SEQUENCE AND TISSUE EXPRESSION OF THE ABCB4 (MDR3) GENE IN THE CANINE

By
ERICK SPENCER

A thesis submitted in partial fulfillment of the requirements for the Degree

MASTER OF SCIENCE IN VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

August 2008
To the Faculty of Washington State University:

   The members of the Committee appointed to examine the thesis of ERICK SPENCER find it satisfactory and recommend that it be accepted.

________________________________
Chair

________________________________

________________________________
AKNOWLEDGEMENTS

I would like to extend my sincerest thanks and appreciation for all of the guidance, mentorship, and encouragement provided by Dr. Katrina Mealey throughout the last several years. I would also like to thank Steve Bentjen for his guidance and instruction in the laboratory. Lastly, I would like to thank the members of my committee, Doctors Kathryn Meurs and Boel Fransson, for their support and guidance.

In addition, I would like to acknowledge Dr. Gary Halderson, Kevin Lahmers, and David Spranklin for their help in obtaining the tissues used in the experiments.
Abstract

By Erick Spencer, MS
Washington State University
August 2008

Chair: Katrina L. Mealey

The ABCB gene subfamily of ABC transporters transports ligands across biological membranes. ABCB4 functions primarily on the apical membrane of hepatocytes to translocate lipids. ABCB4 translocates the phospholipid phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane in people. Functional alterations in the ABCB4 transporter have been associated with a number of cholestatic diseases in people. Furthermore, Abcb4 (-/-) knockout mice have also been demonstrated to develop hepatic and biliary injury.

Approximately 97% of the canine ABCB4 was sequenced and its tissue distribution was determined. There was approximately 99% similarity and identity between ABCB4 consensus sequence reported here and the reported canine sequence with most of the variability occurring within exons 26 and 27. The sequenced ABCB4 gene shared a splicing variant with human ABCB4 variant A. Canine ABCB4 was abundantly expressed in the liver, sparsely expressed in the brain, and not expressed in the duodenum. The plentiful expression of ABCB4 in canine hepatic tissue supports a potential role for the protein in normal biliary function similar to that in people. Tissue distribution, along with characterization, of the ABCB4 gene in the canine
will provide the foundation for future investigation into the physiologic function of *ABCB4* in the dog. As with people, it is likely that mutations in *ABCB4* are responsible for, or associated with, cholestatic disease in dogs.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER ONE. SEQUENCE AND TISSUE EXPRESSION OF THE ABCB4 (MDR3) GENE IN THE CANINE</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>5</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>8</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>9</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>13</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Primers for canine mdr3.................................................................19
LIST OF FIGURES

Figure 1. …………………………………………………………………………………………………………………………………………………20

The predicted amino acid sequence generated by the nucleic acid sequence in exons 26 and 27 of canine ABCB4 and human ABCB4. (A) The predicted amino acid sequence generated by the nucleic acid sequence of exon 26 revealing the seven amino acid splicing variant of the consensus sequence from this study and the human variant A sequence. (B) The ten amino acid variability between the sequence from the Boxer dog and our predicted consensus sequence and the human variant A sequence.

Figure 2. …………………………………………………………………………………………………………………………………………………22

Electrophoresis of PCR products from ABCB4 generated with consensus primers.
INTRODUCTION

The research reported in this thesis was conducted with and under the guidance of the graduate committee members as part of the Small Animal Medicine Residency of the Department of Veterinary Clinical Sciences at Washington State University College of Veterinary Medicine. This research has generated one manuscript (Chapter 1) which will be submitted for publication in the journal *Mammalian Genome*. The format of this chapter is as required by that journal.
CHAPTER ONE

Sequencing and tissue expression of the ABCB4 (MDR3) gene in the canine

Erick S Spencer, DVM
Steve Bentjen,
Kevin K Lahmers, DVM, PhD, Dipl. ACVP
Gary J Halderson, DVM, PhD, Dipl. ACVP
Katrina L Mealey, DVM, PhD, Dipl. ACVIM, Dipl. ACVCP

