensitivity of a histological method in the detection of low-dosage illicit treatment with 17b-estradiol in male calves

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## ABSTRACT

With this study we evaluated the performance of a histological method for the detection of microscopic lesions in the sexual accessory glands of veal calves administered 17b-estradiol valerate for growth promotion; our goal was to determine whether the method might hold potential as an official screening technique according to the Commission Decision 2002/657/EC. The target organs of veal calves treated with low-dose 17b-estradiol valerate and those of control animals were microscopically examined in blinded manner and the histolopathogical features scored by two pathologists. Distinct histopathological differences were seen in the prostate and the bulbo-urethral glands of the treated animals versus the controls. Our findings indicate that, compared with the negative results obtained with the official test, this histopathological method can identify illicit 17b-estradiol valerate treatment up to 15 days after the last drug administration.

Based on the preliminary evidence for the method's high specificity and sensitivity, a validation study could be set up according to Decision 2002/657/EC. Confirmation of its performance could support adoption of histopathology as an official screening method in the National Residue Monitoring Program for the detection of illicit estrogenic treatment.

# INTRODUCTION

Estrogens, of which 17b-estradiol is the most active molecule, constitute a group of steroid compounds known for their importance in the estrous cycle. Conventionally called the female sex hormones, these naturally occurring substances are formed out of androgen precursors, such as 4-androstenedione, through the action of the enzyme aromatase in the ovaries, in the adipose tissue of the adrenal glands and in other organs (Nebbia, 2009). These steroids stimulate the development of the female reproductive structures and secondary sexual characteristics. Besides their function in the reproductive cycle, they also play an important role in a number of other physiological processes, including mineral, fat, sugar and protein metabolism, intestinal motility, blood coagulation, cholesterol metabolism, sodium and water conservation by the kidneys (Noppe, Le Bizec, Verheyden, & De Brabander, 2008), and in all strategically important tissues to improve protein anabolism and mineral retention (Meyer et al., 1995; Sauerwein, Pfaffl, Hagen-Mann, Malucelli, & Meyer, 1995).

Owing to their wide-reaching systemic effects, estrogens are also illegally administered to stimulate growth in calves and boost meat production (Guarda, Biolatti, & Miglietti, 1990; Meyer, 2001; Meyer, Sauerwein, & Mutayoba, 1990; Smith, Wilson, & Swanson, 1999). Since the treatment of cattle with sex steroid hormones exposes humans to an increased intake of residues through the consumption of meat from treated animals, the use of hormonal drugs in animal meat production has been officially prohibited in Europe since 1989 (The Council of the European Union (1996) Council Directive 96/22/EC).

Council Directive 96/23/EC requires Member States to adopt and implement a National Residue Monitoring Plan to control the illicit use of growth promoters for fattening purposes. The Directive sets forth sampling levels and frequency, as well as the group of substances to be monitored for each category of live animals or animal products (The Commission of the European Communities, 1996, Council directive 96/23/EC).

Although 17b-estradiol use in food producing animals has been banned since 2008 (The European Parliament and the Council of the European Union (2002) European Parliament and Council Directive 2008/97/EC) due to its potential carcinogenicity, investigations and analysis of illegal preparations have shown that steroids, natural hormones and b agonists are still widely in use.

Under current legislation, analytical screening and confirmatory methods applied in National Monitoring Plans employ chemical analysis of specific target compounds; however, this approach allows for fraudulent compliance with EC requirements as samples tested a few days after the last drug administration test negative because these molecules are rapidly metabolised or because available tests fail to detect new and/or unknown substances (Cai & Henion, 1997; Hewitt, Kearney, Currie, Young, & Kennedy, 2002; Van Poucke & Van Peteghem, 2002). With regard to 17b-estradiol

investigations, Italian legislation prescribes serum as the matrix to be used for official analysis (Ministerial Decree 14 November, 1996). To control the illegal use of 17b-estradiol and ensure consumer protection, sensitive analytical methods need to be developed. In this context, bioassays which detect estrogens have proven economic, fast and robust techniques as early warning systems for the illicit use of hormonal treatments, and a yeast bioassay has recently been validated for screening purposes (Bovee et al., 2009). Histopathological examination of the sexual accessory glands (prostate and bulbo-urethral glands in the male, Bartholini's glands in the female) is an older monitoring approach that, like bioactivity- based methods, is both economic and quick and can identify the indirect effects of estrogenic treatment on target organs (Groot & Biolatti, 2004; Groot et al., 2007; Groot, Schilt, Ossenkoppele, Berende, & Haasnoot, 1998; Kroes, Berkvens, Loendersloot, & Ruitenberg, 1971; Schilt et al., 1998; Weijman, Zwart, Vos, & Ramaekers, 1996). Despite these advantages, its reliability for screening purposes is still questionable due to the lack of accuracy evaluation studies.

