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A new set of endogenous reference genes for gene expression studies of porcine stomach

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ABSTRACT

Gene expression analysis by Real-Time PCR requires careful selection of endogenous reference genes to obtain accurate results. We evaluated usefulness of six reference genes to expression studies of porcine stomach. We selected genes which were recently described as very stable *(RPL27, RPS29, RPS13, OAZ1)* and two commonly used housekeeping genes (*GAPDH, ACTB*). Our results indicate that *OAZ1, RPS29* and *RPL27* are more suitable reference genes than *ACTB* and *GAPDH* in expression studies of porcine stomach. In our study the most stable genes were *OAZ1* (M=0.856), *RPS29* (M=0.862) and *RPL27* (M=0.892), while *ACTB* (M=1.03) and *GAPDH* (M=1.005) were less stable. *RPS13* (M=1.913) appeared to be highly unstable.

Set of three the most stable genes has been used to compare ghrelin *(GHRL)* expression in two different regions (*diverticulum ventriculi* and *fundus ventriculi*) of porcine stomach. Ghrelin mRNA was highly expressed in both regions of stomach, however level of *GHRL* mRNA was approximately 5-fold higher in *fundus ventriculi* than in *diverticulum ventriculi.*

KEY WORDS: pigs, ghrelin, Real-Time PCR, reference genes, stomach

INTRODUCTION

Real-Time PCR is currently the most precise method for gene expression studies. However, each expression experiment should be preceded by careful

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selection of endogenous reference genes (ERGs) to obtain accurate results (Bustin et al., 2009). Endogenous control is a housekeeping gene, expressed in every cell of the organism. Although genes like glyceraldehyde-3-phosphate dehydrogenase *(GAPDH),* beta actin *(ACTB)* or hypoxanthine phosphor-ribosyltransferase *(HPRT)* are commonly used endogenous controls, their expression level often depends on tissue type, developing stage or other factors.

To date, several studies evaluated usefulness of commonly used housekeeping genes to expression analysis of various porcine tissues (Erkens et al., 2006; Kuijk et al., 2007; Nygard et al., 2007; Svobodová et al., 2008). However, no such a study for porcine stomach has been performed.

Stomach is a place of synthesis of many important substances engaged in regulation of appetite, motility of gastrointestinal track and weight gain as a consequence. Hormones like ghrelin, serotonin and gastrin are a part of a complex hormonal axis which regulate animal's growth and reproductivity. Analysis of expression of genes coding for these proteins is necessary to understand mechanisms which control feed intake and weight gain of farm animals. One of the most interesting hormones engaged in feeding regulation is ghrelin - a novel hormone secreted mainly by stomach mucosa (Kojima et al., 1999; Date et al., 2002). It was proved that plasma ghrelin stimulates food intake in man (Wren et al., 2001) and is involved in weight gain in rat, mice (Tschöp et al., 2000) and pigs (Salfen et al., 2004). Ghrelin is also supposed to play a major role in the regulation of reproduction in pigs (Zhang et al., 2008).

The aim of our study was to evaluate usefulness of six reference genes to expression studies of porcine stomach. We selected genes (*RPL27*- ribosomal protein L27*, RPS29* - ribosomal protein S29, *RPS13*- ribosomal protein S13 and *OAZ1* - Ornithine Decarboxylase Antizyme 1) which were described as very stable after analysis expression data from 13,629 published human gene arrays (de Jonge et al., 2007) and two commonly used reference genes (*GAPDH, ACTB*). Set of three the most stable genes has been used to compare ghrelin expression in two different regions (*diverticulum ventriculi* and *fundus ventriculi*) of porcine stomach.

MATERIAL AND METHODS

Animals for the study (female, prepubertal pigs, with an average body weight of 100 kg) were kept in Pilot Plant of the National Research Institute of Animal Production. In total, twenty four animals were examined (seventeen Pietrain and seven Landrace pigs). Two fragments of stomach (*diverticulum ventriculi* and *fundus ventriculi*) were collected immediately after slaughter and kept in RNA later

(Ambion Inc.) during transportation (in total forty eight samples). For evaluation of the stability of reference genes twenty nine samples, taken from 7 Landrace and 16 Pietrain gilts, were chosen (twenty one *diverticulum ventriculi* and eight *fundus ventriculi)*, while for comparing expression of *GHRL* gene in two regions of stomach, twenty six samples, taken from 10 Landrace and 3 Pietrain gilts, were chosen (two samples from thirteen animals). All animals were fasted 48 h before slaughter.

