DEVELOPMENT OF A NOVEL DIAGNOSTIC TEST USING PODOCYTURIA AS A BIOMARKER FOR DETECTION OF KIDNEY DAMAGE

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

Page

ABSTRACT

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Primary glomerular diseases are the most common cause of progressive chronic kidney disease (CKD) in dogs, a common and significant source of illness that often progresses to kidney failure. It is hypothesized that podocyte cells in the glomerulus are washed away in urine as a result of glomerular damage. Using markers such as podocyte gene products, more sensitive and specific detection of damage to the glomeruli is possible, allowing for earlier treatment of CKD that could potentially prolong the life of dogs. The purpose of this study was to develop novel non-invasive markers of CKD, specifically podocyturia, and optimize primer and probe sets that could be used on urine sediment to detect podocyte gene products effectively and efficiently. Primer-probe sets were developed for both reference genes and podocyte gene products. Primer concentrations were optimized and both reference genes and podocyte genes amplified efficiently under optimal concentrations in kidney tissue.

With additional testing using urine sediment, analysis and statistical correlation between podocyte loss and glomerular damage may be able to provide insight into the pathogenesis and treatment of CKD in dogs.

CHAPTER I

INTRODUCTION

Primary glomerular diseases are a leading cause of chronic kidney disease (CKD) in both humans and animals. These disorders are characterized by abnormal structure and function of the glomerulus, a key compartment of the functional unit of the kidney, the nephron. Blood that travels through the kidney is filtered at the glomerulus. The rate of this filtration, and thus measure of overall kidney function, is the glomerular filtration rate (GFR).

Damage to the glomerulus therefore is a good measure of kidney dysfunction (Bostom et al, 2002). Such damage invariably leads to protein loss, known as proteinuria, and might possibly result in cell sediment shedding. Intermediate to high molecular weight proteins such as albumin, if found in urine and not caused by pre- or post-renal factors, are indicative of kidney damage. Additionally, special glomerular cells, known as podocytes, are also thought to be markers of glomerular damage if found in urine (Petermann and Floege, 2007). Podocytes overlie the glomerular basement membrane (GBM), and along with the GBM and endothelium, the podocyte layer comprises one of the three main components of the glomerular filtration barrier.

While evidence of kidney damage is common, progressive CKD is not recognized as frequently due to the insidious nature of the disease and the lack of sensitive and specific non-invasive diagnostic tests (Petermann and Floege, 2007). Although the magnitude of proteinuria has prognostic significance in dogs, proteinuria can be indicative of either ongoing or previous injury (Jacob et. al., 2005). Podocyturia, on the other hand, is thought to indicate active glomerular

injury, and it might therefore determine whether treatment for glomerular disease is effective and whether the disease is likely to progress (Schmid et. al., 2003). Therefore, measuring cell content in urine can indicate active glomerular injury, and may possibly provide a novel, non-invasive test for determining progression of CKD in dogs.

The main objective of this study was to develop a novel, non-invasive method to detect podocyte injury in dogs. In particular, we designed primers and probes to amplify RNA isolated from urine sediment in for the detection of podocyturia. These biomarkers will eventually be evaluated for their reliability in detecting glomerular injury and determining CKD progression. In addition to these experimental tests, we conducted surveys which were sent to the patients' owner or attending clinician, in order to assess the long-term outcome of each dog and determine the clinical value of both novel and conventional tests of kidney function and damage.

The central hypothesis is that detection of podocyte gene expression profiles in urine will improve detection of glomerular injury and will correlate better with the severity of glomerular damage and prognosis in dogs with CKD than traditional diagnostic tests such as proteinuria. In order to develop a test for podocyturia, we had two main objectives:

1) Determine which reference genes to use as a baseline for accurately measuring podocyturia

- Design sequences for primer-probe sets.
- Verify specific amplification of desired gene using RT-PCR.
- Optimize concentrations of primer-probe sets.

- Perform quantitative RT-PCR (qRT-PCR) on mRNA isolated from banked kidney tissue and a variety of urine sediments from dogs.

2) Develop canine-specific primer-probe sets to be used in detecting podocyte gene products (podocin, nephrin, and actinin-4).

- Similarly design, verify, optimize, and perform qRT-PCR on isolated mRNA to detect podocyte gene products.

Once all primer-probes are optimized and demonstrate amplification using kidney tissue and urine sediment, the correlation of podocyturia and glomerular damage will be performed to determine its diagnostic value in comparison to traditional methods such as the urine protein:creatinine ratio (UPC).