With this present study we evaluated the performance of a histological method for the detection of microscopic lesions in the sexual accessory glands of male veal calves experimentally treated with 17b-estradiol valerate to promote growth. Our goal was to determine whether the method might hold potential as an official screening technique according to Commission Decision 2002/657/ EC (The Commission of the European Communities (2002). Commission Decision 2002/657/EC).

## MATERIALS AND METHODS

### *ANIMALS AND EXPERIMENTAL DESIGN*

In all, 108 male veal calves aged between 15 and 35 days were bought from local breeders, divided into two homogenous groups based on body weight and age, and housed in two pens under controlled conditions for 6 months according to Council Directive 86/609/EEC. Each pen had its own crib, multiple drinking troughs, and a dedicated automated milk feeder system. To protect the animals against infection, all were vaccinated against IBR, Para influenza (PI3), BRSV and BVDV (CATTLEMASTER 4 Pfizer Animal Health, New York, USA).
Clinical evaluation was carried out daily by a veterinarian and comprised a daily observation and, if necessary, a physical assessment. Treatments for occurring infections were performed without using hormonal active substances.
The animals had free access to fresh water and were fed standard milk replacer with an automatic milk feeder until 4 months of age, then 0.5 kg of corn was added to the diet twice a day. Before administration, all feeds, milk replacer and corn were analyzed with an ELISA test to exclude the presence of hormonal active substances. During the sixth month of the study period, 17b-estradiol valerate was administered by intramuscular injection in the neck at a dose of 5 mg once a week for four weeks to 69 randomly selected animals; placebo was administered to the remaining 39 animals used as controls. The trial was conducted in a blinded manner. Fifteen days after the last injection, all animals, aged between 6.5 and 7 months, were slaughtered 10 a day in a CE-certified

slaughterhouse.

All experiments were carried out according to European Economic Community Council Directive 86/609, recognised and adopted by the Italian Government (D.L. 27/01/1992 no. 116).

## CHEMICAL ANALYSIS BY LIQUID CHROMATOGRAPHY E TANDEM MASS SPECTROMETRY (LCEMS/MS)

### SAMPLE COLLECTION

Blood samples of 10 treated animals and 10 controls were collected before the first administration, and at 6 and 12 h after each treatment, at 24 and 48 h after the last treatment, and at the slaughterhouse. The serum was obtained from each sample and stored at   80°C until use.

### CHEMICALS AND REAGENTS

17a-Estradiol, 17b-estradiol, estriol, estrone, formestane (internal standard [IS]), acetonitrile, methanol, tert-butylmethylether, acetone, Dansyl chloride and formic acid (Sigma-Aldrich, St. Louis, MO, USA), sodium hydrogen carbonate were supplied from Merck (Darmstadt, Germany); blank reference serum was obtained from non-treated animals and tested to verify the absence of the analytes.

Stock standard solutions of 17a-estradiol, 17b-estradiol, estriol and estrone were prepared in methanol at a concentration of 1000 mg/L and stored at 20°C in the dark.

Calibration curves at five concentration levels (0, 20, 50, 100 and 500 ng/L for each analyte) were obtained by spiking blank serum with 17a-estradiol, 17b-estradiol, estriol and estrone working solutions.

### SAMPLE PREPARATION

3 mL of serum sample were transferred into a 25 mL glass tube and 30 mL of the internal standard solution   (formestane 10 ng/mL) were added. The sample was subjected to liquid/liquid extraction by adding 10 mL of diethylether. The centrifuge tube was shaken vigorously for 5 min with a vortex multimixer (Tecnovetro, Monza, Italy) and then centrifuged at 3500 rpm for 5 min (Mega-fuge 1.0 Heraeus, ASHI, Milan, Italy). The supernatant organic phase  was transferred into a 10 mL glass tube and evaporated to dryness under  a  gentle stream of nitrogen and heating (50°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was redissolved in 50 mL of hydrogen carbonate buffer 0.1 M (pH 10.5), and 50 mL of Dansyl chloride solution (1 mg/mL in acetone) were added. The sample was shaken for a few seconds, transferred into the heater block and kept at 60°C for 6 min. The derivatized extract was then transferred into the analytical vials for  the LCeMS/MS analysis.

