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Development of a method for the analysis of serglycin proteoglycan gene expression in canine blood samples

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Abstract

Cancer is a highly prevalent disease among canine breeds. Nowadays, mainly histology parameters are used to determine the prognosis and for selecting treatment strategies.
Due to the high degree of morbidity and mortality in cancer affected dogs, there is a strong need to identify additional diagnostic methods and biomarkers.
Serglycin is an intracellular proteoglycan found to be overexpressed in several types of cancer, including aggressive canine mammary cancer. High expression levels of serglycin in cancer cells are considered to correlate with cancer metastases and a poor prognosis for the patient. Detection of serglycin expression may, therefore, be a potential diagnostic marker for metastatic cancer in canine breeds.
A known molecular technology used daily in medical diagnostics is quantitative polymerase chain reaction (qPCR). QPCR is primarily used to identify the expression pattern of specific genes. The aim of this study was to establish a qPCR assay that could be used for detection of serglycin expression in canine blood. The focus of the study was to validate potential reference genes as well as optimization of a functional qPCR assay. For this purpose, blood samples from canine donors with unknown disease history were chosen.
Total RNA from each blood sample was extracted it an optimized TRIzol protocol. The samples were first tested in a step-down PCR, and then further tested in three different qPCR optimization steps. Both the step-down PCR and the qPCR included four genes; *SRGN* and three references genes *EEF2, HPRT* and *ACTIN B.* The qPCR assays showed high specificity and sensitivity for two of the genes, *SRGN* and *EEF2*.Primer pairs for each of the two genes showed efficiency values within the range of 90 % to 105%, and their reflection of linearity was R^2>0.980. The optimal annealing temperature for the *SRGN* and *EEF2* gene was set as 62 °C with a 300 nM primer concentration. Unfortunately, the published primer-design for two of the reference genes, *i.e. HPRT* and *ACTIN B* were poorly designed resulted in amplification of unwanted DNA and primer dimer formation.
In conclusion, the presented method in this study showed evidence that serglycin could be detected in small amounts of canine blood utilizing the described qPCR assay. We concluded that the *EEF2* gene is a stable reference gene for studying gene expression in dog blood with qPCR. However, in order to be able to apply our method in further studies, additional suitable reference genes need to be identified, tested and further validated.

Keywords: Canine cancer, serglycin, biomarker, qPCR, reference genes, blood sample.

Sammanfattning

Elakartad cancer förkommer med hög frekvens bland många hundraser. Olika histologiska och histopatologiska parametrar används huvudsakligen idag för att bestämma prognos och välja behandlingsstrategi. På grund av den höga graden av sjuklighet och mortalitet inom elakartad cancer hos hund, behövs ytterligare diagnostiska metoder och biomarkörer behöver utvärderas för användning på kliniken. Serglycin är en intracellulär proteoglykan som har visat sig vara överuttryckt i flera typer av cancer, inklusive aggressiva juvertumörer hos hund. Höga expressionsnivåer av serglycin i cancerceller anses vara korrelerade med cancermetastaser och en dålig prognos för patienten. Detektion av serglycin-uttryck kan därmed vara en potentiell diagnostisk metod för att bedöma risk för cancermetastaser hos olika hundraser. En känd molekylärteknik som används dagligen inom medicinsk diagnostik är kvantitativ polymeras kedjereaktion (qPCR). qPCR används främst för att identifiera uttrycksmönstret av specifika gener. Syftet med studien var att etablera en qPCR-analysmetod för att kunna detektera serglycin- (*SRGN*-) uttryck i hundblod, med målet att metoden sedan ska kunna användas för prognostisk screening av hundpatienter. Studiens fokus lades vid validering av potentiella referensgener samt optimering av en funktionell qPCR-analys. För detta ändamål valdes blodprover från hundar med okänd sjukdomshistoria. Totala mängden RNA isolerades med hjälp av ett eget designat TRIzol-protokoll. Först testades proverna i en step-down PCR för att sedan testats vidare i totalt tre olika qPCR optimeringsanalyser. Både step-down PCR och qPCR inkluderade fyra gener; SRGN och tre referensgener *EEF2, HPRT* och *ACTIN B.* qPCR-analysen visade sig ha hög specificitet och känslighet för två av generna *SRGN* och *EEF2.* Primerparen för var och en av de två generna visade sig ha effektivitesvärden inom intervallet 90% till 105% och deras R^2 värden låg över 0,980. Den optimala annealing-temperaturen för *SRGN* och *EEF2* generna sattes till 62°C med 300 nM primerkoncentration. Tyvärr, den publicerade primer-designen för två av de valda referensgenerna *HPRT* och *ACTIN B* visade sig vara sämre vilket ledde till amplifiering av oönskat DNA och bildning av primerdimerer. Som slutsats, metodutvecklingen i denna studie visade att serglycin-uttrycket kunde detekteras i små volymer av hundblod med hjälp av qPCR-analys, samt att EEF2 är en stabil referensgen för att kvantifiera genuttryck i hundblod med qPCR. Men för att kunna tillämpa metoden i fortsatta studier behöver ytterligare lämpliga referensgener identifieras och valideras.
Nyckelord: Elakartad cancer hos hund, Serglycin, biomarkör, qPCR, referensgener, blodprov.

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Abbreviations

ACT B	βeta-actin
EFF2	Eukaryotic elongation factor 2
HPRT	Hypoxanthine phosphoribosyl transferase
RT-PCR	Reverse Transcription polymerase chain reaction
SRGN	Serglycin
qPCR	Quantitative polymerase chain reaction

1 Introduction

One of today´s leading causes of death in canine breeds is cancer. High rates of cancer and aggressive tumor development can be found in several canine breeds, *e.g.* around 50% of the mammary tumors found in bitches are malignant tumors (Pawłowski et al., 2013).

The spread of malignant tumors through metastases are often difficult to treat, which unfortunately often leads to the death of the animal (Bonnett et al., 2005). Prognostic parameters being used today to evaluate tumor status are mainly based on histological variables including tumor size, lymph node status, lymphatic or vascular invasion and, the grade of differentiation (Manuali et al., 2012).

Poor diagnostic methods and inefficient treatment strategies can partly account for the high morbidity and mortality rates of malignant tumours found in canine breeds (Manuali et al., 2012). As of today, a few biomarkers have been evaluated for diagnosing canine lymphomas (Bryan, 2016) but, there are still no measurable indicators available to use in the canine clinical practice capable of a prompt detection and prediction of cancer malignancy in blood samples. To improve the survival rate for the cancer patient there is, therefore, a need for the development of suitable and reliable

biomarkers for the early detection of metastatic cancer.
Serglycin is an intracellular proteoglycan consisting of a core protein which carries long glycosaminoglycan chains (GAGs) (Korpetinou et al., 2013). In different cell types, serglycin is decorated with highly divergent structural GAGs, which depends on the glycosylation machinery at work in respective cell type. The GAGs have the capacity to interact with several important biological molecules through high-affinity binding (Korpetinou et al., 2014). It is primarily the negative charge of the GAGs chains that form hydrogen bonds and electrostatic interactions, thus becoming the biologically activate part of the protein (Pejler et al., 2009).

