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Transcriptome of pig muscle assessed by erial analysis of gene expression (SAGE)

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RIASSUNTO – Analisi del trascrittoma del muscolo di suino mediante Serial Analysis of Gene Expression (SAGE). *Lo scopo dello studio è stato di studiare il profilo trascrizionale di mRNA di muscolo suino adattando il metodo SAGE, sviluppato per l'uomo. Allo scopo sono stati prelevati dei campioni di longissimus dorsi da suini di 9 mesi mediante biopsia e, dopo estrazione dell'mRNA e retrotrascrizione in cDNA, sono stati analizzati mediante SAGE. Il metodo utilizzato ha previsto la costruzione di librerie di tag a 17bp (LongSAGE), una variante dell'originale protocollo che produceva tag da 10bp troppo corti per la corretta localizzazione su un genoma ancora poco caratterizzato. Inoltre sono state apportate alcune modifiche alla metodica originale per aumentare il controllo tra le numerose fasi procedurali. Allo stato, sono stati identificati oltre 750 tags corrispondenti a geni espressi nel muscolo, variabili per quantità da 1 a 43 coppie di mRNA trascritto. I risultati preliminari indicano la possibilità di utilizzare il metodo SAGE per gli studi di associazione con i caratteri fenotipici e per confrontare condizioni fisiologiche diverse nel suino.*

KEY WORDS: trascriptome, muscle, pig, SAGE.

INTRODUCTION – Serial analysis of gene expression (SAGE) is a molecular biology technique applied to measure the global gene expression levels, characterise transcriptomes, compare the transcript levels between tissues and uncover new molecules within defined signal transduction pathways (Tutela and Tuteja, 2004). Furthermore, SAGE provides quantitative and comprehensive expression profile of genes in a given cell population. The method works by isolating short fragments of genetic information from the expressed genes that are present in the cell being studied (Le Dai, 2004). These short sequences, called SAGE tags, are linked together for efficient sequencing. The frequency of each SAGE tag in the cloned multimers directly reflects the transcript abundance. Therefore, SAGE results in an accurate picture of gene expression at both the qualitative and the quantitative levels. SAGE does not require a hybridization probe for each transcript and allows new genes to be discovered. This technique has been applied widely in human studies (Kasai *et al.*, 2005), but, at the best of our knowledge, this technique was never applied to swine, since the pre-existing reduced information on pig genome sequence did not allow high matching levels among tags and genes.
The results present constitute a preliminary information of the suitability of the SAGE method to assess whole trascriptome of pig muscle, both from a quantitative and qualitative point of view. Further application will be the investigation of physiological changes of muscle associated with ageing and with meat quality of pigs.

MATERIAL AND METOHDS – Muscle biopsy of *longissimus dorsi* was sampled from two 9 months old pig of commercial lines. The experiment was carried out in accordance with state and local laws and ethical regulations. Samples were immediately frozen at -80°C and later processed to extract total RNA, using TRIZOL Reagent (Life Technologies), a mono-phasic solution of phenol and guanidine isothiocyanate, that is an

improvement to the single-step RNA isolation method (Chomczynski and Sacchi, 1987). Fos SAGE analysis, a commercial kit (I-SAGE™ Long Kit, Invitrogen) was used, following the instruction manual published on the web site (www.invitrogen.com). Briefly, poliadenylate RNA (mRNA) was isolated by means of Dynal Oligo(dT) Magnetic Beads, retrotrascribe to cDNA and digested with restricion enzyme NlaIII. The 3' terminal fragments were isolated with the anchored streptavidin-coated magnetic beads, ligated to LS adapters, modified with an amino group at the 3′ end to prevent self-ligation. Adjacent tags were released by cleavage with the type II restriction endonuclease MmeI e linked together to obtain ditags of ~130bp. These ditags were preliminary amplified by PCR and analyzed by agarose gel electrophoresis. Large-scale PCR amplification and polyacrylamide gel purification were performed and the ditags were again digested with NlaIII. The bands containing the ditags were purified by a polyacrylamide gel, excised and self-ligated to produce long concatamers. The concatamers between 500 and 1500 bp were isolated by agarose gel and extracted by DNA and gel purification kit (Amersham). Three different pools of concatamers were prepared, according to the size: 500-750bp, 750-1000bp and 1000-1500bp. These products were cloned into SphI site of pZErO®-1 vector and were screened by PCR to select inserts for sequencing.