## INSTRUMENTATION

Chromatographic separations were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a Merck LiChroCART - C18 (5 mm) 150 4.6 mm column and a Phenomenex SecurityGuard 4.2 mm pre-column (Phenomenex, Torrance, CA, USA). The chromatographic run was carried out with a binary mobile phase of acetonitrile (A) and 0.05% formic acid in water (B) using the following isocratic conditions: 85% Ae15% B. Total run time was 19 min, the injection volume was 20 mL, and the flow-rate was 0.5 mL/min. The LC was interfaced to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) operating in atmospheric pressure chemical ionization (APCI) e positive ion mode. The other MS parameters were set as follows: curtain gas 15 psi; nebulizer gas 30 psi; probe temperature 350 C; gas for collisional activation $N_2$ at 2 psi; nebulizer current 3 mA; entrance potential 10 V.

Ion acquisition was operated at unit mass resolution in the multiple reaction monitoring (MRM) mode using the transitions from the protonated molecular ion of each analyte to the fragment ion indicated in Table 1.

| Analyte | Ion precursor *m/z* | Declustering potential (V) | Product ions *m/z* | Collision energy (V) | Collision cell exit potential (V) |
|---|---|---|---|---|---|
| 17a-Estradiol | 506.3 | 60 | 506.3∕170.3 | 50 | 10 |
|  |  |  | 506.3∕171.2 | 60 |  |
| 17b-Estradiol | 506.3 | 60 | 506.3∕170.3 | 50 | 10 |
|  |  |  | 506.3∕171.2 | 60 |  |
| Estriol | 522.3 | 50 | 522.3∕170.3 | 45 | 10 |
|  |  |  | 522.3∕171.3 | 60 |  |
| Estrone | 504.3 | 50 | 504.3∕170.3 | 50 | 10 |
|  |  |  | 504.3∕171.2 | 50 |  |
| Formestane | 536.2 | 70 | 536.2∕170.3 | 50 | 10 |
|  |  |  | 536.2∕171.1 | 50 |  |

**Table 1. API4000 mass spectrometer acquisition parameters: ion source and collision cell potentials, product ion acquired for each analite.**

## VALIDATION AND METHOD PERFORMANCE

Specificity, precision (in terms of repeatability and within- laboratory reproducibility), trueness, $CC_a$, $CC_b$ and ruggedness were estimated according to Commission Decision 2002/657/EC, from data collected at 3 different concentration levels (20, 40 and 60 ng/L for each analyte). Validation results match the 2002/657/ EC prescriptions, $CC_a$, $CC_b$ values are reported in Table 2.

**Table 2. Decision limit (CCa) and decision capability (CCb) for 17b-estradiol and its methabolites, evaluated accordingly to the commission decision 2002/657/EC.**

| Analyte | CCa, (ng L [1]) | CCb (ng L [1]) |
|---|---|---|
| 17a-Estradiol, | 26 | 32 |
| 17b-Estradiol, | 28 | 36 |
| Estriol | 28 | 35 |
| Estrone | 29 | 37 |

## HISTOPATHOLOGIC ANALYSIS

Tissue samples of the prostate and sexual accessory glands of each animal were fixed in formalin, trimmed, routinely processed, embedded in paraffin wax, sectioned at 3 2 mm and stained with haematoxylin and eosin (HE).
Histopathologic examination was blindly performed by two veterinarians. The slides were examined for estrogenic effects such as changes in urethra (hyperplasia, metaplasia) and in the glandular tissue (hyperplasia, metaplasia, cistys) in prostate or in ducts (hyperplasia, metaplasia) and glandular tissue (hyperplasia, metaplasia, cistys) in bulbo-urethral glands. Lesions were semi-quantitatively scored on a scale from 0 to 3 (absent, mild, moderate, severe).

## IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was performed with mono- clonal antibodies CK5 (RCK 103 clone, Novocastra heat-induced epitope retrieval pH 6, 1:100) and CK7 (OV-TL 12/30 clone, Dako enzyme retrieval pk, 1:100) to characterize the cells involved in metaplastic change.
Prostate and sexual accessory glands of both treated and control animals were sectioned at $3.2$ mm, mounted on electrostatic slides and put at 37°C for at least 12 h. Then they were deparaffinized with atoxic substitute of xylene and hydrated through a series of decreasing concentrated ethanol solutions and rinsed successively in distilled water.
After hydration slides were incubated with 0.3% ethanol hydrogen peroxide for 30 min at room temperature to inhibit endogenous peroxidase activity and to overcome the effect of fixation heat-induced epitope retrieval pH 6 was performed for monoclonal antibodies CK5, while enzyme retrieval with pk for CK7.
The EnVision System Kit (Dako) for polyclonal and monoclonal antibodies was used as immunohistochemical detection system. Positively stained cells showed a golden dark brown 3.3-diaminobenzidine tetrahydrocloridee $H_2O_2$ reaction product. At the end, after counterstained with haematoxylin, slides were dehydrated through series of increasing concentrated ethanol solutions, put in atoxic substitute of xylene and mounted for examination.
In control sections the primary antibody was replaced by PBS.

## STATISTICAL ANALYSIS

The results of the microscopic examination were entered into an *ad hoc* database, cleaned and analyzed using Stata 10.1 SE (Stata- Corp, College Station, TX, USA). In order to test for statistically significant differences between the treated animals and the controls, and as the data were not normally distributed, we per- formed a test of hypothesis for non-parametric data using the Wilcoxon rank-sum test (Mann-Whitney). The validity of the evaluation of the histopathological lesions was evaluated for those parameters that resulted

significantly associated with the treatment.

# RESULTS AND DISCUSSION

## *CHEMICAL ANALYSIS*

Results of chemical analysis are shown in Table 3. Analysis of serum collected at 12 h after the last treatment showed the presence of 17b-estradiol in all 10 samples (concentration range, 57e224 ng/L; average, 132 ng/L); 9/10 samples tested positive for 17b-estradiol (concentration range, 30-132 ng/L, average: 60 ng/L) at 24 h after the last treatment and 9/10 samples tested positive (concentration range, 28-137 ng/L, average 45 ng/L) at 48 h after the last treatment. Respectively three and four out of 10 samples resulted negative after the second and the third treatment both at 6 and 12 h. Furthermore no 17b-estradiol residues were detected by LCeMS/MS analysis on serum samples collected before the second and the third 17b-estradiol administration, except for one animal; finally all samples taken at the slaughterhouse gave negative results. 17a-estradiol metabolites were detected (57 ng/L) in one sample taken at 48 h after the last treatment. Neither estriol nor estrone were detected in any of the samples.

**Table 3. Results of chemical analysis.**

| Sample | 1st Treatment $17b$-Estradiol (ng L$^{-1}$) | | | 2nd Treatment $17b$-Estradiol (ng L$^{-1}$) | | | 3rd Treatment $17b$-Estradiol (ng L$^{-1}$) | | | 4th Treatment $17b$-Estradiol (ng L$^{-1}$) | | | | | Slaughterhouse |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 6 | 12 | 0 | 6 | 12 | 0 | 6 | 12 | 0 | 6 | 12 | 24 | 48 | |
| 1 | nd | 134 | 107 | nd | nd | nd | nd | nd | nd | 30 | 202 | 167 | 75 | 46 | nd |
| 2 | nd | 50 | 57 | nd | 30 | 29 | nd | 37 | nd | 34 | 219 | 139 | 132 | 137 | nd |
| 3 | nd | nd | 30 | nd | nd | nd | nd | nd | nd | 65 | 42 | 102 | nd | 34 | nd |
| 4 | nd | 106 | 52 | nd | nd | 29 | nd | nd | nd | nd | 116 | 107 | 42 | 28 | nd |
| 5 | nd | 93 | 132 | nd | 32 | 52 | nd | 28 | 43 | nd | 309 | 224 | 60 | 49 | nd |
| 6 | nd | 87 | 85 | 79 | 32 | 29 | nd | 64 | 76 | nd | 132 | 186 | 72 | 30 | nd |
| 7 | nd | 33 | 38 | nd | 73 | 61 | nd | 42 | 37 | nd | ns | 57 | 30 | nd | nd |
| 8 | nd | 35 | 28 | nd | 32 | nd | nd | 29 | 37 | 31 | 80 | 85 | 44 | 42 | nd |
| 9 | nd | 48 | 51 | nd | 28 | 30 | nd | nd | 30 | 33 | 86 | 159 | 41 | 32 | nd |
| 10 | nd | 89 | 80 | nd | 33 | 30 | nd | 31 | 32 | 79 | 106 | 95 | 40 | 28 | nd |