The serglycin gene is well conserved and the protein is expressed in several animal species *e.g.* in human, horses, mice and dogs (Kolset and Tveit, 2008). The serglycin gene is expressed in multiple cell types such as in hematopoietic cells including mast cells, neutrophils and macrophages as wells as in non-hematopoietic cells, for example, endothelial cells (Pejler et al., 2009). In most hematopoietic cells serglycin is involved in granular retention of preformed mediators. For example, in mast cells serglycin is stored intracellularly in the secretory granules, where it seems to play an important intracellular role by regulating the correct storage and secretion of numerous inflammatory mediators such as proteases, histamine, cytokines, and chemokines (Kolset and Tveit, 2008).

Serglycin has also shown to be present and expressed in several cancer types, *e.g.* myeloma, acute myeloid leukemia, breast and glioma (Roy et al., 2017). In tumor cells, serglycin can appear on the tumor cell surface where it plays a role in mediating interactions between the tumor cells and the microenvironment (Korpetinou et al., 2014). The secreted serglycin presented within the tumor microenvironment has been proposed to interact with a cell-surface adhesion receptor called CD44 (Guo et al. 2017). The interaction between the serglycin proteoglycan and CD44 results in triggering the CD44 signal pathway, and this will induce epithelial to mesenchymal transition (EMT), enhance invasiveness and promote metastasis. Recent studies have demonstrated that serglycin expression is essential for metastatic growth and dissemination (Roy et al., 2016). In addition, serglycin seems to play an important role in regulating the protein cargo in tumor-derived exosomes by generating more enriched exosomes. The enriched exosome will deliver its protein cargo to either nearby or distant cells resulting in tumor progression (Purushothaman et al., 2017).

Several scientific studies performed on human tumor cells have concluded that serglycin over-expression correlates with an aggressive tumor cell phenotype, which normally indicates a poor survival prognostic for the patient (Roy et al., 2017; Korpetinou et al., 2013). Some of the more recent studies have supported serglycin as a potential prognostic biomarker for human malignancy (Roy et al., 2017). Importantly, serglycin expression has also been shown to be up-regulated in aggressive canine mammary cancer patients (Pawłowski et al.,2013).

There are several molecular techniques available today that are used to evaluate potential biomarkers. Quantitative polymerase chain reaction, qPCR, also known as Real Time PCR, is one molecular technique commonly used in cancer diagnostics to identify expression patterns of specific genes (Bustin et al., 2005).
qPCR is considered to be an accurate, sensitive and high throughput technique for detection of gene expression. qPCR monitors gene expression in real time using fluorescent reporter molecules. It is possible, then, to quantify simultaneously a specific gene transcript number in several clinical samples (Wong and Medrano, 2005).
During each PCR cycle in the qPCR run, the fluorescence signal will increase over the background fluorescence and reach a point known as threshold cycle (C_t) or quantification cycle (Cq) (Bustin and Mueller, 2005). The Cq-value marks where the target amplification is first detected, and it also represents the starting copy number of mRNA in the original sample (Walker, 2002).
Highly expressed target genes will have a more rapid fluorescence signal increase and will reach the threshold value sooner compared to lower expressed genes (Bustin and Mueller, 2005). A standard qPCR run is normally around 40 cycles, highly expressed target genes will, therefore, have Cq-values between 20-30 while lower expressed genes will have Cq-values between 30-40 cycles. Lower expressed genes will have higher Cq-values because more amplification is needed to detect the fluorescence signal if the amount of mRNA present is low (Walker, 2002). The Cq-values are logarithmic and can, therefore, be used to calculate a quantitative result using either direct (comparative C_t method) or indirect (interpolation to standard curves to create linear values) methods (Wong and Medrano, 2005).

Furthermore, factors that may influence the gene expression output always need to be controlled, *e.g.* cDNA concentration, RNA integrity, enzymatic efficiencies, amplification efficiency and differences between clinical sample types in their overall transcriptional activity (Brinkhof et al., 2006). These factors are often controlled with the help of normalization to internal standards, also known as reference genes or housekeeping genes (Bustin et al., 2005). Reference genes are often used for normalization purposes because their equal expression in each cell of a specific tissue despite of the individual. Reference genes often encode for proteins needed for cell survival, and the ideal gene should have a stable expression pattern under various conditions. There is seldom one gene that is able to meet all criteria, more than one reference gene should, therefore, be included for the normalization control to obtain a correct and reliable end result (Wong and Medrano, 2005). Moreover, some studies have concluded that the expression of commonly used reference genes vary between individuals, tissue type, species and, that the choice of reference genes can have an impact on the outcoming result (Ohl et al., 2005).

To obtain good and reliable quantitative result, optimization of the qPCR assay is a very important step before the assay can be applied to clinical material. qPCR optimization can be performed by establishing the optimal concentration of template, annealing temperatures and primer concentration (Gene-quantification, 2018). Important characteristics of an optimized qPCR assay are: low variability across assay replicates, high amplification efficiency from 90% to 105 % and linear regression line of a strand curve (R^2

>0.980). These values are calculated from a standard curve, obtained by plotting Cq-values from the amplification against the log of chosen dilution series (1/dilution).
An optimized qPCR assay will have a amplification efficiency value (E) at 2, where 2 is equal to 100 % efficiency (Hui and Feng, 2013). So, if the amplification of the template is perfect, duplication will occur after each cycle generating a 2-fold increase.

So far, no qPCR assay has been established for the purpose of detecting serglycin expression in canine blood. It has been shown that qPCR is a sensitive method for detection and characterization of serglycin expression in malignant tumors (Roy et al., 2017), which allows serglycin to function as a prognostic marker for human tumors. The value of detection of serglycin in blood samples via qPCR is based on the fact that malignant tumor cells detach from their primary tissue and circulates in the blood stream (Joosse et al., 2015). Assumptions can, therefore, be made that blood samples from patients with aggressive tumors would show higher expression levels of the serglycin gene than blood samples from patients without metastatic prone cancer. The consistent association pattern between high expression levels of serglycin and aggressive cancer forms, in both animals and humans, suggests that serglycin has the capacity to function as a good biomarker for metastatic cancer.

Therefore, the purpose of this study was to determine if serglycin expression could be detected in a small volume of canine blood, while the aim is to develop a functional assay for screening of canine patients. We here present a potential screening method for detection of serglycin in blood from canine patients utilizing qPCR. We fo-

cused on establishing and validating a model system method, the first step towards a functional qPCR assay that could be useful for detecting serglycin expression in canine cancer patients. Because of the importance of qPCR optimization, several optimization steps were included in the study. For this purpose, we used blood samples from canine donors with unknown disease history with the intention to detect serglycin gene expression and to test published primer pairs for reference genes.