The sequence and occurrence of each of the transcript tags were determined using the software SAGE 2000 software v4.5 (www.sagenet.org). To identify the corresponding transcript, the sequences of 17 bp SAGE tags (21 if we consider NlaIII site plus the adjacent 17bp) were matched with public database (http://www.ncbi.nlm.nih.gov/SAGE/).

RESULTS AND CONCLUSIONS – The SAGE method has been developed by Velculescu *et al.* (1995) to allow the quantitative and simultaneous analysis of a large number of transcripts. SAGE is based on the isolation of unique sequence tags from individual transcripts and concatenation of tags serially into long DNA molecules. The method provides a tool for the quantitative cataloguing and comparison of expressed genes in cells in different conditions and developmental states. Initially, 10 bp tags were used as unique sequence for individual gene identification, but more recently the method was modified and adopted by Saha *et al.* (2002) to obtain a 17 bp length tag (LongSAGE).

Table 1. Example of identified sequence tags and related gene expression

Tag sequence	Tags		Gene transcript associated
	No.	%	
CTTTTCTCCACTCAAAG	43	3.46	Muscle creatine kinase
GAGGCCGTGGCCGCCCA	40	3.22	Phosphoglycerate mutase 2
CCTACTAAGCGGGGAGG	25	2.01	Fructose-bisphosphate aldolase A
AGGATTGAGGAGGCTCT	22	1.77	Phosphopyruvate hydratase
TGGGCAGCCTTCCCTCC	22	1.77	Myosin regulatory light chain 2
ACTTCAACCCGGATGTG	21	1.69	Hemoglobin beta chain
TACCATCAATAAAGTAC	16	1.28	Glyceraldehyde-3-phosphate dehydrogenase
GACGGCTACATCGATGC	14	1.12	Troponin C2 (TNNC2) mRNA, complete cds
GCCAGAAGGTGGCTGAT	11	0.88	Hemoglobin alpha chain
GCGCCCTACAAGGGGGC	11	0.88	Alpha-actinin 3
GGGCTTGCAGGGACAAG	11	0.88	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1
.....	1 - 11		Other genes
AAGAGGTTGTTCTAGTA	1	0.08	Annexin I
AAGCCCGCGGCGAGCAG	1	0.08	Fatty-acid synthase
AAGGAGTTTGAGCGGGA	1	0.08	NADH dehydrogenase
AAGGGTCTCCCTGAGCT	1	0.08	Mitogen-activated protein kinase 12
AGAGCCTTGATTGAAGA	1	0.08	H+-transporting ATP synthase
ATCAGAACACGCTTTCC	1	0.08	alpha E-catenin
CAGCTCAGATCCCGAGT	1	0.08	Lipoprotein lipasi
GAGACTACACCCAAGGT	1	0.08	beta 2-microglobulin

In the present study the LongSAGE method was applied, but the kit developed for human required some modifications, to be adapted to pig. Although the method would apply for all eukaryotic cells, some difficulties were found, which required the use of intermediate controls to consider the NlaIII efficiency, the optimisation of PCR conditions, the use of more appropriated polymerases and ligation effectiveness.
At this state, more than 1242 sequences were identified and associated to 785 different transcripts and in Table 1 the most expressed known transcripts are reported. mRNA abundance varied from 43 tag replications for muscle creatine kinase (3,46%) and 40 tags for phosphoglycerate mutase 2 (3,22%) to 1 tag replication for many genes (beta 2-microglobulin, alpha E-catenin, lipoprotein-lipase). The first 9 most abundant transcripts account for about 18% of the total transcriptome, a typical condition of skeletal muscle tissues (Lanfranchi *et al.*, 1996, Welle *et al.*, 1999).
The preliminary results reported in the present study indicate that SAGE method can be efficiently used to trascriptome analysis of muscle in pigs. The potential use of this technique to associate transcriptome variability with phenotypic traits and differential different physiological conditions in pigs required to be assessed in further researches.

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