

# The Italian strategy to fight illegal treatment with growth promoters: Results of the 2017-2019 histological monitoring plan

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## Abstract

The Histological Control Plan has been introduced in Italy in 2008 as an indirect monitoring tool of illicit administration of sexual hormones and corticosteroids in bovine. Analysis of 2008-2016 results permitted to draw a new plan targeting risk category. This work presents the results of the histopathological monitoring plan that was carried out from 2017 to 2019. The overall prevalence of samples suspected of treatment with corticosteroid was 11.3% [95% confidence interval (CI) 6.6-17.8] in 2017; 10.2% (95% CI 6.6-16.9) in 2018 and 8.9% (95% CI 4.6-15.4) in 2019. The overall prevalence of samples suspected of treatment with sexual hormones was 2.3 % (95% CI 0.5-6.6) in 2017; 6.2% (95% CI 2.7-11.8) in 2018 and 12.4% (95% CI 7.4-19.1) in 2019. Although not targeting and measuring specific molecules, this strategy allows to verify the trend of illicit treatments and identify farms to be submitted to further check.

## Introduction

Growth promoting agents in food producing animals are strictly forbidden in the European Union (EU) by the Council Directive 96/23/EC (Directive 96/23/EC). The term 'Illegal treatment' means 'the use of unauthorized substances or products or the use of substances or products authorized under Community legislation for purposes or under conditions other than those laid down in Community legislation. The prohibition is in place to protect consumers', in fact the ingestion or consumption of residue and or metabolites of unauthorized drugs poses a serious risk to consumers' health.

Every year the European Union focuses its efforts in fighting the illegal use of substances for growth-promoting purposes, by implementing Communitarian Residues Control Plans to monitor the presence of hormonal and veterinary drugs residues in food and feedstuffs (Directive 96/23/EC 1). Despite the intense official control activity, in the 354,517 targeted sample reported to the European Community by the 28 Member States, the percentage of non-compliant samples recovered in 2018 was 0.30% (EFSA, 2020). Particularly in the group of sexual hormones (A3) and corticosteroids (B2F), only 0.16% and 0.15% samples respectively were found to be non-compliant in cattle (EFSA, 2020).

From the analysis of data of National Residue Control Plans (NRCs), in 2013 the European Agency for Food Safety (EFSA) underlined the inefficiency of the screening strategy implemented in the Official Monitoring Plans, suggesting and encouraging the development and use of biologically based methods, in order to improve the effectiveness of the official screening steps of Residues Control (EFSA Scientific Opinion, 2013).

To date this recommendation has been adopted only by the scientific community through the development of untargeted methods to expose ongoing illegal treatments (Dervilly-Pinel *et al.*, 2018) while the legislation in force is not yet adequate. To fill this gap, National Reference Laboratories are involved in the revision of the legislation on residues of pharmacologically active substances controls. In 2008 the Italian Ministry of Health introduced the histological plan as a complementary strategy of control. The objective of this monitoring activity was to verify at national level whether the samples from the slaughterhouses exceeded a predefined prevalence threshold level for each illicit treatment subject to surveillance: corticosteroids and sexual hormones (P=15%).

To achieve this goal, histological analyses were performed on a statistically significant number of slaughtered animals by the Experimental Zooprophyllactic Institutes (IIZZSS) official laboratories. The histopathological examination can detect lesions induced by sexual hormones and glucocorticoids in bovines target organs (*i.e.*, sexual accessory glands and thymus) (Groot *et al.*, 2007, Imbimbo *et al.*, 2012). Sexual hormones are known to cause squamous metaplasia in the epithelium of sexual accessory glands (Pezzolato *et al.*, 2013) while glucocorticoid cause atrophy in thoracic thymus cortex (Bozzetta *et al.*, 2011;

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Zanardello *et al.*, 2018). These changes represent biomarkers that persist far longer than the negativization of the residues in the official matrices analyzed by analytical methods included in the NRCs (Richelmi *et al.*, 2017). These histopathological findings proved to be reliable markers of treatment. In 2008 they were validated to be applied in the histological plan and became part of the Standard Operating Procedures (SOPs) shared among the IIZZSS.

In order to improve the efficiency of the national monitoring plan, data from histological samples collected between 2008 and 2016 have been analyzed to determine on which high risk categories future checks should be focused.

The aim of this study is to present the results of the histopathological monitoring plan carried out from 2017 to 2019 to expose the illicit administration of sexual hormones and corticosteroids in food producing bovines.