2 Materials and Methods.

The clinical material was provided by the Clinical Pathology Laboratory at the University Animal Hospital located at SLU, Uppsala. The clinical material was collected during the period between April 2018 and May 2018. No experimental animals were included for the purpose of this study.

2.1 Blood collection and RNA isolation

Canine blood samples were collected from patients at the University Animal Hospital. Clinical history of all patients was masked. The blood samples were collected with EDTA as anticoagulant and 100 μl of blood was processed for total RNA isolation by immediate addition of 500 μl of TRIzol® Reagent (Invitrogen, Life Technologies). Total RNA isolation was performed using a modified protocol (see Appendix 1, page 45).
Briefly, the RNA was obtained after phenol-chloroform extraction, as a 300-500 μl aqueous phase, and precipitated with an equal volume of isopropanol. To achieve a more efficient precipitation and a higher yield of total RNA, the incubation time after addition of isopropanol

was changed from the proposed 10 minutes to 30 minutes.
RNA pellets were resuspended in 30 μl RNase free water (Autoclaved *Milli-Q* water). All RNA concentrations were further quantified using NanoDrop 8000 (Thermo Fisher Scientific, CA, USA).
The dissolved RNA pellets were frozen and stored at -80°C until further use.

2.2 DNase I treatment and cDNA synthesis

To prevent genomic DNA contamination, a DNase treatment step was included after total RNA extraction. The reaction was performed with the RQ RNase-free DNase I kit (Promega) following the manufacture's protocol. Approximately 1.2 μg of total RNA was treated with DNase I, in a maximum dilution volume of 13.2 μl.
Reverse transcription from RNA to cDNA was then carried out using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, CA, USA). The DNase pre-treated RNA was further mixed with 1.2 μl of dNTP mix [10mM] and 1.2 μl of reverse gene-specific primers [2 μM] in a total reaction volume of 15.6 μl. The samples were incubated at 65 °C for 5 minutes, and immediately placed on ice for at least 1 minute.

A fraction of each reaction mixture (2.6 μl) was then transferred to new tubes as RT-minus controls (i.e., cDNA reaction without addition of Supercript II RT enzyme). The RT-minus controls were included to provide negative controls for the corresponding PCR reactions.
Then, 7 μl of the reverse strand reaction master mix (5x First Strand reaction buffer, 0.1 M DTT) with the SuperScript™ III RT enzyme

were thereafter added to each sample tube (RT–plus samples). In the corresponding RT-minus controls, 1.4 µl master mixture without SuperScript III RT enzyme was added. The cDNA reactions have been scaled up 1.2 times from the original protocol to include the RT-minus controls. The final reaction volume for RT-plus reactions was 20 µl and 4 µl for RT-minus reactions. The samples were incubated at 55 °C for 1 hour and then immediately transferred to 70 °C for 15 minutes for inactivation of the reverse transcriptase enzyme. All samples were placed on ice for additional 5 minutes after the cDNA synthesis was completed and, either used immediately for the PCR reactions or placed at -20 °C for long term storage.

2.3 Primer design and validation of primer pairs

The selection of references genes was based on a previous study describing the use of β-actin (*ACT B*), hypoxanthine phosphoribosyltransferase (*HPRT*) and eukaryotic elongation factor 2 (*EEF2*) as reference genes for metastatic canine mammary cancer (Bulkowska et al., 2017). Serglycin primers were selected based on a known *Canis lupus familiaris* serglycin sequence, available at NCBI Genebank *(Reference Sequence: XM_846674.5).* Primer pairs used in the study are shown in Table 1. To verify the sequences, an alignment search was constructed in BLAST among human, mouse and canine serglycin sequences(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer design was performed using the software Snap Gene 4.1 (http://www.snapgene.com). Primers chosen were positioned between different exons to avoid amplification of genomic DNA. Furthermore, an oligo analysis was performed on the chosen primers

using two different software's, the Oligo analyzer 3.1 (http://www.idtdna.com/calc/analyzer) and the Beacon designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1).

Table 1. *Primers for Canis lupus familiaris used in qPCR and step-down PCR, the primers were designed using the software Snap Gene 4.1*

TARGET GENES	SEQUENCE	TM	Amplicon size
EEF2	ACGCCTTTGGTCGTGTATTC fwd	58°C	173 bp
	GTTCCCACAAGGCACATCTT rev	58°C	
ACTB	TGTGTTATGTGGCCCTGGAC fwd	58°C	164 bp
	TTCCATGCCCAGGAAGGAAG rev	57°C	
HPRT	AAGCTTGCTGGTGAAAAGGA fwd	55°C	219 bp
	CAATGGGACTCCAGATGCTT rev	58°C	
Zhiqiang pair 9 fwd	GTGAAGAGAGCCACGTACCA fwd	59.2°C	147 bp
Zhiqiang pair 9 rev	TCTTGACATTGAAGAACGGTCAG rev	59.2°C	
Claudia primer 1 fwd	TCCAGGGCAGTAGGCTTGTA fwd	55°C	85 bp
Claudia Primer 3 rev	GTGGCTCTCTTCACAGGAGA rev	56°C	

Furthermore, to verify and test the quality of the primer design, a step-down PCR was constructed using cDNA obtained from isolated canine RNA which had been collected from both blood and septal heart wall tissue samples. Total RNA was therefore isolated from a range of 10 μl-100 μl blood to optimize the amount of canine blood needed for the analysis.

Approximately 1 μg of the total RNA was used for cDNA synthesis following the RT2 First Strand kit manufactures instructions (Qiagen). One modification was added to the protocol, RNase free water in the reverse transcriptase mixture was replaced with 3 μl of pooled gene-specific reverse primers [10 μM]. Combining random hexamer and Oligo(dT)-primers with the gene-specific primers ensured coverage of the total mRNA pool, together with the verification of the specific cDNA product expected length and an optimal yield.

The cDNA obtained was later used in a step-down PCR (see settings in table 2). To visualize the RT-PCR products, 2.5% agarose gel was prepared in 1x TAE buffer containing 30 μl of GelRed reagent and 5 μl of a 1 kb plus DNA ladder was used to determine the size of the products.

Table 2. *PCR settings used for the step-down PCR, no 72 °C extension step was included*

Step	Temp (°C)	Time	Note
1	95	5 min	
2	95	30 sec	Repeat step 3x.
	61	3 min	
3	95	30 sec	Repeat step 3x.
	59	3 min	
4	95	30 sec	Repeat step 3x.
	57	3 min	
5	95	30 sec	Repeat step 26x.
	59	3 min	
6	72	10 min	
7	4	∞	

2.4 Real-Time PCR (qPCR)

Each qPCR reaction was performed with a CFX 96® Real-time System (BioRad), using the iQ™ SYBR Green Supermix according to manufacturer's protocol (BioRad, CA, USA) in a total volume of 25 µl, containing 2 µl of cDNA. In addition, all qPCR runs included no template controls (negative control) and, RT-minus controls were included for sample quality check. All samples were run in duplicates, including negative controls and the RT-minus control samples for each gene. Before initializing running, the qPCR plates were centrifuged at 3000 rpm for 2 minutes.