From the Department of Veterinary Clinical Sciences, College of Veterinary Medicine,
Washington State University, Pullman Washington 99164-6610
The ATP binding cassette (ABC) gene family has been identified in all cellular organisms, from prokaryotes to mammals (Dean 2001; Linton 2007). The ABC genes encode transport proteins that are thought to have evolved as complex cellular defense systems that function in the energy-dependent transport of a variety of potentially toxic molecules across the plasma membrane (Sarkadi et al. 2006). Additionally, several aspects of cell physiology such as cell signaling, removal of waste products, nutrient uptake, and energy production rely on ABC proteins for the transport of ligands across biological membranes. These transporter proteins are particularly important in mammals from a medical and pharmacological perspective for multiple reasons. Dysfunction of ABC transporter proteins has been shown to be associated with several genetic disorders in people, including anemia, cystic fibrosis, retinal disease, and hepatic disease.

Furthermore, ABC transporters have the ability to extrude xenobiotics such as chemotherapeutic drugs, antibiotics, hormones, and a variety of other drugs from cells dramatically affecting both efficacy and safety of these agents.

A minimum of four domains are required for an ABC transporter to achieve export. A pair of nucleotide binding domains (NBD) bind ATP whereas the ligand binding sites are provided by two sets of transmembrane domains (TMD) that also provide the specificity. The organization of the domains allows the ABC transporter proteins to be divided into seven distinct subfamilies, A through G.

The ABCB subfamily in humans is composed of eleven transporters, seven of which are half transporters. Half transporters contain only a single TMD and NBD whereas full transporters
contain two NBD and two TMD domains. The ABCB subfamily contains the multidrug resistance (MDR) genes, including arguably the most studied ABC transporter in mammals, the ABCB1 (MDR 1) gene. A 4 base-pair deletion of the canine *ABCB1* gene has been identified in several dog breeds. This polymorphism renders affected dogs extremely sensitive to the toxic effects of several pharmaceutical agents, illustrating the importance of this gene family in dogs. Little is known about other members of the ABCB gene family in dogs, but recent literature involving humans and mice has shed new light on the role of ABCB4.

Human ABCB4 is a full transporter that functions principally on the canalicular (apical) membrane of hepatocytes to translocate lipids (Smit et al. 1994). The phospholipid phosphatidylcholine (PC) is transported from the inner to the outer leaflet of the canalicular membrane by ABCB4 making it available for sequestration of bile salts in mixed micelles. Biliary phospholipids act as carriers and solvents of cholesterol, as well as reduce the detergent activity of bile salts. Because bile salts can induce biliary mucosal injury via their detergent-like actions, phospholipids thereby function to protect the biliary system from the damaging effects of bile salts. Functional changes in the ABCB4 transporter can potentially result in disease of the biliary system. For example, mutations in ABCB4 are responsible for progressive familial intrahepatic cholestasis type 3 (PFIC3) and have been associated with intrahepatic cholestasis of pregnancy, primary biliary cirrhosis, and cholelithiasis in people (Deleuze et al. 1996; de Vree et al. 1998; Dixon et al. 2000; Lucena et al. 2003; Rosmorduc et al. 2003). Furthermore, *Abcb4* (-/-) knockout mice have impaired phospholipid and cholesterol secretion (Smit et al. 1993), as well as portal inflammation, bile duct injury, and fibrosis (Popov et al. 2005; Fickert et al. 2004) and spontaneous cholelithiasis formation (Lammert et al. 2004).
Currently, there is no published information regarding the canine \textit{ABCB4} gene, its expression in tissues, its relation to corresponding human ABCB4 and rodent \textit{Abcb4} genes, or its potential role in canine biliary disease. Interestingly initial screening of the canine \textit{ABCB4} sequence was made by BLAST (http://www.ncbi.nlm.gov/BLAST) using human MDR3 (ABCB4; AC005068.2) against the canine genome to search for the dog cDNA sequence. This yielded a 2,022 base pair sequence, which is approximately half the size of the human ABCB4 cDNA sequence (3,924 base pairs), suggesting that the canine ABCB4 transporter was a half-transporter. This was particularly interesting because several members of the ABC family that are full transporters are thought to have resulted from a gene duplication event. Because canine genes are 2.7 times less likely to have undergone gene duplication as compared to human genes (Lindblad-Toh et al. 2005), we postulated that the canine \textit{ABCB4} gene was a half-transporter. The objective of this investigation was to characterize the canine ABCB4 gene with regard to full cDNA sequence, tissue expression, and comparison to human and rodent homologues.
Materials and methods

