Bulgarian Journal of Veterinary Medicine (2012), **15**, No 1, 13–20

GASTROINTESTINAL TISSUE EXPRESSION OF VILLIN mRNA IN TURKEYS

A. M. HARITOVA

Department of Pharmacology, Physiology of Animals and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Summary

Haritova, A. M., 2012. Gastrointestinal tissue expression of villin mRNA in turkeys. *Bulg. J. Vet. Med.*, **15**, No 1, 13–20.

Villin belongs to the family of actin-binding proteins. Its mRNA expression levels in gastrointestinal tract tissues of turkeys were studied in twelve healthy animals, BUT9 breed, divided in two groups. The first group consisted of untreated birds and served as control. The second group was treated with danofloxacin mesylate at a dose rate of 6 mg/kg/24 hours. Villin mRNA expression was detected in the crop and the proventriculus, and high levels were measured in the small and large intestines. Additionally, the profile of villin mRNA expression was tested after danofloxacin treatment. Its expression pattern was not statistically significantly changed. Moreover, in consideration of previous findings, villin was tested as a reference gene for quantitative RT-PCR experiments. Results showed that it was more stable than β-actin and that together with glyceraldehyde 3-phosphate dehydrogenase, it can be used as housekeeping genes for intestinal tissues such as duodenum, jejunum, ileum, caecum and colon.

Key words: gastrointestinal tract, turkey, villin

INTRODUCTION

Villin is an epithelial actin-binding protein that has been found in significant amounts in the gastrointestinal and renal epithelial cells. It has been identified also in other epithelial cells such as brush cells that line the respiratory tract, taste receptors cells and others. Villin is detected in immature digestive tract and its expression increases with the cells differentiation and moves from the crypt to the tip of the villi (Khurana, 2006). This protein organizes, integrates and regulates multiple epithelial cell functions such as cell morphology, motility and death. It participates in the assembly of the intestinal brush border cytoskeleton and its dynamics. It is required for cell shape and motility and is involved in bacterial entry and cell-cell dissemination (Athman *et al.*, 2002; Silacci *et al.*, 2004). Thus this protein became very important for actin cytoskeleton remodelling due to bacterial infection of intestines. Villin has a role in epithelial cell response to injury and is used as a marker for human colonic diseases (Khurana & George, 2008). Although essential for cell function, villin levels of mRNA expression in the different parts of poultry gastrointestinal tract are not well characterized.

Quantitative PCR is widely used for the quantification of mRNA levels of expression because it is a specific and sensitive tool. In this technique, numerous

variables such as RNA integrity, enzymatic efficiency need to be controlled. Therefore, gene expression measurements require a normalization strategy to allow meaningful comparison to be made across biological samples. Reference genes, the so called housekeeping genes, provide precise normalisation standards if they are equally expressed in different tissues at all stages of development and are unaffected by experimental treatment (Bustin *et al.*, 2005). There are several reports on evaluating the suitability of reference genes for accurate normalisation of gene expression. Results show that it is difficult to use unique reference gene for all tissues or in all of the experimental and clinical protocols (Huggett *et al.*, 2005). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin and 18S-RNA are among the most popular reference genes. Novel evidence indicate that their expression can vary widely across tissues or cells during the differentiation process or due to different response after experimental manipulation. Most of these data are generated for human, rodent (mice and rats), and dog tissues (Brinkhof *et al.*, 2006; Dydensborg *et al.*, 2006, Wang *et al.*, 2010). The studies in poultry are few. GAPDH and β-actin were used as the most suitable reference genes in evaluation of mRNA expression of ATP-binding cassette (ABC) transporter proteins in various poultry tissues (Haritova *et al.*, 2008; 2010). B-actin, ubiquitin and glucose-6-phosphate dehydrogenase were established as the most stable reference genes in blood (De Boever *et al.*, 2008). However, the information about the most suitable reference genes for poultry gastrointestinal tract in islimited. Selection of such genes is not easy because intestinal tissues are very sensitive to treatment, drug-drug or drug-diet interactions.