The qPCR was performed on four occasions with cDNA obtained from 100 µl of blood. At first, the quality of each cDNA sample and the designed primer pairs of each gene were checked in a preliminary qPCR assay. This first assay included four individuals, *i.e.* number 8, 9, 10 and 11. cDNA from each individual was diluted 1:5 with nuclease free water. The chosen PCR cycling conditions included a 95°C heating step of 3 minutes (initial denaturation/enzyme activation). The samples were then cycled 40 times at 95°C for 15 seconds (denaturation), 59°C for 30 seconds (annealing) and 72°C for 30 seconds (extension).
This procedure was then repeated with three of the primer pairs for genes, *EEF2*, *SRGN*, and *HPRT.*

To optimize the template concentration, the best performed sample, individual 11, was chosen and a 1:4 cDNA dilution series (1:4, 1:16, 1:64 and 1:256) was prepared for amplification of *EEF2* and *SRGN* transcripts. This second qPCR reaction was performed under the same PCR cycling conditions as the previous run, described above. Next, a primer concentration and annealing temperature optimization for the amplification of *EEF2, SRGN* and *HPRT* transcripts was performed. cDNA from individual 11 diluted 1:64 was tested against three different concentrations, 200 nM, 300 nM and 400 nM for each primer pair. To optimize the annealing temperature, a gradient approach was used: 63 °C, 62.7 °C, 62.2 °C, 61.2 °C, 59 °C, 58.5 °C and 58 °C. Furthermore, at the end of each run a melting curve from 65°C to 95°C was obtained in a 0.5 °C increment for 0.05 seconds while all the other cycling parameters were kept the same as in previous assays.

3 Results

3.1 Total RNA extraction

A modified protocol of the TRIzol® Reagent (Invitrogen, Life Technologies) was applied. Table 3 is showing the total extraction RNA result before (sample 1, 3, 6, 7) and after (sample 8, 9, 10, 11) the modification was added to the original protocol. The NanoDrop measurement reveled a higher yield and quality of RNA obtained with the modified protocol for the majority of the samples. Notably, optimal 260/230 and 260/280 ratios should have values between 1.8 to 2.0 to be considered as pure RNA. In this case, 260/230 and 260/280 ratios were both lower despite the modifications introduced in the protocol, suggesting that phenolic reagents and genomic DNA might have been present. As a precaution a DNase treatment step was therefore included in the cDNA protocol to avoid amplification of unwanted DNA.

Table 3. *RNA concentration and quality measurements performed on a Nanodrop 8000.*

Sample ID	Concentration (ng/μl)	260/280	260/230
1	12.9	1.23	0.34
3	28.5	1.54	0.21
6	36.8	1.37	0.32
7	18.0	1.26	0.35
8	294.2	1.75	0.63
9	25.6	1.54	0.12
10	194.3	1.91	0.78
11	960.1	1.89	1.64

3.2 Step-down PCR

To investigate the potential to detect serglycin in blood and to verify the primer design of each primer pair, total RNA extracted from 100 μl of a canine blood sample and septal heart wall tissue total RNA (kindly provided by Åsa Olsson, HGEN Dept, SLU) was used in down step PCR. By using gel electrophoresis analysis, the size and overall quality of the PCR products were determined. This analysis revealed that serglycin transcripts (ZSGRN and CSRGN primer pairs) together with the three reference genes transcripts (ACTB, EEF2 and HPRT primer pairs*)* could be successfully amplified in a normal PCR assay with the designed primer pairs. Figure 1 shows products within the expected size range for each of the transcripts. The *ZSRGN* primer pair did not generate a good quality product in the septal heart wall PCR run and was therefore excluded from further experiments.

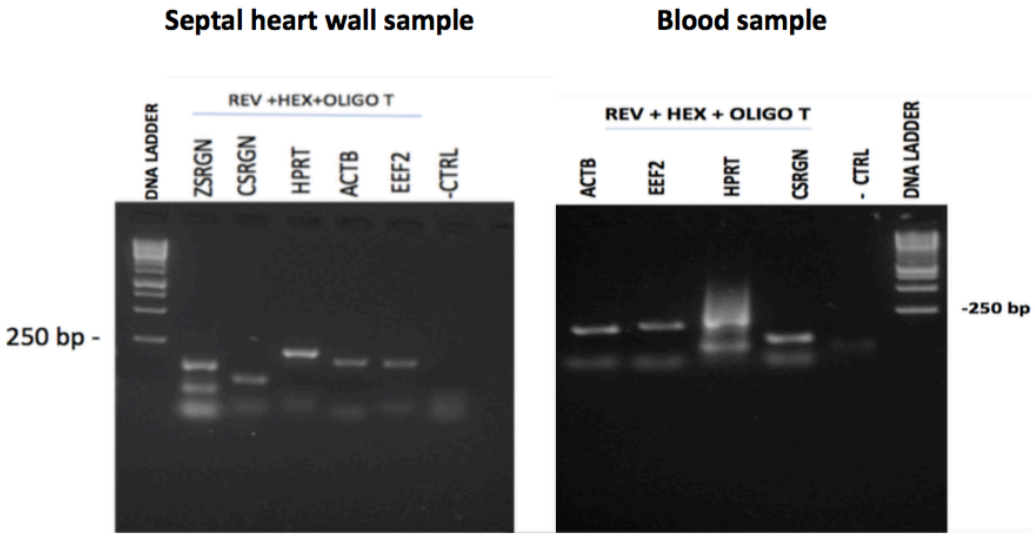

Figure 1. 2.5 % agarose gel analysis of the amplicons produced from the serglycin and four reference gene transcripts. The left image represents RNA obtained from septal heart wall tissue, and the right image RNA from the100 μl canine blood sample. Both PCR products were generated by a step-down PCR reaction.

3.3 qPCR optimization

The first qPCR assay provided data with different Cq-values for the four tested individuals *i.e.* number 8, 9, 10 and 11. Individual 11 had the lowest mean Cq-value for each gene and was thereby selected as the best performing sample (see Table 4). The qPCR test included no template controls (NTC) and RT-minus controls for each gene. Table 4 shows no significant differences between the RT-minus and RT-plus Cq-values obtained for the reference genes *ACT B* and *HPRT*. In addition, the NTC (No Template Control) for both genes also showed amplification of unwanted products that generated signals in the qPCR assay.

