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The physiological way: Monitoring RNA expression changes as new approach to combat illegal growth promoter application

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The use of growth-promoting agents in food-producing animals is forbidden in the European Union (EU). Therefore a strict control programme has been developed, detecting residues of all known growth-promoting agents using chromatographical methods in combination with mass spectrometry or immunoassays. New designed xenobiotic substances or hormone cocktails are difficult to identify with these methods and therefore the development of new sensitive test methods is important.

A promising indirect approach is the detection of physiological effects of the administered growth promoters on the molecular level using 'omic' technologies. The analysis of the transcriptome on mRNA and miRNA level and thereby identifying biomarkers for the use of anabolic agents is one possible strategy for developing a new screening method. This paper describes the technologies available for gene expression profiling and summarizes the efforts made in the analysis of the transcriptome in order to identify potential gene expression biomarkers for the use of growth promoters in cattle. © 2012 John Wiley & Sons, Ltd.

Keywords: growth promoters; omic technologies; transcriptomics

Introduction

Anabolic substances, like steroid hormones or β-agonists, are known to increase muscle mass and to decompose lipid tissue and are therefore useful for growth-promoting purposes in animal husbandry in order to increase weight gain and to produce more lean meat. Due to proven side-effects of some of these substances affecting the consumer, the use of all growth-promoting agents is strictly forbidden in the European Union (EU; Directive 96/23/EC). In some toxicological reports, it is stated that the use of selected anabolic substances in meat production has no effect on the consumer and so the use of these specific substances is licensed in countries like USA, Brazil, Mexico or South Africa.[1] Swan *et al.* described the reduction of sperm quality of male offspring as positively correlating with the mother's beef consumption during pregnancy.[2] This reports leads to the assumption that residues of applied growth promoters influence the development of male reproductive organs *in utero* and that the ban of growth promoters in the EU can be considered reasonable.

Within an EU control programme, residues of all known anabolic agents are detected using immunoassays or chromatographical methods in combination with mass spectrometry (MS).[3–5] With these methods, newly designed anabolic drugs cannot be identified until their chemical structure is discovered. Another problem is the application of 'hormone cocktails' including multiple substances, each in low concentrations. These cocktails have additive effects of its components and show comparable physiological effects like a single drug applied in high concentration. Due to their low amount they cannot be measured using the usual techniques.[6] Therefore it is necessary to develop new sensitive screening methods to detect a broad range of substances independent of their structure and concentration.

In the process of designing xenobiotic anabolic drugs, the structure of the new agent is changed, but the desired physiological effect – weight gain with an increase of muscle mass and a decrease of fat tissue – is the same as that of the natural substance. The technical detection of these effects on the animal's physiology is a promising way for the development of new methods to screen for the misuse of anabolic agents. The question is: how can these effects be quantified?

There are different methods available to monitor physiological effects on the transcriptomic, proteomic, or metabolomic level. These methods are called 'omic' technologies and each can be differentiated in targeted and untargeted methods. With targeted methods, it is possible to quantify single, elected factors, like the expression of single RNAs, proteins, or metabolites. Untargeted methods screen for a high amount of differentially expressed factors at once.[7] Normally, untargeted methods are used to screen for potential biomarker candidates which are then verified using targeted methods.

Within the omic technologies, the analysis of the transcriptome is a very promising approach to finding biomarkers for the use of anabolic substances, because it is known that steroid hormones directly influence gene expression upon binding to their receptors which act as transcription factors for specific genes.[8] By activating a G-protein coupled phosphorylation cascade, β-agonists indirectly affect the transcription of specific genes.[8] Nowadays transcriptomics not only include the quantification of gene expression, connected to the analysis of mRNA. The analysis of another non-coding RNA species, namely the microRNAs (miRNA), is becoming

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more and more important, especially in the identification of disease specific biomarkers.[9] miRNAs are involved in post-transcriptional regulation and are expressed in a tissue and disease or physiology specific manner.[10] Due to these properties, the analysis of miRNA expression changes is also an interesting approach to finding biomarkers for the use of anabolic agents.

This paper describes the technical and analytical requirements for the quantification of mRNA and miRNA expression changes and shows the efforts made up to now in the identification of gene expression biomarkers for the misuse of anabolic agents in cattle.

Available methods for the quantification of gene expression changes

Methods for the quantification of gene expression can generally be subdivided to untargeted and targeted analytical strategies.[7] Untargeted methods use the hypothesis-free analysis of changes in the expression of all genes of the specific species, which means that all changes on transcriptomic level that are caused by anabolic treatment are detected in one experiment. Due to the high amount of resulting data, the statistical analysis of these experiments is time-consuming and requires special software packages. A summary of analytical methods for gene expression analysis is given in Table 1.