The aim of this study was to evaluate mRNA expression levels of villin and to test its suitability as a candidate reference gene for gastrointestinal tissues in turkeys. Additionally, the effect of fluoroquinolone treatment on the stability of villin mRNA levels was tested and compared to the most commonly used reference genes GAPDH and β-actin.

MATERIALS AND METHODS

Animals

Twelve turkeys (BUT 9, 8 weeks old, 5 males and 7 females) were obtained from the experimental poultry farm at the Institute of Animal Husbandry, Stara Zagora. The body weight of the male turkeys varied between 1.9 and 2.6 kg and that of the females between 1.8 and 2.4 kg. The animals were kept at room temperature, close to 25 ^{o}C. Free access to water and standard commercial feed, without additives (Provimi Zara, Stara Zagora, Bulgaria) were provided.

Four animals (two female and two male) served as controls and remained untreated. The other 8 animals (five female and three male) were treated with danofloxacin mesylate (Advocin 180, Pfizer, NL 9945 UDA, Part. No 2058806, V0704, 18% sterile solution) at a daily dose rate of 6 mg/kg for 5 consecutive days. At the end of the treatment all animals were euthanized and tissue samples from crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum and colon were immediately collected, snap-frozen in liquid nitrogen and stored at –70 °C until analysis.

RNA isolation and cDNA synthesis

Total RNA was isolated from individual tissues using Trizol Reagent (Invitrogen

life technologies, Cat No 15596-018) according to manufacturer's instructions. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies). Samples were stored (<7 days) at –70 °C prior to analysis. First strand cDNA's were synthesized from 1 μg total RNA with the iScripttmcDNA Synthesis Kit (Bio-Rad Laboratories, USA), containing both oligo (dT) and random hexamer primers. Shortly, to a master mixture (prepared *ex tempore*), containing 5× iScript Reaction Mix and iScript reverse transcriptase, 1 μg of total RNA in sterile nuclease-free water was added to give a final volume of 20 μL. This reaction mixture was incubated for 5 min at 25 °C, and at 42 °C for 45 min followed by heat inactivation of the enzymes at 85 °C for 5 min and a final fast cool step. The cDNA samples were stored at –20 °C until use.

qRT-PCR analysis

Primers complementary to turkey villin (Schrickx *et al.*, 2004), GAPDH and β-actin cDNA's were designed and commercially synthesized (Isogen Bioscience BV, The Netherlands). Specific primers are presented in Table 1. The reaction mixture for the qPCR containing 10 μL of the diluted cDNA, was mixed with 15 μL iQTM SYBR Green Supermix (Bio Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol ⁄μL for each primer) and sterile water according to the manufacturer's instructions. Quantitative RT-PCR was performed using the MyIQ single colour real-time PCR detection system (Bio-rad, Hercules, CA) and MyiQ System Software Version 1.0.410 (Bio Rad Laboratories Inc., USA). A standard dilution series of pooled cDNA including a blank control was run in the same plate as the samples. Samples of all birds and tissues were individually analysed. All analyses were run in triplicate.

Following an initial hot-start for 3 min, each reaction went through a PCR cycle with a denaturation step at 95 °C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72 °C for 30 s. After 35 cycles a melting curve was obtained by increasing the temperature with 0.5 °C every 10 s from 65 °C to 95 °C.

Data analysis

Only cycle threshold (Ct) values <35 were used to calculate the PCR efficiency from the slope generated with the MyiQ System

Table 1. Nucleotide sequences of the primers used for real-time quantitative PCR analysis

Gene	NCBI accession number	Forward primer 5'→ 3'	Reverse primer 5'→ 3'	Ta (°C)
Villin	AY825266	GGCACCAACGAGT ACAACACCA	TGCAGCCCTTCCCATACCAGA	63.5
GAPDH	U94327	ATGTGCCAACCCCC AATGTCTC	AGCAGCAGCCTTCACTACCCTCTT	64.5
β-actin	AY942620	ATGTGGATCAGCAA GCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	64.0

GAPDH – glyceraldehyde 3-phosphate dehydrogenase; NCBI – The National Center for Biotechnology Information; Ta – optimal annealing temperature.