The Italian legislation provides (Ministerial Decree 14 November 1996) a 17b-estradiol serum concentration threshold value of 40 ng/L in order to

discriminate between endogenous and exogenous origin of the estrogen. The LCeMS/MS method described in the present work and used for the chemical analysis gives a Decision limit or CCa (minimum concentration at and above which the analyte can be detected and quantified with known accuracy and precision) sharply below the legislation limit (see Table 2) our data show that official sampling performed on serum in the farm, would have little chance to detect the illicit treatment with low-dosage 17b-estradiol, and no chance at slaughterhouse.
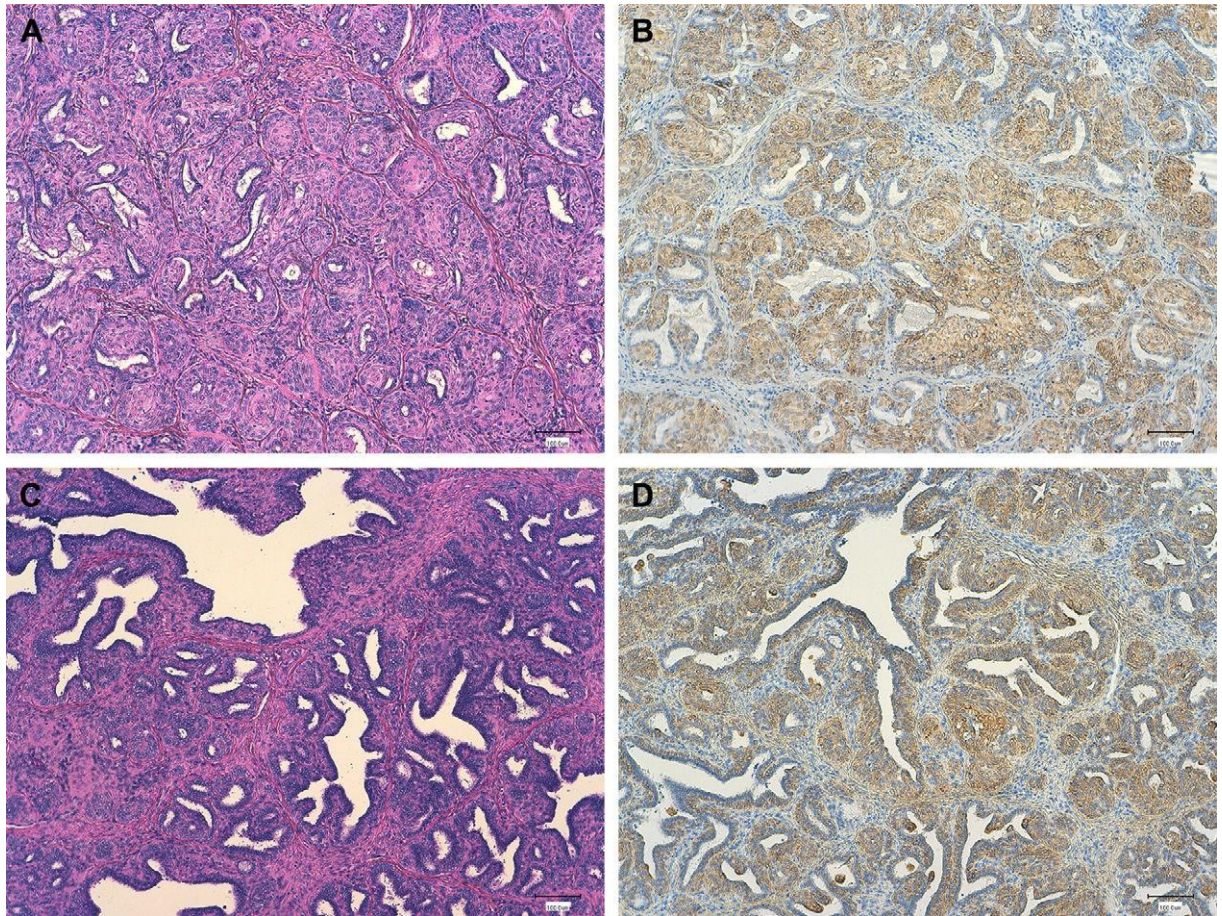