The total RNA was extracted using SV Total RNA Isolation System (Promega), according to manufacturer protocol. The quantity of extracted RNA was estimated by BioPhotometer (*Eppendorf*), and its quality was evaluated by gel electrophoresis. The purity of RNA was checked by Real-Time PCR with No-RT samples.

The RNA (1 µg) was reverse transcribed into cDNA at 37ºC using High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems).

Primers and probes for *RPS13, RPL27, RPS29, OAZ1* and *GAPDH* were designed by Agata Piestrzyńska-Kajtoch (unpublished data), whereas primers and probes for ghrelin (*GHRL*) and beta actin (*ACTB*) were purchased as a TaqMan gene expression assays from Applied Biosystems company (Table 1).

Relative quantification of the expression was performed on 7500 Real-Time PCR System using Gene Expression PCR Master Mix (Applied Biosystems). Reactions, in a total volume of 25 µl, were performed in triplicate. The protocol included two initial steps: 50ºC for 2 min (UNG incubation) and 95ºC for 10 min (AmpliTaq Gold activation) and 40 cycles of 95ºC for 15 s (denaturation) and 1 min at 60ºC (annealing/extending). The results were analysed using Sequence Detection System software v. 1.4 (Applied Biosysytems).

The obtained Ct values were converted into input data for the geNorm application for identification of the most stable reference genes and normalization factor (NF - geometrical mean of the most stable genes) calculation. Stability of reference genes was assessed on the basis of M value, which is calculated as the average pairwise variation for that gene with all other genes tested (Vandesompele et al., 2002). Lowest M value indicates the most stable gene. Descriptive statistics (Ct min, Ct max, standard deviation, coefficient of variation) of the results were calculated with Bestkeeper software as well as coefficient of correlation with Bestkeeper index (geometrical mean of Ct of analysed genes) (Pfaffl et al., 2004).

Samples of *fundus ventricul and diverticulum ventriculi* from 13 animals were used for comparison of ghrelin expression level. Relative quantity of ghrelin mRNA was calculated according to Pfaffl (2001). Significance of difference between expression level of mRNA ghrelin in group of *fundus ventriculi* and *diverticulum ventriculi* was compared by using two sample T test (Satterthwaite method). Normal distribution of the data was verified by Kolmogorov-Smirnov test. These computations were performed by the use of procedure T Test of the SAS package programs (2006).

Table 1. Primer and TaqMan probes used in the study and PCR efficiencies

	Gene	Primers sequence	Probe sequence	Amplicon length. bp	Reference sequences accesion numbers	PCR efficiency
GHRL	Ghrelin	Taqman Gene Expression Assay ID: Ss03392359_g1 (Applied Biosystems)		58	NM_213807.1	1.97
ACTB	Beta actin	Taqman Gene Expression Assay ID: Ss03376563_uH (Applied Biosystems)		79	AY550069.1; DQ845171.1	2.02
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F CGATGCTGGTGCTGAGTATGTC R AGTGAGCCCCAGCCTTCTC	TGGAGTCCACTGGTGTC	71	AF017079	1.87
OAZ1	Ornithine decarboxylase antizyme 1	F TGCAGCGGATCCTCAACA R TGGGTTTATCCCCCTCCTTCT	CCACTGCTTCGCCAGA	57	EU545195	2.01
RPL27	Ribosomal protein L27	F CGCTACTCCGGACGCAAA R GGTCTGAGGTGCCATCATCA	CGGTCATCGTAAAGAA	58	NM_001097479	2.00
RPS13	Ribosomal protein S13	F CCGCCAAATTGGAAATACGA R CAACCAACAAGTTTATGCAACCA	CATCCACAGCCTCTG	63	AY610459	2.06
RPS29	Ribosomal protein S29	F CGGAAATACGGCCTCAATATG R GCCAATATCCTTCGCGTACTG	CCGCCAGTGCTTC	60	NM_001001633	2.01

RESULTS

Twenty nine samples of stomach (21 *fundus ventriculi* and 8 *diverticulum ventriculi*) were used for evaluation of the stability of reference genes. According to geNorm software the most stable was *OAZ1*(M=0,856), *RPS29*(M=0.862) and *RPL27*(M=0.892). *ACTB* (M=1.03) and *GAPDH* (M=1.005), commonly used housekeeping genes were less stable, while *RPS13* (M=1.913) appeared to be highly unstable (Table 2). The best correlation between BestKeeper index and candidate reference gene was obtained for *OAZ1, RPS29* and *RPL27*. *GAPDH* and these three genes had also the lowest standard deviation (SD) and the coefficient of variation (CV), while *RPS29* and *ACTB* had higher SD and CV (Table 2). Our results shows that *OAZ1, RPS29* and *RPL27* are more suitable reference genes than *ACTB* and *GAPDH* in expression studies of porcine stomach.

