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Quantitative Real Time PCR approach to study gene expression profile during prenatal growth of skeletal muscle in pig of Duroc and Pietrain breeds

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RIASSUNTO – Applicazione della real time PCR allo studio dell'espressione genica nel tessuto muscolare scheletrico di suino durante lo sviluppo embrionale nelle razze Duroc e Pietrain. *La PCR quantitativa "real time", è stata utilizzata per analizzare i livelli d'espressione di geni muscolari di suino durante lo sviluppo embrionale. A tale scopo è stato utilizzato il LightCycler™ system per quantificare il numero di copie di mRNA di geni del tessuto muscolare scheletrico e confrontarne il pattern di espressione nelle razze suine Duroc e Pietrain, molto diverse per caratteristiche produttive e per la qualità della carne e della carcassa. Il livello d'espressione di questi geni è stato monitorato con quantificazione relativa, basata sul rapporto tra il numero copie cDNA/µl di ciascun gene target rispetto al gene di riferimento β2-microglobulina (B2M).*

KEY WORDS: quantitative real time-PCR, gene expression, pig embryos, skeletal muscle genes.

INTRODUCTION – The quantitative real time-PCR (QRT-PCR) is a very sensitive method used to quantify mRNA level in gene expression analysis. Combining amplification, detection and quantification in a single step, allows a more accurate measurement compared to the traditional PCR end point analysis (Pfaffl, 2001; Bustin, 2002). This innovative molecular technology is increasingly used in different fields of animal science owing to its relevant potential for analytical and quantitative applications. In particular, because of its high sensitivity quantitative real time-PCR can be a powerful technique to quantify physiologically significant changes in gene expression. Moreover it represents a suitable tool for verification of putative candidate target genes found in array experiments (Bai *et al.*, 2003; Pfaffl *et al.*, 2003). The knowledge on specific pig genes level in muscle tissue during embryo development is missing at this time in literature. It could be relevant a better understanding of the molecular basis of physiological processes influencing muscle growth and metabolism and consequently both meat quality and carcass traits. The aim of this study was to develop and optimize a quantitative real time-PCR assay to perform expression studies during prenatal muscle development of genes of known functional relevance in embryos of two porcine breeds Duroc and Pietrain.

MATERIALS AND METHODS – *RNA source, total RNA extraction and cDNA preparation*. We extracted total RNA from a minute skeletal muscle tissue sample from Duroc and Pietrain embryos of 21, 35, 49, 63, 77 and 91 days using a modified TRIZOL (Invitrogen Corporation, Carlsbad, California) protocol. For each developmental stage and each breed, 8 samples were analysed. RNA integrity was verified on agarose gel by ethidium bromide staining and by spectrophotometer analysis (OD260/OD280 nm) with an absorption ratio threshold >1.8. After quantification total RNA (10µg) was treated with DNAse (Invitrogen Corporation). 1µg of treated RNA was retrotranscribed according to the manufacturer's instructions using Improm-II™ Reverse Transcription System and Oligo-dT primers (Promega Corporation, Wisconsin USA).

Optimisation of PCR and QRT-PCR conditions. Cycling conditions for all PCR primers were optimised in a thermal cycler (MJ Research PTC 100 Thermal Cycler, Minnesota, USA) with regard to *Taq* DNA polymerase, $MgCl_2$, dNTP, forward and reverse primers concentrations, and annealing temperatures (51-61°C). Gene specific PCR products were checked by electrophoresis. Optimal PCR condition was transferred on real time PCR protocols and for quantitative PCR of cDNAs the LightCycler™ system (Roche Applied Science Mannheim, Germany) was used. The optimised PCR reaction mixture were prepared (QuantiTech SYBR green PCR kit - Qiagen, Hilden, Germany or Fast Start DNA Master SYBR Green I - Roche Diagnostics) and used to fill the LightCycler capillaries. 2 µl of cDNA were added as PCR template. Quantitative PCR cycling was initiated and the real time amplification was monitored using LightCycler software version 3.3 (Roche Diagnostics). The quantification was carried out with the 'Second Derivative Maximum' method. Relative quantification was performed following the basic procedure described in http://www.gene-quantification.info/.