A BLAST analysis constructed for both the *ACT B* and *HPRT* primer pairs gave multiple hits for several different species, posing a higher risk for amplification of genomic DNA or potential pseudogenes. Furthermore, all the HPRT qPCR products were further analyzed with both a melt curve and agarose gel analysis (see Figure 2). Both analytical methods revealed unwanted product amplification. The melt curve analysis showed a peak that did not correspond to the HPRT product. Furthermore, the gel analysis revealed amplicons with a molecular weight under 100 bp, much smaller than the *HPRT* amplicon expected size (219 bp). This unwanted amplification product could either be an indication of primer-dimer formation, or a DNA contamination of the primer stock solution. The qPCR result obtained for both the *ACT B* and *HPRT* genes were therefore regarded as inconclusive and were excluded from further experiments.

Table 4. *Average quantification values (Cq-values) obtained from the first qPCR assay, showing both RT-plus and RT-minus for each gene and for individuals 8 to 11*

INDIVIDUAL	*SRGN*	*EEF2*	*HPRT*	*ACT B*
8 (+) RT	30.8	0.0	32.2	22.8
8 (-) RT	0.0	0.0	32.2	22.6
9 (+) RT	32.6	34.3	32.01	26.3
9 (-) RT	0.0	0.0	32.5	26.0
10 (+) RT	25.8	26.0	28.3	20.9
10 (-) RT	0.0	0.0	32.3	22.34
11 (+) RT	24.0	26.7	28.0	21.0
11 (-) RT	0.0	0.0	32.8	22.5

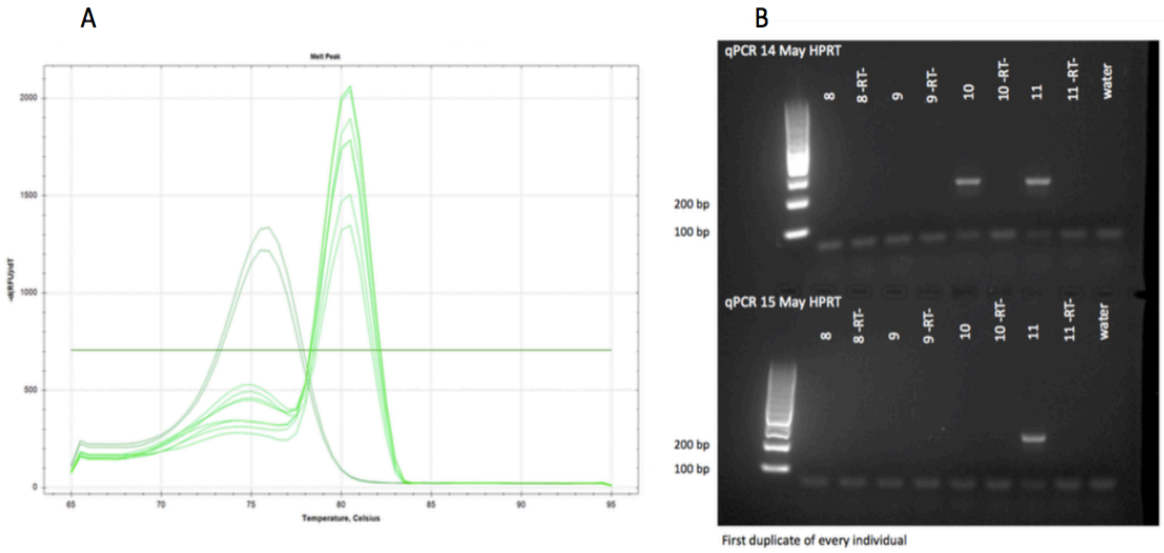

Figure 2. Melt-curve and 2.5% agarose gel analysis of the HPRT reaction products obtained from the preliminary qPCR assay. **A**, the melt curve analysis showing two peaks with melting temperatures at 75.5°C and 80.5°C. **B**, Agarose gel analysis of the amplification products including both plus-RT, minus-RT and NTC controls for each of the individual samples. Bands under 100 bp corresponds to the first peak and bands over 200 bp corresponds to the second peak in the melting curve (A).

To adjust the template amount necessary to give an appropriate Cq-value for the clinical application a 4-fold dilution series of cDNA from individual 11 were prepared to use for amplification reactions, including primer pairs for the *SRGN* and *EEF2* genes. To estimate the qPCR assays efficiency, regression curves were plotted for both genes as mean Cq-values against the log (1/dilution), (Figures 3 and 4). The R^2 values were then calculated, and the slope of the linear regression line was used to determine the amplification efficiency, E, for each of the genes ($E=10^{-1/slope}$). In addition, the percentage ampli-

fication efficiency was calculated for both genes using the following formula: %Efficiency = ($10^{-1/slope}$ -1) x 100 %.

The plotted regression curve showed that both the *SRGN* and *EEF2* genes had a R^2>0.980, a reflection of linearity.

Notably, the 1:64 dilution points did fit perfectly into the linear regression line between two dilution points. An accurate %E value should lie between 90%-105%, this if the qPCR assay should be considered as robust and reproducible. When looking at the data, both the *SRGN* and *EFF2* had values within this range (see table 5). At the end of each cycle, *SRGN* showed 2.03-fold increase while *EFF2* showed 2.04-fold increase of amplicon copy number.

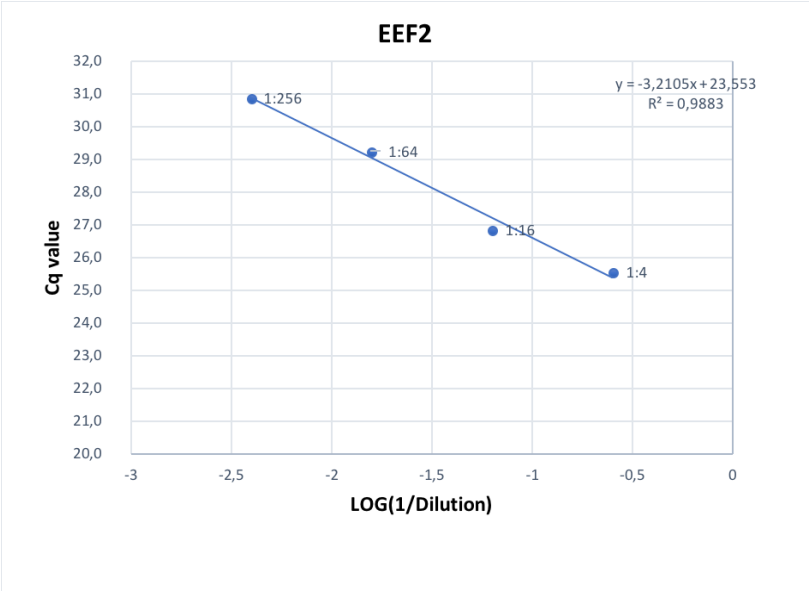

Figure 3. The plotted regression curve as, mean Cq-value vs log(1/dilution) for the EEF2 gene. Generated from the second qPCR assay with a 1:4 serial dilution of cDNA template from sample individual 11. The calculated equation for the regression line and R^2 value is shown.