With gene expression microarrays, all sequenced genes of a species can be analyzed in one sample. Therefore DNA fragments of all known genes called probes, which represent specific coding regions of target genes, are immobilized on a solid surface (glass slide, membrane), whereas one gene is represented by more than one probe sequence and one probe is present in multiple copies.[11] To analyze gene expression changes between two RNA samples, they are reverse transcribed to cDNA and labelled with two different fluorescent dyes. The samples are mixed and hybridized to the slide whereupon the cDNAs bind to their complementary probes. The amount of bound cDNAs can be quantified by the intensity of emission of the two fluorescent dyes.[11] Using specific software tools, array data can be analyzed and gene expression changes can be measured. Up to now, microarrays are the gold standard method to screen for all gene expression changes between two samples. Nowadays there are also microarrays for the identification of miRNAs available. Different companies also offer the possibility to design individual microarrays with selected target genes or miRNAs, offering also a targeted approach of microarray technology to screen for changes in the expression of different genes and miRNAs.[12] The disadvantage of microarray technology is that only the expression of genes whose sequence is already known can be analyzed. Another problem is the high background level and the limited dynamic range of detection.[13]

A further method for untargeted gene expression analysis is serial analysis of gene expression (SAGE) based on the generation of unique sequence tags of about 9–10 base pairs length from present mRNAs and the connection of these tags to long molecules for sequencing.[14,15] The length of 9 base pairs is sufficient to distinguish up to 4^9 transcripts. Older sequencing technologies are time consuming and therefore, ligating more of these short sequence tags and cloning them into a vector enables sequencing of numerous mRNAs in one reaction. Due to the use of oligo dT primers for reverse transcription, this method can only be used for the identification of mRNAs.[14,15]

During the last few years, new, fast technologies for deep sequencing called next-generation sequencing (NGS) have come into focus. With this technology, whole genomes and transcriptomes can be sequenced in a short time. One application of this

Table 1. Comparison of available methods for gene expression analysis.

Method	Type	Advantages	Disadvantages
Hybridization microarrays	Untargeted	Analysis of the expression of all identified genes in one experiment, also applicable for the analysis of miRNA	Only the expression of genes with a known sequence can be analyzed, high background evel, limited range of detection, complex ata analysis
Custom-made microarrays	Targeted	Analysis of a high number of defined genes in one experiment, also applicable for the analysis of miRNA	Only the expression of genes with a known sequence can be analyzed, high background level, limited range of detection, complex data analysis
SAGE	Untargeted	Detection of all regulated genes, very sensitive	Only analysis of mRNA, complex data analysis
RNA-Sequencing	Untargeted	Detection of all regulated genes, *de novo* assembly possible, no upper limit of quantification, high dynamic range of detection, nearly no background signal, very sensitive, also applicable for the analysis of miRNA	Expensive, complex data analysis
Northern blot	Targeted	Sensitive	Time-consuming, generation of robes necessary, quantification of ingle genes
RT-qPCR	Targeted	Very sensitive, fast method, cheap, also applicable for the analysis of miRNA	Quantification of single genes th known sequence
RT-qPCR Panels	Targeted	Very sensitive, fast method, cheap, also applicable for the analysis of miRNA	Quantification of a panel of 96/384 genes, belonging to the same physiological pathway

technology for the analysis of RNA expression is RNA-Sequencing (RNA-Seq). From the extracted RNA, a cDNA library is produced and to each fragment, specific adapters are attached. Each cDNA fragment is sequenced in a high throughput manner resulting in a high number of short sequences that will either be aligned to a reference genome or assembled *de novo* without knowing the genome sequence of the species.[13,16] With these NGS platforms the analysis of miRNA is also possible. This method has no upper limit of quantification like microarrays, shows a high dynamic range of expression levels, has nearly no background signal and allows the detection of 'one single RNA molecule'.[13]

For the analysis of chosen candidate genes, targeted methods are useful. The first method developed for targeted analysis of RNA expression is northern blot. Northern blotting involves the electrophoretical separation of RNA and the capillary transfer of RNA from the agarose gel to the positive charged blotting membrane. Identification of specific RNAs occurs via hybridization probes composed of labelled single stranded nucleic acids, either RNA or DNA, with a complementary sequence to the RNA of interest. Probes are either labelled with radioactive isotopes, fluorescence dyes or with chemiluminescent markers.[17]