## Materials and methods

### 2017-2019 Monitoring Control Plans

The objective of this monitoring activity was to verify at national level whether the batches from the slaughterhouses exceeded a predefined prevalence threshold level for each illicit treatment subject to surveillance: corticosteroids and sexual hormones (P=15%). According to this design, samples of thymus and sexual accessory glands of beef cattle and veal calves were analyzed by histopathology according to the SOPs in use in the histopathological laboratories of IIZZSS.

Results were reviewed in order to obtain further information's regarding high-risk categories to be accounted for in the sampling design of the new National Monitoring Plan of the present study.

To confirm that the observed prevalence of sexual hormones illegal treatments in veal calves is equal or above the 10% threshold, 147 batches (first stage) were calculated to be sampled and at least 20 batches had to be evaluated as not compliant.

The number of animals (second stage) estimated to be sampled within each batch was equal to: all animals in the case of batches consisting of less than 5 animals; 5 in the case of batches consisting of 5 to 7 animals; 6 in batches consisting of more than 7 animals.

To verify that the observed prevalence of corticosteroid illegal treatments in beef cattle is equal or above the 13% threshold, 104 batches were calculated to be sampled and at least 14 batches had to be evaluated as not compliant.

Based on these criteria, the number of animals estimated to be sampled within each batch was equal to all animals in the case of batches consisting of less than 5 animals; 5 in the case of batches consisting of 5 to 7 animals; 6 in batches consisting of more than 7 animals.

The objective of the new monitoring

plan was to verify whether at national level the batches sent to the slaughterhouse exceed a predefined prevalence threshold level for each illicit treatment subject to surveillance, separately for the beef cattle and veal calves (P=13% for beef, P=10% for veal). The activity from 2017 to 2019 was planned on veal calves to search for illicit treatments with sexual hormones and on beef cattle to expose corticosteroid treatments. A two-stage sampling was used to demonstrate a treatment free status respectively from sexual hormones in veal calves and corticosteroids in beef cattle. The first stage consisted in selecting a sample of batches; the second stage involved selecting a sample of animals from each batch of stage one. Parameters used to estimate the sample size to demonstrate the absence of treatment with sexual hormones in veal calves (sampling and analysis of sexual accessory glands: prostate and urethral bulb glands) are shown in Table 1.

The cut-point number of suspected animals to demonstrate batch sexual hormones treatment free status, calculated using imperfect tests and allowing for small populations, is shown in Table 2: one batch was considered suspected if a certain number of animals equal or greater than the cut-point number was found in the random sample collected from the batch.

Parameters used to estimate the sample size to demonstrate corticosteroids treatment free status in beef cattle (thymus sample and analysis) are shown in Table 1.

The cut-point number of suspected ani-

mals to demonstrate batch freedom from corticosteroid treatment are the same reported in Table 2.

### Sampling process

Sampling was performed by official veterinarians in all Italian regions: a training course was organized to ensure that the sampling criteria were adhered by all field workers. To guarantee the representativeness of the sampling the operators were asked to perform a random sampling, avoiding criteria in the choice of batches, and applying an even geographical and temporal distribution of the sampling during the year. The thoracic thymus and sexual accessory glands (prostate and bulbo urethral glands) were collected at the slaughterhouse, fixed in neutral buffered formalin, and sent to the laboratories of the IIZZSS to be analyzed according to the dedicated SOPs.

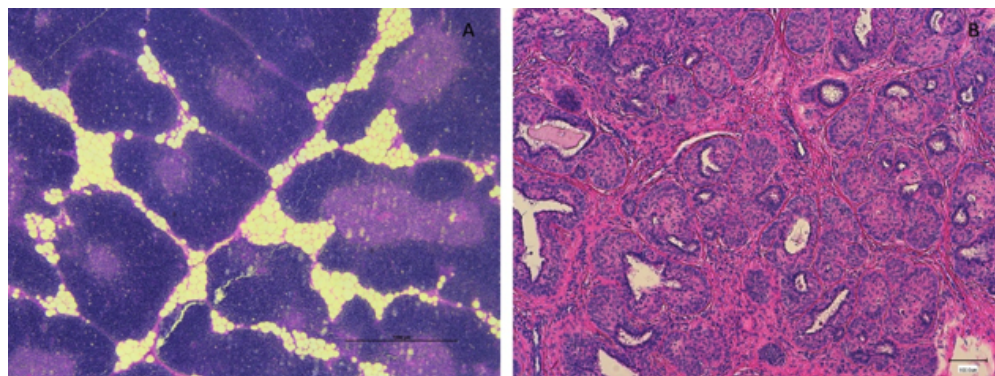
### Histopathology

Samples were trimmed, routinely processed, embedded in paraffin wax and serially sectioned at 3 +/-2 µm for the histological evaluation. Slides were stained with Hematoxylin and Eosin and observed by optic microscopy (ZEISS Axio Scope.A1, Köln, Germany)