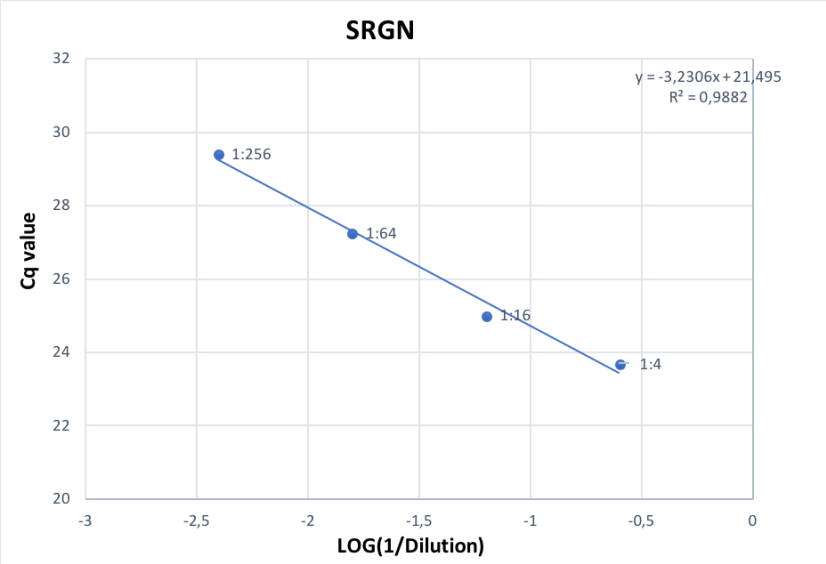

Figure 4. The plotted regression curve as, mean Cq-value vs log(1/dilution) for the SRGN gene. Generated from the second qPCR assay with a 1:4 serial dilution of cDNA template from sample individual 11. The calculated equation for the regression line and R^2 value is shown.

Table 5. *Calculated amplification efficiencies values (E) for both the SRGN and EEF2 genes*

GENES	$E=10^{-1/SLOPE}$	%E= $(10^{-1/slope} -1) \times 100$ %.
EFF2	2.04	104%
SRGN	2.03	103%

The qPCR assay was further optimized for annealing temperatures and concentration for each primer pair both for *SRGN* and *EEF2*. For this purpose, a range of annealing temperatures (5°C range) based on the primer pairs T_m values were selected together with three different primer concentrations: 200 nM, 300 nM and 400 nM. An annealing at 62.7 °C with a primer concentration of 300 nM gave

the lowest Cq value (28.78) for the *EFF2* gene, while for the *SRGN* gene, the lowest Cq-value (27.36) was obtained at 62.2 °C with a primer concentration of 400 nM, (see Figure 5). Notably, for both *SRGN* and *EEF2* no significant difference between Cq-values was observed using this temperature range and primer concentration, see Figure 6 and 7. However, the Cq-values for *SRGN* increased a little with a decreasing annealing temperature and the 400 nM primer concentration for *EEF2* had a tendency to be least effective. Therefore, the optimal annealing temperature and primer concentration selected for further assays was 62 °C with a 300 nM primer concentration for both genes. Moreover, a melt-curve analysis was analyzed to exclude potential co-amplification of nonspecific product or primer dimer formation. Figure 6 shows two separate single peaks, one with T_m at 81.0 °C corresponding to *SRGN* and one with T_m at 84.5 °C corresponding to *EEF2*, thus revealing that only the specific products had been successfully amplified.

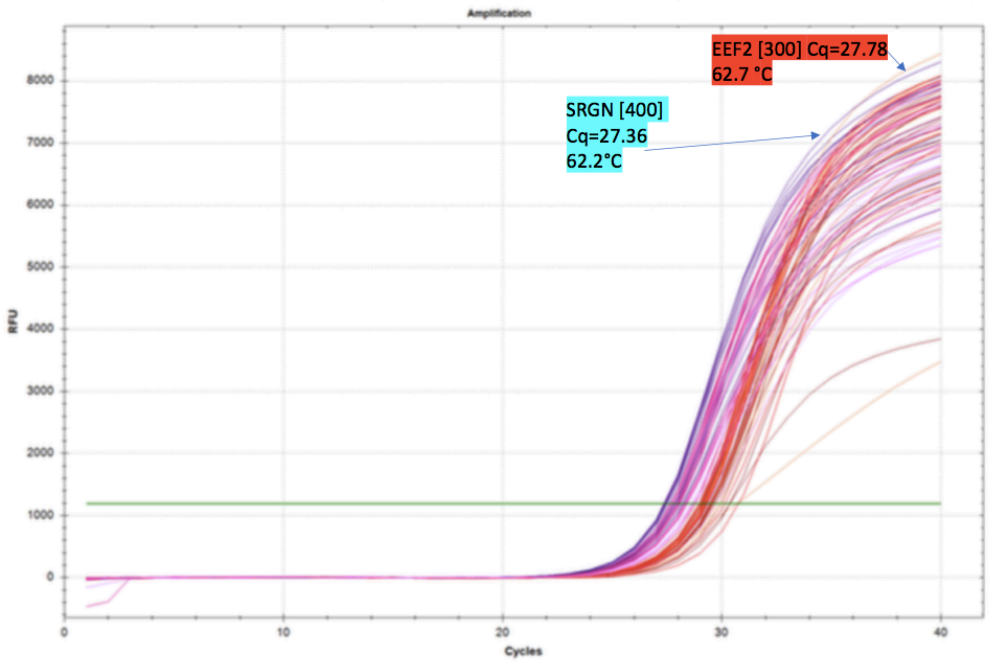

Figure 5. Annealing temperature optimization, showing an annealing temperature gradient from 58 °C to 62.7 °C performed with SRGN and EEF2 primer pairs at three different concentrations. *EEF2* gave the lowest Cq value at 62.7 °C with a 300 nM primer concentration and *SRGN* had the lowest Cq-value at 62.2 °C with a 400 nM primer concentration.

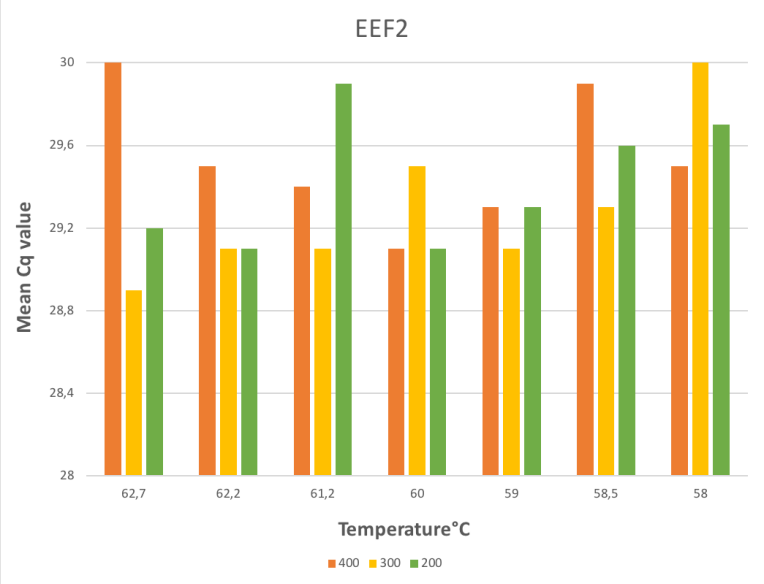

Figure 6 .Mean Cq-values for *EEF2* at different temperatures and primer concentrations.