Software. All PCR assays displayed efficiencies between 92 and 105%. Additionally, efficiencies for each reaction were estimated by LinRegPCR 7.0 software. Gene expression data were presented using the algorithms outlined by Vandesompele *et al.* (2002) and the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/). The housekeeping genes were characterized according to the lowest M value.

Statistical analysis

Results for expression levels of villin in the tested tissues were presented as median and minimum/maximum (Statistica 6.1 for Windows, StatSoft, Inc., USA, 1984-2002). Statistical evaluation of mRNA expression level of villin was done with Friedman ANOVA, and the effect of treatment on mRNA levels of expression was assessed with the Mann-Whitney test at a level of significance $P<0.05$.

RESULTS

Levels of expression

Villin mRNA expression was detected in crop and proventriculus. In these tissues villin mRNA levels were significantly lower than those found in the intestines ($P<0.02$). High levels were found in small and large intestines (Fig. 1). The statistical analysis of the villin mRNA expression levels in the different parts of the gastrointestinal tract revealed that the expression in small and large intestinal tissues was constant ($P>0.05$). Insignificantly lower expression was found in the caecum compared to small intestine and colon. Danofloxacin treatment did not result in statistically significant changes in mRNA level of expression in the intestines ($P>0.05$).

geNorm results

Values of M were very high and indicated a low stability of tested genes when the crop and the proventriculus were analysed with other tissues. Therefore, these tissues

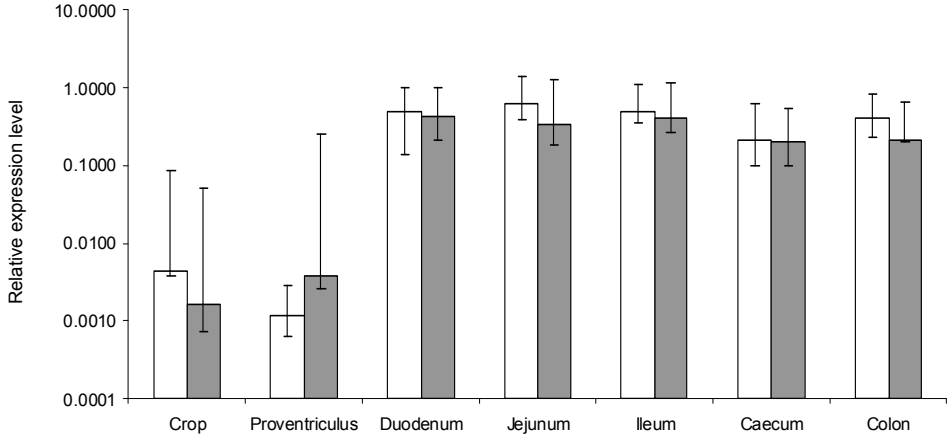

Fig. 1. Relative levels of expression of villin mRNA in the tissues of turkeys' gastrointestinal tract, determined by the delta-Ct method. Data are presented as median and maximum–minimum. White columns present data for untreated animals (n=4) and gray columns – for turkeys (n=8) treated with danofloxacin mesylate at a dose rate of 6 mg/kg for five days.