RNA extraction. Tissue samples were acquired, following IACUC procedures, from dogs donated to the Washington State University College of Veterinary Medicine. Approximately 100 mg of liver tissue was added to one milliliter of Trizol (Gibco BRL, Grand Island, NY). The liver was then homogenized with thumb forceps. RNA isolation was achieved following the manufacturer’s instructions. The concentration of RNA was measured on a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA).

Primer development. Canine sequence for the ABCB4 gene was made by cross-species comparison using a BLAST of human ABCB4 (MDR3; AC005068.2) to the canine archives (http://www.ncbi.nih.gov/genome/guide/dog/). Primers were randomly designed and are listed in Table 1.

RT-PCR. Each 100 µl reverse transcriptase reaction was made using 20 µl Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI) 5x buffer, 20 µl dNTPs mix, recombinant RNasin ribonuclease inhibitor, 4 µl reverse transcriptase, 36 µl water, 4 g of oligo dT, and 8 µg of RNA. The reverse transcriptase polymerase chain reaction (RT-PCR) was performed on a GeneAmp PCR system 2400 (Perkin Elmer, Waltham, MA) using the following program: 42°C for 60 minutes, 95°C for 5 minutes, and then hold at 4°C.
**PCR.** Polymerase chain reaction was performed on a MJ research PTC-200 peltier thermal cycler (MJ Research, Waltham, MA) with Promega 2.5 µl buffer, 2.5 µl Mg, 2.0 µl dNTPs, 11.0 µl water, 0.1 µl taq, 4.5 µl RT PCR mix, and 2 µl of the corresponding primer mix. Amplification parameters were: 95°C for 60 seconds, 95°C for 15 seconds, 56°C to 62°C for 30 seconds, 72°C for 60 seconds, 37 cycles of 60°C to 2°C, and then hold at 4°C. Amplification products were electrophoresed on a 1% agarose gel.

**Sequencing** Primer pairs that yielded the expected product size were identified and selected for purification. Products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) according to the protocol provided by the manufacturer. All PCR products had a volume of approximately 11 µl prior to the clean-up procedure however the volume following the purification was 50 µl each. Six microliters of each PCR product and the corresponding forward primers were submitted to the Core Genomics laboratory at Washington State University for sequencing. Sequences received from the core genomics laboratory were compared to the expected *ABCB4* dog sequence using BLAST.

**Brain and intestine expression of *ABCB4.** Brain and duodenal tissues were harvested as described above for liver. RNA extraction of brain was performed as previously described for liver. A mortar and pestle were cooled with liquid nitrogen and approximately 100 grams of duodenum was added to the mortar filled with liquid nitrogen. A pestle was then used to crush and homogenize the duodenum. RNA isolation, RT-PCR, PCR amplification of RNA, gel
electrophoresis, purification, and sequence processing were performed as described above for liver.
Results

Sequence of \textit{ABCB4} gene. Approximately 97\% (or 3570 base pairs) of the 3684 base pair \textit{ABCB4} was sequenced in four individual mixed breed dogs. With the exception of six single nucleotide polymorphisms the nucleic acid sequence of the four dogs sequenced was identical. There was approximately 99\% similarity and identity between the published canine mdr3 gene sequence and the \textit{ABCB4} consensus sequence identified in our study. Almost all of the variation between the reported canine \textit{ABCB4} sequence and the consensus sequence reported here occurred within exons 26 and 27 (figure 1). In addition, there were differences in the nucleic acid sequence between the published canine \textit{ABCB4} sequence and the \textit{ABCB4} consensus sequence in our study in both exons 17 and 23.