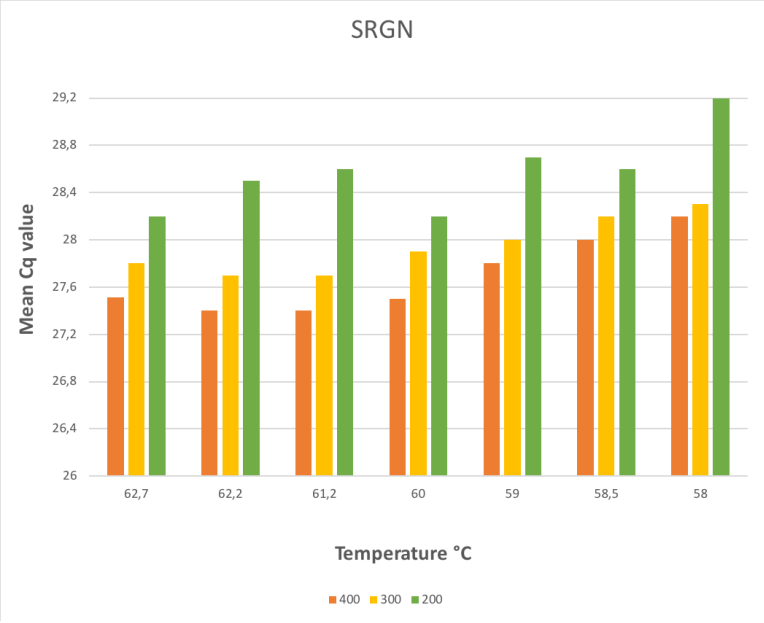

Figure 7. Mean Cq-values for *SRGN* at different temperatures and primer concentrations.

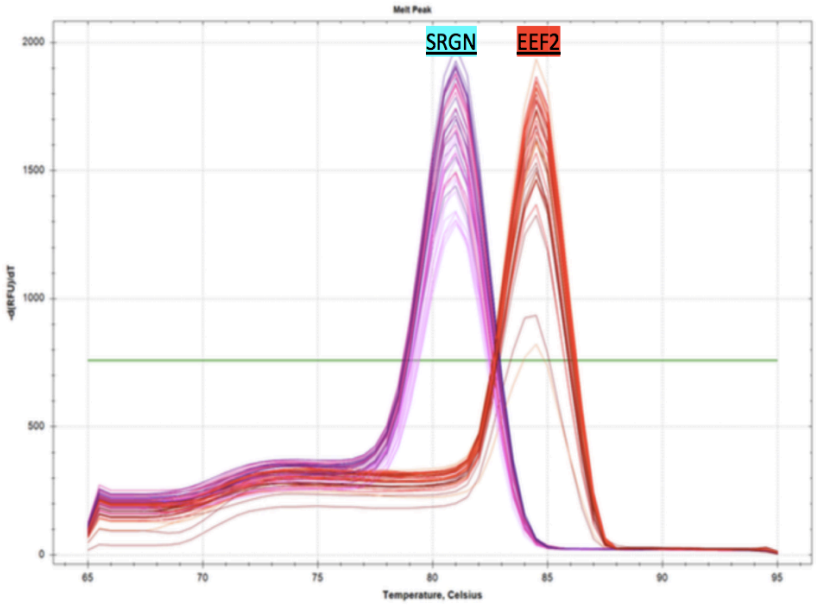

Figure 8. Melt-curve analysis, showing two separate melting peaks at 81.0 °C and 84.5 °C. The first peak represents the *SRGN* gene while the second peak represents the *EFF2* genes.

4 Discussion

Canine cancer is one of the most common causes of death in certain dog breeds (Pawłowski et al., 2013). The high mortality rates of malignant tumours have been correlated with poor diagnostic predictions and inefficient treatment strategies (Manuali et al., 2012). The possibility of using serglycin as potential biomarker for detection of canine cancer was therefore evaluated, with the aim to develop a functional qPCR assay that in the future could detect serglycin expression levels in a small sample volume of blood from canine cancer patients. The focus of this study has been to test important parameters for a functional qPCR assay, *e.g.* the RNA extraction method, primer design, concentration of cDNA and primers, and optimal annealing temperatures.

To extract RNA from small volumes of canine blood samples a modified TRIzol method was established. This method proved to be a more efficient method for canine blood RNA extraction than the original method. The result shows that with our optimized protocol we could increase the concentration of total RNA yield, and that 100 μl of canine blood is enough to isolate a sufficient amount of RNA. Although, RNA could be successfully extracted from most of the in-

dividual samples, the 260/230 and 260/280 ratios were not optimal suggesting contamination of phenolic compounds or DNA. Therefore, DNase treatment is recommended after the RNA extraction to avoid contamination that might affect the interpretation of results. For this reason, a DNase treatment step has been included in our standard protocol. Furthermore, more sensitive methods are available for checking the RNA integrity *e.g.* lab-on-chip technologies like Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the Experion (Bio-Rad Laboratories, USA)(Fleige and Pfaffl, 2006). These technologies have proven to be stable and less sensitive against contamination, providing better information about the RNA integrity than a NanoDrop measurement. This kind of method could, therefore, be included as an extra RNA quality check for more accurate values.

Two of the four primer pairs tested in the qPCR assay showed sufficiently good efficiency to obtain validated results, SRGN and EFF2. Notably, two of the reference genes, *ACT B* and *HPRT*, proved to be poorly designed. When the *ACT B* and *HPRT* genes were further analyzed, several pseudogenes for each gene and multiple hits for several species for each primer pair were found.

A reference gene should not have pseudogenes and all primers should be designed so that they are separated by an intron, to avoid genomic DNA amplification.