**Fig. 1. TREATED ANIMAL-effect of 17b-estradiol on glandular tissue of the bulbo-urethral gland.** A: Severe metaplasia of the glandular tissue of the bulbo-urethral gland (HE 10X); B: Strong immunopositivity of metaplastic basal cells (CK5 10X); C: Mild metaplasia of the glandular tissue of the bulbo-urethral gland (HE 10X); D: Mild immunopositivity of metaplastic basal cells (CK 5 10X).
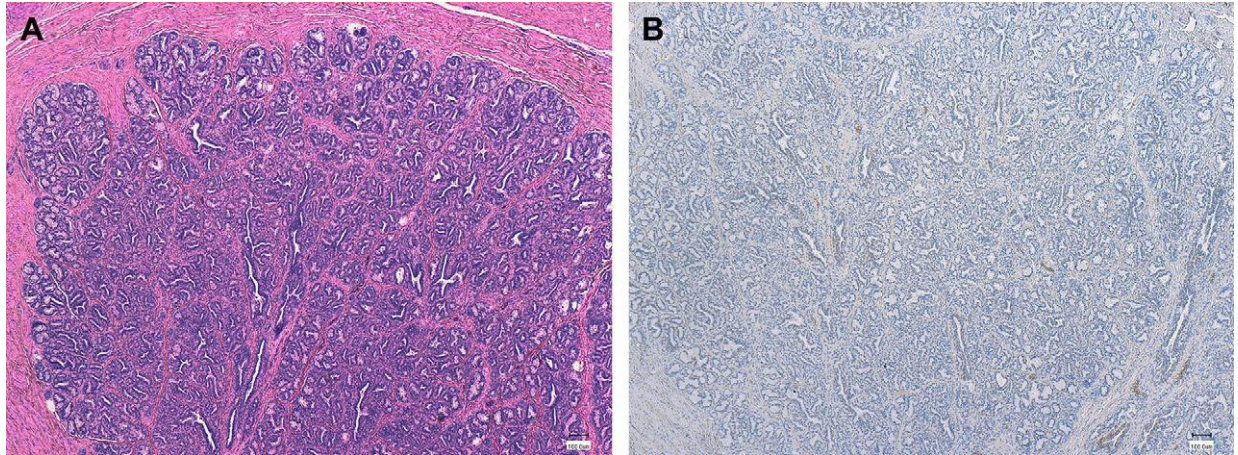
**Fig. 2. CONTROL ANIMAL e bulbo-urethral gland.** A: Normal glandular tissue of the bulbo-urethral gland with cubic epithelia (HE 4X); B: Normal staining pattern of basal cells (CK5 4X).

*HISTOPATHOLOGY ANALYSIS*

Normal histology of the disseminated prostate gland is characterised by alveoli lined with a low epithelium constituted of cylindrical and cubical cells, while the urethra is covered with a transition epithelium. Bulbo-urethral glands are an agglomerate of glandular lobules made up of mucinuos, acinous tubulo-alveolar glands with a central excretory duct. The epithelium covering the glandular tissue in male veal calves is cubic or simple prismatic while the ducts are covered with a simple bathyprismatic epithelium.

The most striking feature of the lesions in the treated animals was the dramatic metaplasia of the glandular tissue of the prostate and of the ducts and glandular tissue of the bulbo-urethral glands. Metaplasia in the glandular tissue of the prostate was observed in 51/69 treated animals as compared to normal epithelium in all the control animals; mild-to-severe duct metaplasia in the bulbo-urethral glands was noted in 67/69 treated animals and in the glandular tissue of 66/69 animals versus mild metaplasia seen in only one control animal. Histological patterns observed in the controls aided in clarifying which microscopic findings can be related to physiological conditions of veal cattle, thus narrowing the spectrum of lesion features related to illicit drug treatment. To date, pathologists have described all organ lesions but without identifying the most significant. With this new approach, histological results can be standardized, which would permit simplification of microscopic interpretation and limit the margin of subjectivity implicit in the histological method. Furthermore, accuracy evaluation, as a fundamental parameter for the application of the technique as an official screening method, could be performed.