Expression of canine \textit{ABCB4} mRNA in brain but not duodenum.

Expression of \textit{ABCB4} mRNAs was examined in brain and duodenal tissue from 4 healthy, adult, mixed breed dogs. \textit{ABCB4} was expressed in brain tissue from all four dogs, but was not expressed in duodenal tissue (Fig. 2). The sequence of \textit{ABCB4} from brain tissue was identical to that of \textit{ABCB4} found in the liver.
**Discussion**

In people the ABC transporter ABCB4 translocates PC to the outer from the inner leaflet of the apical membrane of the hepatocyte. Although expression of ABCB4 mRNA has been found outside of the canalicular membrane of the hepatocyte expression of the MDR3 protein is thought to be limited to the hepatocyte (Smit et al. 1994; Sakardi et al. 2006). Translocation of PC across the canalicular membrane helps minimize the detergent activity of bile salts thereby protecting the biliary and hepatic epithelium from the deleterious affects of bile salts. Mutations in the ABCB4 gene have been demonstrated to be responsible for cholestatic disease in people such as PFIC3 (Deleuze et al. 1996; de Vree et al. 1998). Moreover, mutations in the ABCB4 gene have been associated with a number of cholestatic diseases in people including ICP (Dixon et al. 2000; Lucena et al. 2003) and choledolithiasis (Lucena et al. 2003; Rosmorduc et al. 2003).

Characterization of the abcb4 gene in dogs and determination of its tissue distribution may help to elucidate the role of canine MDR3 protein.

The initial online search for the canine abcb4 sequence yielded a gene that appeared to be half the size of the human ABCB4 gene. Because the human ABCB4 gene is thought to have resulted from a gene duplication event, we postulated that the canine ABCB4 gene might actually be a half-transporter. In fact, it has been reported that dogs are approximately 50\% less likely to have gene duplication events than humans (Bailey 2006). Our results, as well as subsequent searching of the canine genome has yielded an expected canine sequence of 3,684 base pairs which is comparable to the human ABCB4 gene.
There were six single nucleotide polymorphisms amongst the four dogs sequenced in this study occurring at nucleotides A1134G, G1195A, G1885A, G1925T, T1987C, and C2908T. Only the polymorphism at nucleotide A1134G is likely to result in a change in the predicted amino acid sequence producing either lysine or arginine. As both of these amino acids are basic, hydrophilic, and cationic it is unlikely to alter the structure or protein of the final protein.

The consensus sequence for the canine \textit{ABCB4} gene that we obtained from 4 dogs was comparable to that of the human ABCB4 gene. The four dogs used were virtually identical to each other however they differed from the canine \textit{ABCB4} sequence obtained from the canine genome project. Most of the variation between the dogs sequenced in this experiment and the reported canine sequence, from the Boxer dog in the canine genome project, occurred in exons 26 and 27 (fig. 1). With the exception of a twenty-one nucleotide splicing variant at the 5’ end of the exon, exon 26 was identical in the reported canine sequence and the dogs sequenced in this experiment. Human ABCB4 variant A has the same twenty-one nucleotide splicing variant as our canine sequence.