Table 2. Values of average expression stability (M) and pairwise variations (V2/3) according to the geNorm software analysis

geNorm parameter	All tested tissues		Small intestine		Large intestine	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
M value for GAPDH	–	–	0.728	1.038	0.819	0.920
M value for β-actin	–	–	1.076	1.317	0.823	1.256
M value for villin	–	–	0.779	0.985	0.678	0.893
Mean M value (GAPDH+β-actin+villin)	2.460	2.320	0.861	1.110	0.773	1.023
Pairwise variations (V2/3)	–	–	0.352	0.423	0.255	0.408
Mean M value (GAPDH+villin)	3.504	3.136	0.431	0.706	0.674	0.557

were excluded from the analysis. The lowest M values were obtained after separate analysis of small intestinal and large intestinal tissues (data not shown). When expression levels in tissues were analysed separately in untreated and treated turkeys, the genes in the control group displayed higher stability (lower M values) compared to danofloxacin-treated poultry (Table 2). geNorm also calculates the pair wise variation (V2/3) between two reference genes reflecting the effect of including more than two genes in the normalisation. A high V2/3 value denotes a significant effect of including an additional gene in the calculations. Pairwise comparison values were lower for tissues from untreated turkeys.

DISCUSSION

Villin belongs to the family of actin-binding proteins. It is expressed in epithelial cells and is identified in significant amounts in the gastrointestinal, renal and urogenital epithelial cells (Khurana & George, 2008). This study indicated that in turkeys, villin mRNA was constantly expressed from the duodenum to the colon. It was only detected with high inter-individual variability in tissues unique for poultry such as the crop and the proventriculus. Villin mRNA was found at detectable levels in the stomach of rats and at high levels in duodenum, jejunum, ileum and colon (Zhu & Altmann, 2005). Although the crop and the proventriculus do not have analogues in mammals, it can be concluded that in both poultry as in mammals, villin mRNA was highly expressed in the tissues distal to the stomach. This finding is not surprising if the physiological function of villin is considered. It is presumed that villin function in the intestines of turkeys includes regulation of actin dynamics, cell morphology, signal transduction, epithelial-to-mesenchymal transition, cell migration, cell invasion and apoptosis (Silacci *et al.*, 2004; Khurana & George, 2008). Since villin is recognized as a multifunctional actin regulatory protein in the intestinal cells, questions arise about the stability of its mRNA levels of expression. In the current investigation, the effect of treatment with fluoroquinolone drug danofloxacin mesylate was of particular interest. Results showed that mRNA villin levels of expression were not changed by the treatment or were statistically insignificantly downregulated mainly in the jejunum and the colon. It can be assumed that villin

mRNA expression was not affected by danofloxacin mesylate treatment and that the damage of epithelial intestinal cells was not related to the administration of this antibacterial drug. Similar results were obtained in studies with slides from small and large intestines (van de Kerkhof *et al.*, 2007). The cited authors found that villin mRNA expression in rat large intestinal slides was not affected by 3-methylcholantrene, β-naphthoflavone, indirubin, tetrabutylhydroquinone, dexamethasone and phenobarbital. Incubation with these compounds for more than 8 h resulted in downregulation of villin mRNA in small intestines which was attributed to the normal apoptosis of cells in tissue slides (van de Kerkhof *et al.*, 2007). These data suggested that villin can serve as reference gene in qRT-PCR studies and motivated the evaluation of its stability as a housekeeping gene.