Nowadays, the analysis of the expression of single genes is done by RT-qPCR. This method is based on the original Polymerase-Chain-Reaction (PCR) which is used to amplify defined parts of the DNA using the action of DNA polymerases in a defined temperature protocol. Normal PCR is used to amplify defined pieces of DNA and end point analysis of the PCR product is possible using agarose gel electrophoresis, but in this way the quantification of the amount of starting material is not possible. In 1992, Higuchi *et al.* developed a method called real-time PCR that enables the amplification and the monitoring of the amount of product during the course of the reaction.[18] There are two ways for visualization of the amplification course. Non-specific fluorescence dyes like SYBR Green I that are able to intercalate with double-stranded DNA are added to the PCR reaction. Intercalation causes fluorescence of the dye which can be measured after each PCR cycle. An increase in PCR product leads to an increase in fluorescence signal and with specific analytical strategies it is possible to calculate the amount of starting material.[19,20] However, these dyes bind to all double-stranded DNAs independent of their sequence and thus also bind unspecific PCR products or primer dimers. A more sensitive method is the use of fluorescently labelled DNA probes, giving a fluorescence signal when binding to the specific PCR product.[19] To analyze the expression of specific genes using this method, reverse transcription of RNA to cDNA is necessary before real-time qPCR can be performed (RT-qPCR). Nowadays multiplex qPCR enables the quantification of a set of genes in one reaction. Therefore DNA probes for the different genes of interest are labelled with different dyes emitting light measurable at different wave lengths.[21] Another high throughput application is custom-made PCR pathway panels. Therefore assays for different genes of a biological pathway have to be designed to run at the same conditions and primer pairs are lyophilized and fixed in the wells of a 96- or 384-well plate. After adding of the PCR reaction components and the sample, qPCR can be performed and many genes can be quantified in one biological sample in one run, enabling the screening for gene expression changes of more target genes.[22] RT-qPCR is also a standard method for the quantification of specific miRNAs and qPCR panels for miRNA detection are also available on the market.

Efforts made in the analysis of the transcriptome in order to identify potential gene expression biomarkers for the use of growth promoters in cattle

For the identification of potential gene expression biomarkers for the abuse of anabolic substances, the performance of animal trials is essential, because the involvement of the kinetics of the specific compound in the body has to be taken into account. Cell culture models are useful to study the physiological action of a drug on a special subgroup of cells but do not include the interaction of different organs and matrices that occurs when applying the substance to the living organism.

There are already different publications available dealing with the identification of gene expression changes caused by different anabolic substances in order to identify potential biomarkers. Thereby the choice of the right tissue, dependent on the receptor concentration for the specific substance, is an essential point. Regarding the administration of steroids, organs of the reproductive tract will be the first choice due to the dependency of most physiological procedures on steroid hormones. In male animals, testis and prostate will be candidate tissues for the analysis of the influence of steroids on gene expression.

Lopparelli *et al.* (2011) examined the expression profiles of 12 candidate genes in the testis of cattle after administration of dexamethasone applied orally or intramuscularly, alone or combined with 17β-estradiol using RT-qPCR.[23] When dexamethasone was applied intramuscularly, $P450_{C17}$ and MR-like were significantly down-regulated, whereas in animals that received dexamethasone orally, $P450_{C17}$ was up-regulated. HSD17β1, a regulator of corticosteroid action was up-regulated in animals that received dexamethasone orally alone or in combination with 17β-estradiol. The combined administration of dexamethasone plus 17β-estradiol also up-regulated the expression of HSD17β3, a factor involved in steroidogenesis. In conclusion no single biomarker could be identified for the administration of dexamethasone alone or in combination with 17β-estradiol, but first potential candidates could be identified.[23] In the same study, the influence of the prohormones dehydroepiandrosterone (DHEA) and boldione on gene expression in testis was monitored, whereas DHEA administered alone, up-regulated the expression of aromatase and in combination whith boldione, DHEA significantly alters the expression of $P450_{scc}$, HSD17β1 and the androgen receptor.[23]

In the prostate of veal calves treated either with a combination of estradiol benzoate plus testosterone enantate or estradiol benzoate plus boldenone undecylenate, gene expression changes of 12 selected genes were quantified using RT-qPCR.[24] They identified seven genes, whose expression was changed either by treatment one or two, or by regarding and analyzing both treatment groups as one group of treated animals. They also analyzed samples taken at two different time points, 6 and 14 days after the last treatment dose. Two out of these seven genes, HMGCS1 and AR, were regulated regarding all treated animals 6 and 14 days after the last treatment dose and also by comparing control animals to the two treatment groups separately. Therefore these two genes represent potential biomarker candidates in testis for the application of an estrogen combined with an androgen.[24]