CHAPTER II

METHODS

Collection of kidney tissue and urine sediment

Banked kidney tissue was previously obtained post-euthanasia from dogs affected with X-linked hereditary nephropathy (XLHN) after they reached end-stage CKD. Tissue was cut into 0.5-1 cm fragments, placed in RNAlater, and stored at either 4^{o}C or -80^{o}C until processing. Voided urine samples (~5-10 ml) were also collected from dogs with XLHN and centrifuged at 500xg for 10 min, allowing any cell sediment to form a pellet at the bottom of the tube. Urine supernatant was carefully removed, to avoid disturbing the sediment pellet. Next, the sediment was resuspended in 1 ml RNAlater to stabilize the RNA, and the samples were either stored at either 4^{o}C or -80^{o}C until RNA isolation.

Isolation of RNA from kidney tissue and urine sediment and conversion to cDNA

For urine sediment, we used TRI Zol (Sigma TRI Reagent) to isolate RNA from urine sediment, using two chloroform separation steps to increase RNA quality as described below. First, the urine sediment suspended in RNAlater was centrifuged to pellet any cellular material. The supernatant was removed and the sediment was lysed with 750 ulTRI Reagent by repeated pipetting. Samples were incubated for 5 minutes at room temperature and then 150 ul of chloroform was added. The sample was covered tightly, shaken vigorously for 15 seconds, and incubated for 2–3 minutes at room temperature. The resulting mixture was centrifuged at 12,000 x´ g for 15 minutes at 4 °C. Centrifugation separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase

(containing RNA). Using the aqueous phase, the chloroform step was repeated once. Next, the aqueous phase was transferred to a fresh tube followed b addition of 400 ul isopropanol. The sample was incubated for 10 minutes at room temperature then centrifuged at 12,000 x´ g for 10 minutes at 4 °C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed by adding 750ul of 75% ethanol. The sample was vortexed briefly and then centrifuged at 7,500 x g for 5 minutes at 4°C. Lastly, the RNA pellet was air dried for 5–10 minutes, taking care not to let the RNA pellet dry completely. The RNA pellet was resuspended in 10ul RNase-free water. To facilitate dissolution, repeated pipetting with a micropipette at 55–60 °C for 10–15 minutes was performed. RNA isolated from kidney tissue and urine sediment was reverse transcribed into cDNA using Quantitect 's Reverse Transcription Kit. RNA samples and kit contents were bought to room temperature. To eliminate genomic DNA, 4ul gDNA wipeout buffer and 23ul DNase-free water were added to each 1ul of RNA. Samples were incubated for 2 minutes at 42°C and placed on ice. RNA then was reverse transcribed into cDNA by adding 2ul RT enzyme, 2ul of a primer mix, and 8ul RT 5x buffer to the samples and incubated for 15 minutes at 42°C. The RT enzyme was inactivated by heating the samples for 3 minutes at 95°C. RNA and cDNA was stored at -20°C.

Primer-probe design and verification: reference genes & podocyte genes

Design of primers and probes

In order to more accurately quantify the presence of podocyturia among samples, appropriate reference genes were needed that showed stable, non-fluctuating levels in both normal and abnormal kidney tissue and urine sediment in dogs. Tested genes included Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 40S ribosomal protein S18 (RPS-18), ribosomal protein

L32 (RPL-32), beta actin (ACTB) and glucuronidase beta (GUSB-1). Primers and probes were either proprietary (ordered from a company, e.g., RPS18, RPL32), obtained from the literature (e.g, GAPDH, GUSB), or designed using Primer3 software (e.g ACTB.). Canine-specific primers for podocyte gene products, (nephrin, podocin, and actinin-4) were obtained from previously published sequences (Kobayashi 2011) or were designed using Primer3software. All probes were designed using Primer3.