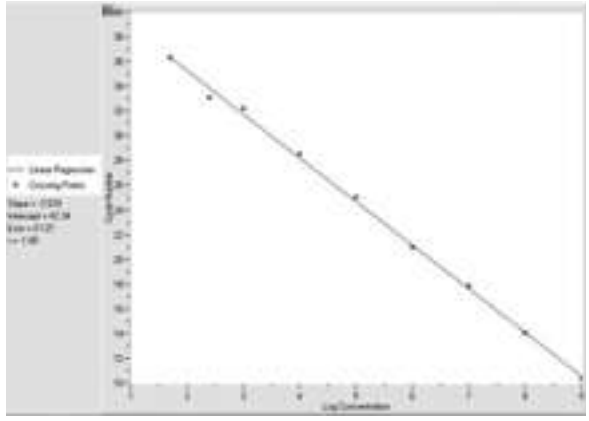
Standard curve preparation. For each analysed transcript and for the reference gene we obtained a calibration curve by cloning a fragment a fragment of the gene in a plasmid vector. After amplification and purification of the cloned DNA fragment, PCR product concentration was measured by spectrophotometer at 260nm obtaining at least 10^9 copy/µl serially diluted to 50 copy/µl in order to have 8-10 points for the standard curve preparation. Copy number of cDNAs was empirically determined using the equation described by Reischl, 2000.

RESULTS AND CONCLUSIONS – This study provides a first application of quantitative real time technology to investigate gene expression level in skeletal muscle tissue of porcine embryos. Real-time reverse transcription PCR assays were developed allowing a relative quantification of mRNA molecules of muscle genes with a sufficiently high sensitivity and repeatability.

To carry on real time PCR technique, we optimized some critical steps. Firstly we carefully checked the integrity of purified RNA, DNA free, and the efficiency of cDNA synthesis because they are sources of most of the inter-assay variation in an *QRT-PCR* experiment. Reliable quality control of cDNA synthesis is thus essential. In order to check for specificity of the PCR product, for each analysed gene we performed a melting curve analysis that allowed recognizing the specific target product by melting temperature (76-88°C). In our analysed samples, we checked PCR efficiency because it is an important criterion for reliable comparison between samples.

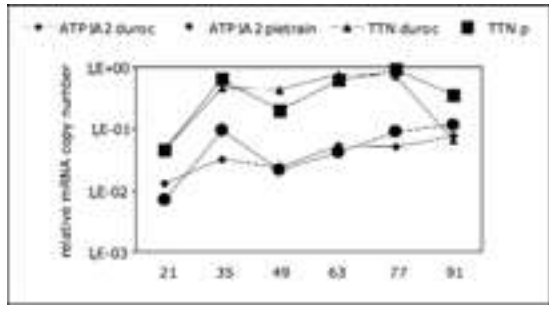
There are three parameters to be respected for an optimal standard curve preparation: slope (it represents the PCR efficiency and has an acceptable range from -2.9 to -5.7 with an optimum of -3.3), error (mean square error) and r (regression coefficient). The real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E=10^{[-1/slope]}$. For each gene, we analysed these parameters and E values obtained ranged from -3.253 to -4.559. Normalisation of target gene expression levels must be performed to compensate for intra and inter *QRT-PCR* variation.

Figure 1. Standard curve for B2M gene.



For relative quantification, we analyzed different housekeeping genes according to the literature (LightCycler technical note No. 15/2002; Warrington *et al.*, 2000; http://www.gene-quantification.info/). We have chosen B2M (beta-2-microglobulin) because its high expression level (10^5 molecules/µl) resulted the most stable in all the embryonic development stages. For B2M we obtained a slope=-3.529 (E=1.92 according to the reported formula) with an error=0.121 and r=-1.00 (Figure 1). Coefficient of variability (CV) was calculated analysing three standard concentrations in triplicate in the same LightCycler run (intra-CV assay) and in three different runs (inter-CV assay). According to Reischl, 2000, values lower than 5.00% were accepted. For B2M gene we obtained an intra-CV of 0.56% and an inter-CV of 2.34%. We applied the optimised real-time PCR to examine the different expression patterns in prenatal muscle development in Duroc and Pietrain embryos for ATPase, Na^+/K^+ transporting, alpha 2(+)polypeptide (ATP1A2) and titin (TTN), two potential candidate genes highly expressed in muscle (Figure 2).

Figure 2. Example of relative expression of gene level.



For both these genes we found a quite high level of transcription in both breeds and in the entire considered period: from $2E^3$ to $3E^4$ molecules/µl for ATP1A2 and from $1E^4$ to $2E^5$ molecules/µl for TTN. It is notable to show that at key developmental stage 35, where the primary myofibrils begin to be produced (Lefaucheur, 2001), there is a higher expression level for both analysed genes. This increment is more evident for ATP1A2 in Pietrain breed. These preliminary expression data support the hypothesis that the target genes expression during muscle growth is regulated and it is characterised by physiological changes following the main steps of the tissue development. The development of an optimised technique for reliable mRNA quantification represents a powerful tool to investigate on the relation between gene expression and both muscle growth, meat quality and carcass traits in pig.

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