Reiter *et al.* described changes in gene expression of heifer tissues caused by treatment with Melengestrol Acetate (MGA), Finaplix-H® (Trenbolone Acetate) or Ralgro® (Zeranol). Thereby mRNA expression in uterus, two different muscles and liver tissue

were examined. Candidate genes were chosen by their physiological action in different biological pathways in the specific tissue and were quantified using RT-qPCR. They identified 11 significantly regulated genes in neck muscle, 8 significantly regulated genes in hind limb muscle, and 18 significantly regulated genes in the liver, whereas none of these genes was up- or down-regulated more than 2-fold. In uterine tissue, 13 significantly regulated genes could be identified, whereas the expression changes were up to 6-fold.[25] These results again indicate that hormone-dependent tissues are potential targets for the identification of gene expression biomarkers to screen for the misuse of anabolic steroids.

Another publication describes gene expression changes in uterine endometrium and ovary tissue of heifers following treatment with a combination of trenbolone acetate plus estradiol. Thereby uterine endometrium was subdivided to uterus horn and uterus corpus. Nine genes could be identified as significantly regulated in uterus horn and 12 genes in uterus corpus, whereas the expression of 4 genes, namely AR, BMF4, Caspase 3 and complement factor C7, were influenced in both regions.[26] Comparing these results with those published by Reiter *et al.*, it can be observed, that AR and Caspase 3 were also significantly regulated by Finaplix® indicating that these genes could act as first biomarkers in uterine tissue for treatment with Trenbolone Acetate.[25] Regarding the results obtained in ovary, where 22 genes were significantly regulated, it can be concluded that ovary will be a more potential organ for the identification of gene expression changes in order to find biomarkers for the abuse of anabolic steroids.[26] Another new approach in the same animal trial was the quantification of gene expression in vaginal smear containing vaginal epithelial cells. This matrix can be taken in a non-invasive form from the living individual and due to the direct hormone responsiveness of the vaginal epithelium, expression changes of different genes, especially of factors involved in keratinization, cell growth, and apoptosis could be expected. From the 27 quantified genes, 13 genes could be identified as significantly regulated whereas nearly all 27 genes showed a trend to regulation but not reaching the level of significance.[27]

A further interesting tissue for the quantification of gene expression changes upon administration of growth promoters is muscle as a physiological target organ of the treatment. As already described above, Reiter *et al.* examined the expression of different genes in hind limb muscle and neck muscle upon treatment with MGA, Finaplix®, and Ralgro®, whereas they found eight and eleven significantly regulated genes, respectively.[25] De Jager *et al.* used microarray analysis to identify differently expressed genes in longissimus dorsi muscle after implantation of Revalor-H® (Trenbolone Acetate plus Estradiol). In this experiment, 121 out of 16 944 quantified genes were differentially expressed. One gene that would not be expected to be involved in anabolic functions, but was shown to be highly regulated in this trial, was oxytocin (up to 97-fold). The plasma levels of oxytocin protein were also measured and found to be 50-fold increased, making oxytocin a new, very promising biomarker candidate on mRNA and protein level.[28]

The liver is involved in different hormonal pathways and therefore it is attractive for the identification of potential gene expression biomarkers for the use of anabolic agents. There are already different publications available describing changes in RNA expression in bovine liver upon administration of growth promoters. Giantin *et al.* analyzed the influence of dexamethasone administered orally or intramuscularly, alone or in combination with 17β-estradiol on the expression of 18 selected genes in bovine liver. They identified 11 significantly regulated genes; four of them – namely CYP2B6-like, CYP2E1, GSTA1 and SULT1A1–like– regulated in all different treatment groups. Therefore these four genes represent first biomarker candidates for the use of dexamethasone alone or combined with estradiol.[29] Rijk *et al.* performed a microarray experiment regarding the influence of dexamethasone taken orally or intramuscularly on gene expression in liver. They identified 23 genes whose expression was altered by the oral administration of dexamethasone, and 37 genes altered when administration was performed intramuscularly. Only one of these genes – DMBT – was regulated in both groups.[30] Becker *et al.* analyzed expression changes in the liver of heifers treated with trenbolone acetate plus estradiol. They quantified the expression of 34 genes belonging to metabolic pathways controlled by steroid hormone action. Eleven of these genes could be identified as significantly regulated.[31] They also tried to quantify changes in miRNA expression using a ready-to-use PCR panel, enabling the measurement of 730 miRNA in two PCR runs. Fourteen miRNAs could be identified as significantly up-regulated and 22 miRNAs showed a significant down-regulation.[22] This shows the potential of the additional analysis of miRNAs to identify new biomarkers for the misuse of anabolic substances.