Table A1. Gene Sequences for Primers and Probes

Ref. Gene	Forward	Reverse	Probe
GUSB-1	GGTGCGTCGGGA-TAAGAAT	CCCAGGTCTGCTTCATAGTTG	6FAM-CACTTCCTTCCTGAAGCCTG-BHQ
GAPDH	CGGATTTGGCCG-TATTGG	GACAATATCCACTTTGCCAGA-GTTAA	6FAM-CGCCTGGTCACCAGGGC-BHQ
RPS-18	Proprietary	Proprietary	Proprietary
RPL-32	Proprietary	Proprietary	Proprietary
B-actin	CCAAAAGCACTCT-TCCAACC	GCACTGTGTTGGCATACAGG	
Pod. Gene	Forward	Reverse	Probe
Nephrin	GGTGCGTCGGGA-TAAGAAT	CCCAGGTCTGCTTCATAGTTG	6FAM-CACTTCCTTCCTGAAGCCTG-BHQ
Podocin	CGGATTTGGCCG-TATTGG	GACAATATCCACTTTGCCAGA-GTTAA	6FAM-CGCCTGGTCACCAGGGC-BHQ
Actinin-4	CAATGCACTGATC-CACCGG	TTCACGATGTCCTCCGCA	6FAM-CACAGACCAGAGCTGATCGA-BHQ

Verification of primer specificity

Primer sets were validated by performing simple PCR reactions using cDNA from canine kidney tissue, visualizing the PCR products on a 1.5% agarose gel, and then sequencing the PCR products. BLAST online software was used to confirm that the product matched similar published gene sequences for the target gene in the NCBI database. PCR reactions were performed using BioRad iCycler IQ. A PCR reaction mix was prepared as outlined in Table B1,

preparing enough volume to run at least two replicates of each sample. All components were combined (on ice) except the sample cDNA. This master mix then was aliquoted into different tubes, followed by addition of the appropriate cDNA type and volume to each. Tubes were briefly centrifuged to ensure homogenization. The following thermal cycling conditions were used: polymerase activation and DNA denaturation for 2 min at 95oC once, then 40 cycles of denaturing for 15 sec at 95oC and annealing/extension for 30sec at 60oC.

Table B1. Simple PCR Reaction Mix

Reaction Component	Volume (ul) per Sample
SsoFast Probes Supermix (2X)	10.0
Primer-probe mix	1.0
cDNA sample	1.0
Water	8.0
Total	20.0

A 1% agarose gel was used to detect the amplified PCR product. First, the appropriate amount of agarose was calculated and measured. For example, for a small, 8-well gel, 0.5 g of agarose was added to 50ml of 1x TAE buffer, whereas for a 15 or 20-well gel, 1.0 g of agarose was added to 100ml of 1x TAE buffer. The dry agarose was added to a 250ml flask followed by the appropriate volume of TAE, swirling to mix. The solution was microwaved in short increments and mixed frequently, taking care not to let the solution bubble over. The agarose dissolved completely, resulting in a transparent solution. Next, 2.5ul GelRed nucleic acid stain was added to the flask and immediately mixed to avoid clumping. The mixture was cooled to lukewarm (~15 minutes) then poured into the gel apparatus, pushing any air bubbles to the side with a fine tip. Once the gel solidified (~30 min to 1 hour), well were loaded with 10ul of a PCR reaction

mixed with 1ul of 6x loading buffer. A 1kb+ ladder (5ul) was used to verify size of the PCR product. Gels were run at 90 V for 30 minutes, or until the dye front reached the end of the gel.

Reverse transcriptase polymerase chain reaction (rt-pcr) and primer-probe optimization

Both the reference genes and podocyte gene primer and probe sets were tested on cDNA from canine kidney cortex obtained from dogs with CKD due to XLHN to verify their performance using a two-step qRT-PCR reaction. In step one, RNA isolated from urine sediment was reverse transcribed into cDNA using Quantitect's Reverse Transcription Kit as described above. In step two, we analyzed gene expression using a BioRad CFX96 real-time PCR detection system. We prepared 20uL samples using the PCR reaction mix described in Table C1. We used the following PCR cycling protocol: initial denaturation 95oC for 2 min; and 40 cycles of denaturation 95oC for 15 sec, annealing/ extension 55oC for 30 sec.

Once amplification and detection were confirmed, primer concentrations were optimized to obtain the most efficient results. A PCR reaction mix was prepared (shown in Table C1) to run four replicates of each of the nine conditions shown in Table D1. A 96-well PCR plate was loaded similar to the layout of Table D1, with four replicates for each sample. Wells were sealed with transparent film and the plate was centrifuged at 1200 xg for 5 min. The plate was placed in the qRT-PCR machine, and the thermal cycling conditions given above were followed.

Probe concentration also was optimized for the reference genes following the same PCR reaction and thermal cycling steps above, with four replicates each of 50nM, 150nM, and 250nM concentrations using the optimal primer concentrations.