Samples were analyzed to highlight severe atrophy (Richelmi *et al.*, 2017) (Figure 1A) in thymus and metaplasia in sexual accessory glands (Figure 1B) (Pezzolato *et al.*, 2013) according to the SOP criteria in place.

**Table 1. Parameters used to estimate the sample size for veal calves' population.**

Parameter	First stage (batches) %	Second stage (animals) %
Confidence level	95	95
Sensitivity	95	90
Specificity	90	80
Expected prevalence	10	80
Statistical power	90	90



**Figure 1. Thymus samples of veal calves suspect for corticosteroid treatment with severe atrophy. Haematoxylin–eosin staining (A). Sexual accessory gland of veal calves suspects for sex steroid treatment showed severe metaplasia. Haematoxylin–eosin staining (B).**

The data on the presence of lesions for each animal were recorded into a database (DB).

### Statistical analysis

Results registered in the DB were used to calculate the total percentage of non-compliances. An extension of the Wilcoxon rank-sum as developed by Cuzick (1985) was used to verify a statistically significant variation of treatment over years. All analysis were performed with Stata 16.1 (StataCorp LLC, 4905 Lakeway Drive College Station, Texas 77845-4512 USA).

## Results

According to the results from the 2008-2016 plans, a new plan was designed and a sample size of 147 batches was calculated in order to increase the performance of the surveillance system.

The total number of beef cattle batches sampled for corticosteroids was 141 in 2017, 127 in 2018 and 123 in 2019 for a total of 340, 325 and 304 beef cattle analyzed respectively each year.

The total number of veal calves' batches sampled for sexual hormones was 129 in 2017, 130 in 2018 and 137 in 2019 for a total of 426, 386 and 390 veal calves analyzed respectively each year.

The overall prevalence of treatment with corticosteroid was 11.3% (95% confidence interval (CI) 6.6-17.8) in 2017; 10.2% (95%CI 6.6-16.9) in 2018 and 8.9% (95% CI 4.6-15.4) in 2019.

Suspected batches for corticosteroid treatment are reported in Table 3.

The overall prevalence of treatment with sexual hormones is 2.3 % (95% CI 0.5 -6.6) in 2017; 6.2% (95% CI 2.7-11.8) in 2018 and 12.4% (95% CI 7.4-19.1) in 2019.

Suspected batches for sexual hormones treatment are reported in Table 4.

The tests for trend were not statistically significant (Figure 2).

## Discussion and Conclusions

Application of growth promoting agents like steroid hormones or anabolic hormones cocktails are well known to increase muscle mass in beef cattle. The main purpose is to reduce lipid mass and increase muscle tissue. These agents promote overall tissue growth, by increasing growth rates, improve the conversion of nitrogen in feed into muscle protein and hence increase the amount of lean and valuable meat in the carcass (Stephany, 2010).

The application of any growth promoting agents is strictly forbidden in livestock production within the EU. Estrogens are known to cause cancer in humans while androgens are related to the development of different neoplasms in animals. Corticosteroids are dangerous for human health, causing in vulnerable people hyperglycemia, diabetes, high blood pressure and maximizing risk of infection (Kavanaugh and Wells, 2014).

At present, these growth promoting

drugs are screened within an EU control program, using sensitive immunological-based test systems (e.g., ELISA, enzyme-linked immunosorbent assays) or chromatographic methods in combination with mass spectrometry (Meyer and Hoffmann, 1987; Meyer *et al.*, 1991; Scippo *et al.*, 1994). Using these established methods, newly designed anabolic drugs cannot be identified until their chemical structure is known, or a specific and high affinity antibody is available for the immune tests. A further problem is the identification of a single low dosage drug mixed in a drug cocktail. These undefined cocktails due to their low concentrations are barely detectable and difficult to quantify in a reproducible manner using conventional detection methods (Cantiello *et al.*, 2007). Therefore, development of new screening methods is required to detect the use of such substances, independently of their structure and/or concentration (Nebbia *et al.*, 2001).