However, despite the fact that both primer pairs for each gene were designed so they were separated by introns and that a DNase treatment step was included as an extra precaution, it could not be excluded that amplification of genomic DNA for both the *ACT B* and

HPRT genes had occurred. Since the primer pairs for both genes gave multiple hits for several species with no observed mismatches, the risk for non-specific target amplification was increased, affecting the data analysis. There are some important aspects that separate our results from other studies performed with *HPRT* and *ACT B* as reference genes. Bulkowska and co-authors implied that both primer pairs for the *ACT B* gene and the *HPRT* gene should serve as good controls for validation of genes involved in metastatic canine mammary cancer (Bulkowska et al., 2017). Their study does not include any validation data for these primer pairs and therefore, is not possible to exclude genomic DNA amplification. However, the use of non-validated reference genes for normalization purposes is a known problem but seldom taken into consideration (Ohl et al., 2005). Such disregard can lead to highly misleading effects, which in turn will affect the credibility of the end results. Therefore, our result suggests that the *ACT B* and *HPRT* primer pairs proposed in Bulkowska et al. (2017), are not good reference genes for validating canine gene expression studies. Instead, new primers with improved target specificity should be tested. Because mismatches at the 3end have shown to have a higher likelihood to prevent unwanted amplification than mismatches at the 5end (Ye et al., 2012), primer pairs whose sequences contain three or more mismatches at the 3end are more recommended. Furthermore, the experimental set up should always include a quality control for each primer pair used. The control should exclude potential genomic DNA amplification, as well as prove that the amplicons are within the expected size range. Alternatively, the amplicons could be sequenced to verify that the correct cDNA sequences are amplified. Inclusion of a melting curve analysis at the end of qPCR assay makes it possible to confirm that the pri-

mer pairs only amplified a single product. The right amplicon size can further be confirmed by running the qPCR amplification products on an agarose gel (Gene-quantification, 2018).

In contrast, when performing a BLAST analysis with the primer pairs for reference gene *EEF2* it did not give multiple hits for several species, only canine breeds, and the melting curve analysis confirmed that the primer pair for *EEF2* had only amplified a single product. This result together with an accurate %E value (104%) allowed us to conclude that, out of the three reference genes tested, the *EEF2* gene is the best suited to use as reference gene for gene expression studies in canine blood.

The primer pairs for the *EEF2* and *SRGN* genes performed well under the described qPCR reaction conditions as showed by the E values (within the range of 90%-105%, see table 6), and therefore indicating a robust, reproducible qPCR assay.
Because both E values obtained were >100% some pipetting error in the serial dilutions may have occurred which is known to have a big impact in the outcome. For more conclusive result new dilution series should be constructed.

In addition, both the *SRGN* and *EEF2* genes showed R^2 values that were higher than 0.980. These results showed that the amplification efficiency is the same for the different concentrations of starting cDNA template and that no significant differences between the Cq-values between replicates can be detected. Knowing that the experimental data fits the regression line, the assay can be considered accurate and reproducible for our samples. In addition, the cDNA

with a 1:64 dilution gave a good signal in the qPCR assay. This relatively high cDNA dilution suggests that a rather low limit of detection can be assumed for this assay.

Furthermore, the annealing temperature that seemed most advantageous for both primer pairs was 62°C with a primer concentration of 300 nM. By choosing a higher annealing temperature for both primer pairs than the calculated (55°C/56°C *SRGN* and 58°C /58°C *EFF2*) specificity can be improved, and primer dimer formation be further avoided. However, no significant differences between Cq values could be observed among the different annealing temperatures and primer concentrations. This result may have been affected by the relatively narrow temperature range (5°C) in the annealing gradient. Because of the limitation of time for this study, it was not possible to repeat the gradient approach with higher temperature differences (*i.e.* in the range of 10°C), but continuation of the optimization regarding this point is recommended for a more accurate result.

In conclusion, the study demonstrated a functional method that can be applied for detection of serglycin expression in the blood of canine patients. Our results showed that a relatively low amount of blood (around 100 μl) from the canine patients is enough to get a reliable result on the expression level of serglycin when utilizing qPCR. We validated the use of one stable reference gene *(EEF2)* and predict that this reference gene can be useful for canine blood expression studies. However, to apply the method in further studies, more suitable reference genes need to be tested for the normalization purpose. We therefore concluded that validation of all reference

genes should be included as an important step of the experimental set up.

As a continuation of this study, and with the purpose of comparing the serglycin expression levels between healthy and cancer patients, it would be interesting to apply the optimized qPCR assay presented here on canine cancer patients with a known history of malignant tumors. Due to the limitation of time for this project, no tumor tissue or blood from cancer patients were obtained. However, the designed method and results in this study will be important for further research on this subject.

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Appendix 1

Protocol RNA extraction

Revised 2018-05-16 Experiment: Assay date:

This protocol is a modification of the TRIzol™ Reagent (Invitrogen, Life Technologies) manufactures protocol for total RNA from canine blood sample.

Reagents
Invitrogen TRIzol™ Reagent
Chloroform
Isopropanol
75% ethanol
RNAse free water

In the original TRIzol™ protocol prescription of RNA is performed by adding 0.5 mL of isopropanol to the aqueous phase, per 1 mL of Trizol and the incubation time proposed is 10 min. We changed the volume of isopropanol to the same volume as the aqueous phase obtained from the chloroform extract, and the incubation time after addition of isopropanol to 30 min. This to obtain a better precipitation and higher yield of RNA.

All individual samples should be prepared in duplicates, to obtain back up RNA samples.

species: Canine (Dog).

Table 1, Regnant calculations using excel to obtain the correct volumes.

Animal Id	*sample Nr*	*blood volume µl*	*volume of Trizol µl*	*volume of Chloroform µl*	*volume of Isopropanol µl*	*volume of 75 % ethanol µl*
	1	100	500	100	volume of aqueous phase obtained from the chloroform extract.	500
	2	100	500	100	volume of aqueous phase obtained from the chloroform extract.	500
	3	100	500	100	volume of aqueous phase obtained from the chloroform extract.	500

Procedure
Always work in sterile hoods and use sterile pipettes/tips/tubes etc.

Start centrifuged

In sterile hood

1. Mark x tubes with sample Nr from above and, then add transfer the proper amount of blood into the clean tubes.

2. Add x µl TRIzol™ Reagent to each Eppendorf tube (volume according to the table above).

3. Vortex the tubes vigorously, 2 min, and incubate at room temperature, 15 min

4. To each Trizol treated tube add x µl of chloroform (volume according to the table above), vortex and incubate at room temperature for 5 min.

5. Centrifuge the sample for 15 min at 12,000 x g at 4 °C.

6. Mark x tubes with sample Nr and RNA, then transfer the colorless aqueous phase containing the RNA into the new tubes*

7. Add the same volume of isopropanol as the aqueous phase obtained from the chloroform extract, and incubate at room temperature, 30 min. (Around 300-500 µl)

8. Centrifuge the sample for 10 min at 12,000 x g at 4 °C.

9. Discard the supernatant with micropipettes, avoid the RNA pellet (a white gel-like pellet at the bottom of the tube).

10. Wash the RNA pellet, add x µl 75% ethanol (volume according to the table above).

11. Vortex briefly the sample to resuspend the RNA pellet, then centrifuge for 5 min at 7500 x g at 4 °C.

12. Discard the supernatant with micropipettes.

13. Air dry the RNA pellet for 5-10 min, and then resuspend the pellet by adding 30 µl of RNAse free water*

    Stored at -80 °C until further use.

*Important to avoid the interphase, therefore do not pipette to close.
*If the RNA pellet does not dissolve properly, vortex until it's have become fully dissolved in the water.