Table C1. PCR Reaction Mix	
Reaction Component	Volume (ul) per Sample
SsoFast Probes Supermix (2X)	10.0
Primer-probe mix	1.0
cDNA sample	1.0
Water	8.0
Total	20.0

Table D1. Optimization of Primers and Probes

	Forward Primers		
Reverse Primers	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

CHAPTER III

RESULTS

Reference gene optimization

Results from PCR testing showed successful amplification of reference genes in kidney tissue for GUSB-1 (Ct.=30.0), GAPDH (Ct.=25.0), and RPS-18 (Ct=21.0). Two other reference genes, RPL-32 and beta-actin, amplified but had higher Ct. values, (34 and 32 respectively). These primer-probe sets appear to be specifically amplifying the desired gene product based on amplification of RNA isolated from kidney tissue.

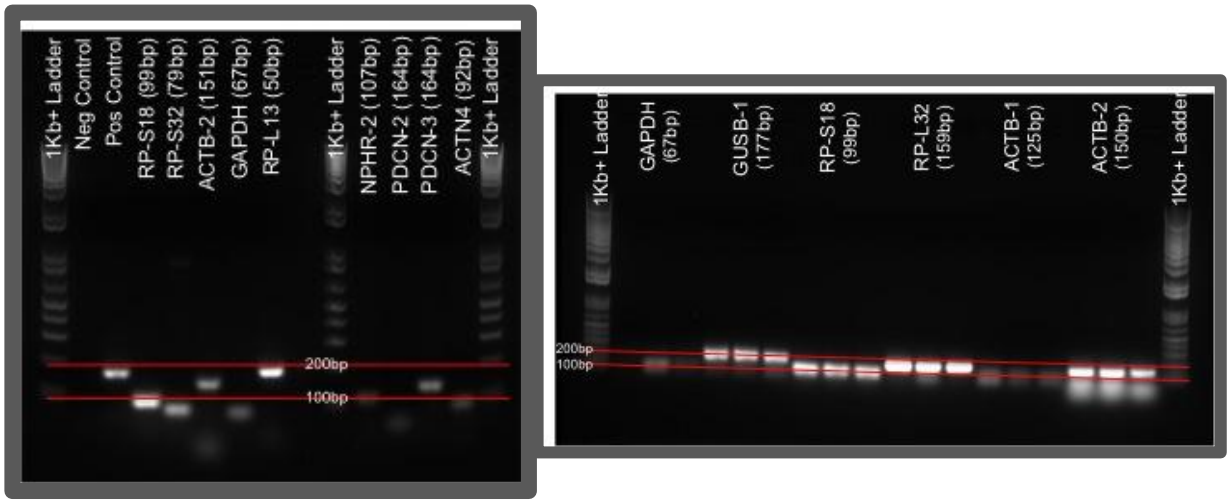

Fig. 1. 1.5% Agarose Gel of Reference and Podocyte Gene PCR Products

Optimal probe concentration for all reference genes was 50nM/ul. Optimal concentrations for primers were available for GAPDH and GUSB-1: forward = 50nM/uL, reverse = 900nM/uL (GAPDH) and forward = 300nM/uL, reverse = 900nM/uL (GUSB-1).

Podocyte primer optimization

After using different sequence variations for nephrin, podocin, and actinin-4 primers, nephrin and actinin-4 showed successful amplification in kidney tissue, with Ct values of 33 and 29, respectively. Optimal primer concentrations for nephrin were 900nM/uL forward and 300nM/uL reverse. Optimal primer concentrations for actinin-4 were 50nM/uL forward and 300nM/uL reverse. Further testing is still in progress to determine whether amounts of these gene products fluctuate with progression of CKD.

CHAPTER IV

CONCLUSIONS AND ANALYSIS

Based on our findings, our hypothesis still holds true for the potential of using podocyte gene products found in urine as valuable markers in the diagnosis of Chronic Kidney Disease. RNA was successfully isolated from urine sediment and appropriate primer-probe sets were designed for multiple reference genes and podocyte-specific genes. These primer-probe sets were also optimized for qPCR with regard to primer-probe concentrations, and successful amplifications of targeted gene products show promising applications of these primer-probe sets. This sets a good foundation for future work in evaluating the usefulness of podocyturia in the detection and monitoring of CKD in dogs, and continued testing of these primers-probes on urine sediment may be able to provide a better scope and analysis of this novel diagnostic test.

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