The determination of microscopical changes caused using growth promoting and anabolic substances based on the analysis of target organs changes is a simple but effective long known method (Groot *et al.*, 2007, De Maria *et al.*, 2009; Cannizzo *et al.*, 2001), to detect permanent changes related to these illegal treatments. Thanks to the process of validation of few reliable

**Table 2. The cut-point number of suspected animals to demonstrate batch freedom from treatment.**

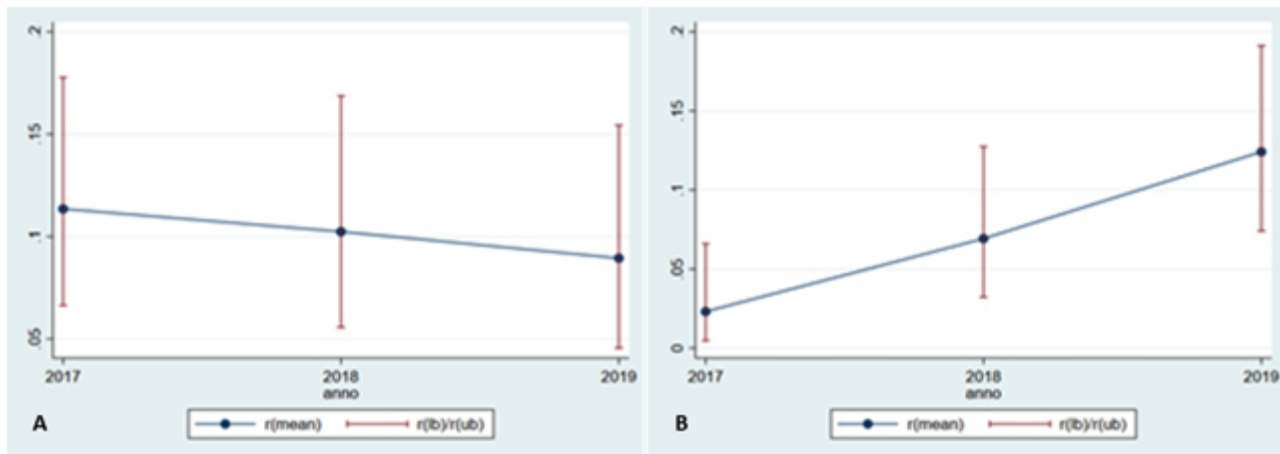
Sample of animals	Cut-point number
1	1
2	2
3	3
4	3
5	3
6	3

**Table 3. Suspected batches for corticosteroid treatment and sex steroid treatments.**

	Corticosteroid			Sexual hormones		
	Batches sampled	Batches suspected	%	Batches sampled	Batches suspected	%
2017	141	16	11.3	129	3	2.3
2018	127	13	10.2	130	9	6.2
2019	123	11	8.9	137	17	12.4

**Table 4. Parameters used to estimate the sample size for beef cattle population.**

Parameter	First stage (batches) %	Second stage (animals) %
Confidence level	95	95
Sensitivity	95	90
Specificity	90	80
Expected prevalence	13	80
Statistical power	90	90



**Figure 2.** Trend of prevalence respectively for corticosteroid treatments in beef cattle (A) and for sexual steroid treatments in veal calves (B). Vertical bars show confidence interval width.

microscopic markers, a monitoring plan for both sexual hormones and corticosteroids could be drawn in Italy in 2008; the efficacy and effectiveness of this plan has recently improved. This is currently applied in the frame of a strategy to highlight farms to be controlled with standard analytical methods in order to fight the misuse of anabolic agents in cattle.

The results achieved by the monitoring histological plan applied from 2017 to 2019 are in contrast with those reported by the Italian NRCP and demonstrate that the phenomenon of illicit treatments is far from being eradicated (EFSA, 2020). Even if histology is not able to identify the administered treatment, the results obtained by the monitoring plan can help directing official controls. In the past few years most of the farms selected through the histological plan have proved to be noncompliant (data not shown) during official controls.

This study also highlights possible consumers' health risks as illicit cattle treatment may result in illegal/dangerous residues in meat.

In conclusion this work shows how monitoring plans based on an untargeted method can support the fight to drug abuse in farm animals. Due to the rather homogeneous situation regarding veterinary drugs residues all over European countries, we can expect that the adoption of alternative strategies suggested by EFSA could disclose the real prevalence of illegal treatments and help to address official controls.

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