Villin is considered as housekeeping gene for enterocytes in previous studies (Engman *et al.*, 2001). GAPDH and β-actin are also widely used as reference genes in qRT-PCR because they are widely expressed in most cell types and have a role as a glycolytic pathway enzyme and nonmuscle cytoskeletal protein, respectively (De Boever *et al.*, 2008; Haritova *et al.*, 2008, 2010). Although that GAPDH and β-actin are commonly used as reference genes, it is known that their mRNA levels can be influenced by experimental conditions, both *in vivo* and *in vitro* (Bustin, 2000; Ho-Pun-Cheung *et al.*, 2009). According to other studies, β-actin mRNA levels are more constant in comparison to GAPDH mRNA in human intestinal epithelial cells (Dydensborg *et al.*, 2006). Published data about the stability of reference genes are controversial, therefore GAPDH, villin and β-actin were evaluated as possible housekeeping genes in our experimental conditions. Reference genes stability is determined with specialised computer programmes. geNorm software provides a ranking of the reference genes based on their expression stability. It identifies the two most stable reference genes or a combination of multiple stable genes for normalisation (Vandesompele *et al.*, 2002). The stability of GAPDH, β-actin and villin mRNA levels of expression was tested for tissues from healthy untreated turkeys as well as for tissues from treated animals with danofloxacin mesylate by using geNorm. GAPDH showed little variation in mRNA level of expression between tissues as well as before and after the treatment. GAPDH was with lower M value than β-actin in small and large intestines. Its individual M value was similar or higher than the respective value for villin. GAPDH remained constant after incubation of slices of small intestines and colon of rat with different inducers of CYP450 enzymes (van de Kerkhof *et al.*, 2007). Data about M value indicated that villin mRNA expression was more stable in large intestinal tissues than in small intestine of turkeys. Treatment with danofloxacin resulted in insignificantly lower stability of villin. However, villin mRNA showed the lowest M value and the highest stability in intestines of turkeys under the current experimental conditions in comparison to other tested genes. Villin mRNA levels remained constant in rat colon tissue slices after incubation with phenobarbital, an inducer of CYP450 enzymes (van de Kerkhof *et al.*, 2007). In small intestines, the villin mRNA expression was constant up to 8 h after incubation with inducers of CYP450 and decreased afterward (van de Kerkhof *et al.*, 2007). The stability of villin has to be related to the lost of enterocytes. It can be considered as a reference gene after

analysis of possible damage and apoptosis of epithelial cells in gastrointestinal tract. High variability in mRNA levels of expression of villin can be expected in gastrointestinal tissues due to diseases. In our experiments, β-actin, another widely used reference gene showed clear upregulation and a higher variability after treatment with danofloxacin mesylate. Among the tested genes, it was the least stable reference gene in gastrointestinal tract of healthy untreated turkeys with the highest M values (Fig. 2). Therefore, villin can be considered as a better reference gene under the tested experimental conditions in our study. β-actin mRNA was also upregulated in neoplastic diseases of intestines (Ho-Pun-Cheung *et al.*, 2009). It varies among tissues in animals and even during the enterocytic differentiation process (Wang *et al.*, 2010). These data suggest that β-actin is not among the most suitable reference genes for qRT-PCR studies of poultry intestines.

Although only three genes were tested in the current study, pairwise variation (V2/3) of reference genes was estimated. geNorm defines 0.15 as the cut-off value, below which the inclusion of an additional reference gene is not necessary (Vandesompele *et al.*, 2002). The analysis of results from experiments with turkeys indicated that the V2/3 value was higher than the accepted cut-off for small and large intestinal tissues and that a third reference gene was necessary for normalisation of qRT-PCR results , but other than β-actin. Optimally, genes from different biological pathways should be selected to minimize the effects of gene co-regulation (Bustin *et al.,* 2009).

In conclusion, villin mRNA is expressed in small and large intestines of turkeys and it is only detected in the crop and the proventriculus. Together, villin and GAPDH are more stable as reference genes in comparison to β-actin in tissues such as small and large intestines under the investigated conditions. They can be applied for normalisation of unspecific variations related to manipulation with mRNA although the inclusion of a third gene, other than β-actin, is required for more precise results.

ACKNOWLEDGEMENTS

The author would like to thank Prof. J. Fink-Gremmels and Dr. J. Schrickx, Faculty of Veterinary Medicine, Utrecht University for their help in the development of specific primers for villin and their support in conducting the current study.

REFERENCES

Athman, R., D. Louvard & S. Robine, 2002. The epithelial cell cytoskeleton and intracellular trafficking. III. How is villin involved in the actin cytoskeleton dynamics in intestinal cells? *American Journal of Physiology – Gastrointestinal and Liver Physiology*, **283**, 496–502.