Table 2. Expression stability of analysed genes after geNorm and Bestkeeper analysis

Item	OAZ1	RPL27	RPS29	RPS13	GAPDH	ACTB
Min, Ct	20.891	20.013	19.433	22.157	30.216	17.212
Max, Ct	23.327	23.135	22.51	28.559	33.838	21.122
Standard deviation, ± Ct	0.528	0.601	0.562	1.589	0.711	0.822
Coefficient of variation, % Ct	2.399	2.831	2.731	6.403	2.231	4.387
Coefficient of correlation, r (Bestkeeper)	0.82	0.857	0.88	0.579	0.666	0.747
M value, geNorm	0.856	0.892	0.862	1.913	1.005	1.03

Geometrical mean of three the most stable genes *OAZ1*, *RPS29* and *RPL27* were utilized for calculation of normalization factor which was further used for expression level of *GHRL* gene evaluation. Ghrelin mRNA was highly expressed in both regions of stomach, however level of *GHRL* mRNA was approximately 5-fold higher in *fundus ventriculi* than in *diverticulum ventriculi* (Figure 1).

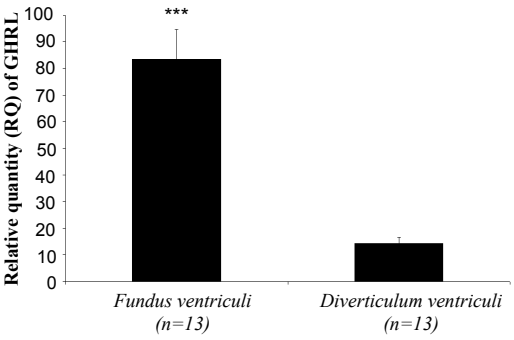

Figure 1. Relative expression of ghrelin (GHRL) in two regions of porcine stomach; *** - P<0.001

DISCUSSION

Our evaluation of stable endogenous reference genes in porcine stomach is in an agreement with general tendency to normalize Real-Time date against new ERGs rather than traditional (like *ACTB* or *GAPDH*) which is seen in recent publications (Jonge et al., 2007; Kwon et al., 2009). It is very probable that genes selected by us are stable in other organs and tissues of pigs, since they are among top 15 candidate genes after analysis expression data from 13,629 published human gene arrays (de Jonge et al., 2007). One of them, *OAZ1,* is also present in 13 novel ERGs, selected after analysis of publicly available human gene expression datasets (Kwon et al., 2009). Nevertheless stability of these genes should be carefully analysed in various tissues and experimental conditions, since *RPS13* (No 1 in top 15 candidate housekeeping genes (de Jonge et al., 2007)) was unstable in porcine stomach.

Ghrelin is a peptide of 28 amino acids, first identified in rat stomach (Kojima et al., 1999). It plays a crucial role in regulation of many biological functions like food intake, gastrointestinal motility, hormone secretion, glucose and enzymes release. In porcine stomach, ghrelin-immunoreactive cells are most abundant in oxyntic and cardiac glands while less numerous in pyloric glands (Govoni et al., 2005). On the other hand, Hayashida (2001) reported that immunoreactive cells are abundant in all gastric mucosal areas. To date, expression of ghrelin in various parts of porcine stomach has not been studied by Real-Time PCR technique. In our study we compared two regions of stomach, both covered with cardiac glands (Popesko, 1989), however there is a much lower density of cardiac glands on *diverticulum ventriculi* mucosa than on *fundus ventriculi.* Our results reflects these differences in cardiac glands distribution on stomach mucosa.

CONCLUSIONS

In conclusion, we recommend usage of three genes: *OAZ1, RPS29* and *RPL27* as an endogenous control in gene expression studies of porcine stomach. Our set was validated by analysis of ghrelin expression, which revealed 5-fold higher expression of ghrelin mRNA in *fundus ventriculi* than in *diverticulum ventriculi.*

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