Most of the tissues described can only been taken at the slaughter house. To enable controls directly at the lifestock

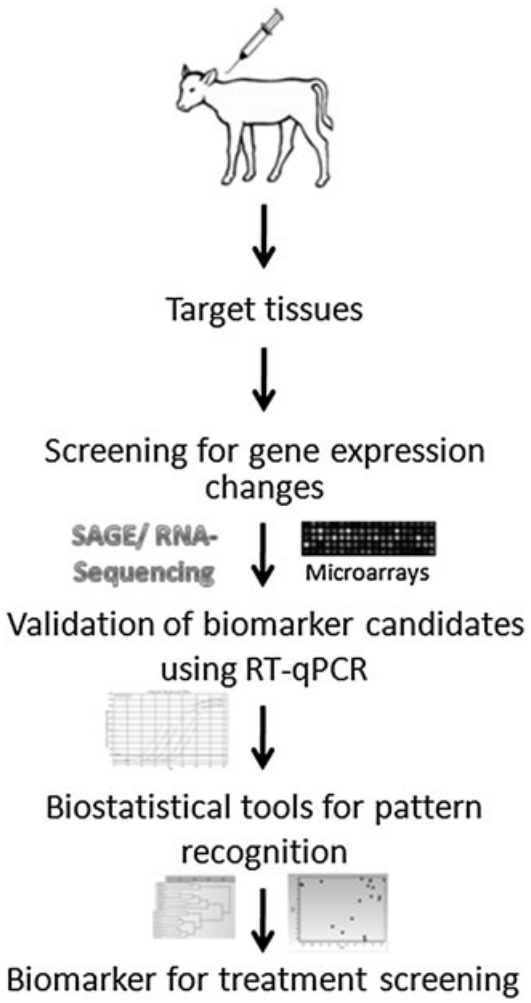

Figure 1. Workflow for the identification of gene expression biomarkers for the use of anabolic substances.

farms – tissues or matrices that can be taken from the living individual in a non-invasive form, independent of gender – will be interesting. Herein blood will be a potential matrix; there are different publications available describing the effects of hormones or other growth-promoting agents on gene expression in blood cells. But most of these experiments are performed in cell culture. Cantiello *et al.* described the influence of the administration of a cocktail of 17β-estradiol, dexamethasone and clenbuterol on the expression of four different cytokines in primary blood lymphocytes, whereas only IFNγ was significantly regulated.[6] The effects of trenbolone acetate in combination with estradiol on the expression of 36 candidate genes, different time points after administration was quantified by Riedmaier *et al.* Eleven of these genes could be identified as significantly regulated, whereas only two genes – GRα and IL-1α – were regulated at two sampling time points.[32]

All these studies show that the identification of single gene expression biomarkers for the misuse of different anabolic agents is difficult. Another way will be the identification of an expression pattern and the extraction of the intended information from the resulting data set using biostatistical methods for pattern recognition like principal components analysis (PCA) or hierarchical cluster analysis (HCA).[8] These methods were already successfully used in bovine uterus, ovary, vaginal smear, liver and blood to visualize a separation between untreated controls and treated animals.[22,26,27,30–32] These results show that the analysis of gene expression changes with biostatistical pattern-recognition methods will be a promising approach to identifying a biomarker pattern to screen for the misuse of anabolic agents in cattle.

Conclusions

The determination of physiological changes caused by the use of anabolic substances based on the analysis of gene expression changes is a very promising way for developing new, very sensitive screening methods in the combat of the misuse of anabolic substances in cattle. For the validation of promising biomarker candidates and for the identification of new gene expression changes, the performance of more animal trials applying different growth promoters will be necessary. The most promising approach will be the use of untargeted screening methods to identify new candidate genes and to validate those using targeted methods like RT-qPCR. Analyzing the resulting data set with biostatistical methods for pattern recognition helps to extract the intended information and to visualize separation between untreated and treated animals (Figure 1).

The analysis of another non-coding RNA species – namely the miRNA – has also been shown to be a promising approach. Circulating miRNA in blood plasma – which are already recognized to function as potential biomarkers in cancer research – will be an especially attractive target. Due to its sensitivity, RNA-sequencing is an upcoming method for holistic RNA expression analysis and therefore is a potential method for future experiments.

Conflicts of interest

Irmgard Riedmaier received financial support from OECD to cover travel costs to the Workshop. The remaining authors have no potential conflicts to declare.

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