Brinkhof, B., B. Spee, J. Rothuizen & L. C. Penning, 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Analytical Biochemistry,* **356,** 36–43.

Bustin, S. A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*, **25**, 169–193.

Bustin, S. A., V. Benes, T. Nolan & M. W. Pfaffl, 2005. Quantitative real-time RT-PCR – a perspective. *Journal of Molecular Endocrinology*, **34**, 597–601.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele & C. T. Wittwer, 2009. The MIQE guidelines: Minimum infor-

mation for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, **55**, 611–622.

De Boever, S., C. Vangestel, P. De Backer, S. Croubels & S. U. Sys, 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. *Veterinary Immunology and Immunopathology*, **122**, 312–317.

Dydensborg, A. B., E. Herring, J. Auclair, E. Tremblay & J. F. Beaulieu, 2006. Normalizing genes for quantitative RT-PCR in differentiating human intestinal epithelial cells and adenocarcinomas of the colon. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, **290**, 1067–1074.

Haritova, A., J. Schrickx, L. Lashev & J. Fink-Gremmels, 2008. Expression of MDR1, MRP2 and BCRP mRNA in tissues of turkeys. *Journal of Veterinary Pharmacology and Therapeutics*, **31**, 378–385.

Haritova, A. M., J. Schrickx & J. Fink-Gremmels, 2010. Expression of drug efflux transporters in poultry tissues. *Research in Veterinary Science*, **89**, 104–107.

Ho-Pun-Cheung, A., C. Bascoul-Mollevi, E. Assenat, F. Bibeau, F. Boissière-Michot, D. Cellier, M. Ychou & E. Lopez-Crapez, 2009. Validation of an appropriate reference gene for normalization of reverse transcription-quantitative polymerase chain reaction data from rectal cancer biopsies. *Analytical Biochemistry*, **388**, 348–350.

Huggett, J., K. Dheda, S. Bustin & A. Zumla, 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity*, **6**, 1–6.

Khurana, S. & S. P. George, 2008. Regulation of cell structure and function by actin-binding proteins: Villin's perspective. *FEBS Letters*, **582**, 2128–2139.

Khurana, S., 2006. Structure and function of villin. *Advances in Molecular and Cell Biology*, **37**, 89–117.

Schrickx, J. A., A. Haritova & J. Fink-Gremmels, 2004. Characterization of villin in turkey. NCBI No: AY825266, http://www.ncbi.nlm.nih.gov/nuccore/AY825266 (11 July 2011 date last assessed).

Silacci, P., L. Mazzolai, C. Gauci, N. Stergiopulos, H. L. Yin & D. Hayoz, 2004. Gelsolin superfamily proteins: Key regulators of cellular functions. *Cellular and Molecular Life Sciences*, **61**, 2614–2623.

van de Kerkhof, E. G., I. A. M. de Graaf, M. H. de Jager & G. M. M. Groothuis, 2007. Induction of phase I and II drug metabolism in rat small intestine and colon *in vitro*. *Drug Metabolism and Disposition*, **35**, 898–907.

Vandesompele, J., K. De Peter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe & F. Speleman, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* **3**, research 0034.1–0034.11.

Wang, F., J. Wang, D. Liu & Y. Su, 2010. Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine. *Analytical Biochemistr*y, **399**, 211–217.

Zhu, L. & S. W. Altmann, 2005. mRNA and 18S–RNA coapplication–reverse transcription for quantitative gene expression analysis. *Analytical Biochemistry*, **345**, 102–109.

Paper received 11.07.2011; accepted for publication 11.11.2011

Correspondence:

Assoc. Prof. Anelia M. Haritova
Department of Pharmacology, Physiology of Animals and Physiological Chemistry,
Faculty of Veterinary Medicine,
6000 Stara Zagora, Bulgaria
tel: 00359 42 699 696
e-mail: haritova@uni-sz.bg