Four alternative splicing variants of human ABCB4 have been described (Van der Bliek et al. 1987; Lincke 1991). The variation in ABCB4 is located within exon 26. The reading frame is left intact in all of the splicing variants. Interestingly enough, the Human variant A of the ABCB4 gene has an identical twenty-one nucleotide splicing variant as the dogs sequenced in this experiment. This splicing variant has not been shown to alter function of the MDR3 protein. It is possible that the Boxer dog sequenced in the canine genome project is similar to Human variant B as this variant contains a 21 nucleotide insertion at the 5’ end of exon 26.
In people, expression of P-glycoprotein, the product of the ABCB1 gene has been demonstrated in many tissues with excretory function such as biliary canalicular cells (Thiebaut et al. 1987), capillary endothelial cells of the brain (Cordon-Cardo et al. 1989), proximal renal tubular cells (Hori et al. 1993), and the brush border membrane of small intestinal enterocytes (Li et al. 1999). Along with its expression in multiple tissues, P-glycoprotein is known to have multiple substrates, suggesting that this protein serves a variety of functions. Tissue expression of canine P-glycoprotein has been demonstrated to be similar to that in people, and canine P-glycoprotein appears to have a similar substrate specificity. In contrast to P-glycoprotein, ABCB4 protein is expressed principally in the liver in humans. The ABCB4 protein in people is thought to be expressed primarily on the canalicular membrane of hepatocytes however mRNA transcripts have been detected in some other tissues (Smit et al. 1994; Chin et al. 1989). Although transcripts of ABCB4 mRNA have been detected outside of the liver the ABCB4 protein has not been demonstrated outside of the liver (Oude Elferink and Paulusma 2007) thus it has been speculated that this protein functions only in the liver. Indeed, the only known substrate for ABCB4 protein is PC. Canine ABCB4 mRNA is expressed in canine liver suggesting that it functions similarly in dogs as it does in rodents and people. ABCB4 expression was not detected in canine intestine.

A recent study suggests a potential role for ABCB4 protein in brain lipid transport and/or lipid homeostasis. ABCB4/Abcb4 mRNA expression is low but detectable in human brain and rat choroid plexus (Choudhuri et al. 2003; Langmann et al. 2003). In our study, ABCB4 mRNA
expression was also detected in canine brain tissue. Whether or not \textit{ABCB4} has a functional role in neural tissue in dogs is not known.

Based on the homology of canine \textit{ABCB4} compared to the human and rodent orthologs, it seems likely that the \textit{ABCB4} gene product functions in a similar manner in dogs. Specifically, ABCB4 is a lipid translocator that is essential for normal biliary excretion. Mutations in the ABCB4 gene cause a number of diseases in people. Progressive familial intrahepatic cholestasis, propensity for gallstone formation, and cholestasis of pregnancy have been shown to be caused by specific mutations in ABCB4. Furthermore, \textit{Abcb4}(-/-) mice have been shown to have impaired cholesterol excretion. The altered cholesterol excretion has been demonstrated to result in bile duct injury, portal inflammation, and fibrosis (Fickert et al. 2004; Popov et al. 2005) in mice. It is reasonable to assume that mutations in \textit{ABCB4} might be associated with cholestatic disease in dogs.

This report describes the sequence and tissue expression of canine \textit{ABCB4}, providing the critical foundation for further investigation into the physiologic function of \textit{ABCB4} in dogs. The abundant expression of \textit{ABCB4} in canine hepatic tissue supports a potential role for the protein in normal biliary function. Further, it suggests that mutations in canine \textit{ABCB4} may play a role in canine cholestatic disease. Further investigation is necessary to determine if alterations in \textit{ABCB4} gene expression or if \textit{ABCB4} mutations are directly associated with cholestatic disease in dogs.
References:


Table 1. Primers for canine mdr3

<table>
<thead>
<tr>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
<th>Expected Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCC ATA GCT CAT GGA TCA GG</td>
<td>CTG CCT GGA TTT AGC ATT GA</td>
<td>180</td>
</tr>
<tr>
<td>GCC ATA GCT CAT GGA TCA GG</td>
<td>ACC GTG TAT TAA GTT CAG TGG T</td>
<td>340</td>
</tr>
<tr>
<td>GTT TGA TGT CAA TGA CAC CAC TG</td>
<td>GCA TAT ATT AAC AGG AAG GCG ATG</td>
<td>437</td>
</tr>
<tr>
<td>CTG TGG CGG AAG AGG CTC</td>
<td>AGC AGC CTG TCC CAC ACT GAA G</td>
<td>292</td>
</tr>
<tr>
<td>CTG ATT GGA GCC TTC AGT GTG</td>
<td>ATC CTG CCC ATC AAT GTT AAT C</td>
<td>357</td>
</tr>
<tr>
<td>ATG GCT GGA AAT CTC GCA TA</td>
<td>GCA CAT GGT GCC TAT CAC AA</td>
<td>184</td>
</tr>
<tr>
<td>TGG CCT TGA TGT AGA AAG CA</td>
<td>CTG GTC CAA AAA CCG CTA TC</td>
<td>188</td>
</tr>
<tr>
<td>CTT CAG CCA GCG TTT TCC</td>
<td>GAG GTT AGC TGT GTT CTG GG</td>
<td>356</td>
</tr>
<tr>
<td>GAT GAG GTT GGC TTT AAT TGC C</td>
<td>GCA CCA AAT CGA AAA CAA CCG</td>
<td>392</td>
</tr>
<tr>
<td>CGG AAG GCA CAC ATC TAT GG</td>
<td>CCT TCT TCA CCT TAA GAC TCA GC</td>
<td>390</td>
</tr>
<tr>
<td>CTG AGT CTT AAG GTG AAG AAG GG</td>
<td>TG TTT TTG ACC TCC TGA GAG C</td>
<td>376</td>
</tr>
<tr>
<td>GCT CGA GCC CTC TTA AAA ACC</td>
<td>TAA GTT CTG TGT TCC AGT CGT G</td>
<td>257</td>
</tr>
<tr>
<td>CTG AGT CTT AAG GTG AAG AAG GG</td>
<td>TAA GTT CTG TGT TCC AGT CGT G</td>
<td>647</td>
</tr>
<tr>
<td>CTC AAC CTG AAG GTT CAG AGC</td>
<td>GTG AAG CAG AGG TAC AGG C</td>
<td>464</td>
</tr>
<tr>
<td>GAA ACA GAG AAT TGC CAT TGC G</td>
<td>GCA CAT GGT GCC TAT CAC AA</td>
<td>547</td>
</tr>
<tr>
<td>CTC AAC CTG AAG GTT CAG AGC</td>
<td>GCA CAT GGT GCC TAT CAC AA</td>
<td>914</td>
</tr>
<tr>
<td>CTC AAC CTG AAG GTT CAG AGC</td>
<td>CTG GTC CAA AAA CCG CTA TC</td>
<td>987</td>
</tr>
</tbody>
</table>
Reported Boxer dog sequence

Our consensus sequence

Human variant A sequence

**A** Exon 26

\[\text{fvdfgfqlldgqekkl}q\text{wlrahlgivsqepyf}dc\text{siaeniaygdn}r\text{avsqdeinv}\text{aakaanihpfietlph}\]

\[\text{Ildgqekkn|qwlratlgivsqepylf}dc\text{siaeniaygdn}r\text{avsqdeinv}\text{aakaanihpfietlph}\]

\[\text{Ildgqekkn|qwlratlgivsqepylf}dc\text{siaeniaygdn}r\text{avsqdeipv}\text{aakaanihpfietlph}\]

**B** Exon 27

\[\text{k}y\text{etrvdkgkqtls}ggq\text{nkrcy}rr\text{alr}k\text{lkicke}d\text{eatsaldtesek}\]

\[\text{kyetrvdkgkqtls}ggqkq\text{r}i\text{aara}l\text{ir}q\text{pqilldeatsaldtesek}\]

\[\text{kyetrvdkgkqtls}ggqkq\text{r}i\text{aara}l\text{ir}q\text{pqilldeatsaldtesek}\]

**Fig. 1** The predicted amino acid sequence generated by the nucleic acid sequence in exons 26 and 27 of canine \textit{ABCB4} and human ABCB4. (A) The predicted amino acid sequence generated by the nucleic acid sequence of exon 26 revealing the seven amino acid splicing variant of the consensus
sequence from this study and the human variant A sequence. (B) The ten amino acid variability between the sequence from the Boxer dog and our predicted consensus sequence and the human variant A sequence.
Fig. 2. Electrophoresis of PCR products from ABCB4 generated with consensus primers.

<table>
<thead>
<tr>
<th>Ladder</th>
<th>Liver</th>
<th>Duodenum Dog d</th>
<th>Duodenum Dog c</th>
<th>Duodenum Dog b</th>
<th>Brain Dog d</th>
<th>Brain Dog c</th>
<th>Brain Dog d</th>
<th>Ladder</th>
</tr>
</thead>
</table>