## IMMUNOHISTOCHEMISTRY

In the prostate and sexual accessory glands of the treated and the control animals, the basal cell layer was immunoreactive with monoclonal antibody CK5 (RCK 103 clone, Novocastra, Newcastle upon Tyne, UK) and the urothelial cells were immunoreactive with monoclonal antibody CK7 (OV-TL 12/30 clone, Dako, Glostrup, Denmark).
Immunohistochemical analysis highlighted basal cell (CK5þ; CK7) involvement in the metaplastic change but no involvement of the urothelial cells (CK7þ; CK5) (Figs. 1 and 2). The detection of metaplastic changes in the epithelial cells appeared to be facilitated by immunohistochemistry with cytokeratin antibodies, as previously described for prostate analysis (Weijman et al., 1996).

## STATISTICAL ANALYSIS

Statistical differences in the microscopic patterns of the treated and the control animals emerged on the Wilcoxon rank-sum test. Table 4 reports lesion characteristics and their sensibility and specificity.
Glandular metaplasia of the bulbo-urethral gland was found to be the most sensitive and specific parameter (Se 95.7%, 95% CI 87.8-99.1); (Sp 97.4%, 95% CI 86.5-99.9).

**Table 4. The diagnostic performances of each single histopathological feature are expressed in terms of Sensitivity and Specificity. The difference between treated and control group is measured by a non-parametric test (Wilcoxon rank-sum test).**

|  |  | Wilcoxon rank-sum test (ManneWhitney) |  |  | 95% CI |  |
|---|---|---|---|---|---|---|
| **Prostate Urethra** | **Metaplasia** | *p* value <0.0001 | S e | 97.10% | 89.90% | 99.60 % |
|  |  |  | S p | 59.00% | 42.10% | 74.40 % |
| **Glandular tissue** | **Metaplasia** | *p* value <0.0001 | S e | 76.80% | 65.10% | 86.10 % |
|  |  |  | S p | 100.00% | 91.00% | 100.00 % |
| **Bulbo-urethral glands ducts** | **Metaplasia** | *p* value <0.0001 | S e | 97.10% | 89.90% | 99.60 % |
|  |  |  | S p | 89.70% | 75.80% | 97.10 % |
| **Glandular tissue** | **Metaplasia** | *p* value <0.0001 | S e | 95.70% | 87.70% | 99.10 % |
|  |  |  | S p | 97.40% | 86.50% | 99.90 % |

Iperplasia, secretion and cysts were not significantly associated with the treatment.

## CONCLUSIONS

The object of this study was to investigate how estrogen could influence target organs histology and which microscopical parameters are a reliable marker of treatment.

Until now only one European country, The Netherlands, performed histological screening to reveal estrogenic illicit treatment in veal calves giving positive on squamous metaplasia of prostate. Nevertheless, a large amount of other microscopical parameters, such as thickened urethra, dysplasia of the epithelium; dilated tubules, hyperplasia secretion and vacuolisation in glands, have been considered to be related to the treatment (Schilt et al., 1998). Our findings show that by identifying a single histological feature (metaplasia of the bulbo-urethral glands) the illicit use of 17b-estradiol can be exposed even after 2 weeks of suspension of low-dosage treatment. This method has a high sensitivity value compared with the official chemical methods, which, when applied to serum samples collected at slaughterhouse detected neither the molecule nor its metabolites. The histological method could provide a rapid and inexpensive tool of valuable interest to overcome current limitations in the control of growth promoters' abuse and it could be used to detect illicit treatment with a potentially carcinogenetic molecule at the slaughterhouse. Accuracy data suggest widening the experiment to obtain the sample size needed for validation in order to comply with the Commission Decision 2002/657/EC requirements for biological screening. Following the validation it could be considered as a new promising approach in term of screening to evidence the diffusion of estrogen illicit treatment in farm animals in order to orientate controls by confirmatory methods.

## ACKNOWLEDGMENTS

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