Expression of monocarboxylate transporters I and IV and the ancillary protein CD147 in the intestinal tract of healthy horses and ponies

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OBJECTIVE
To characterize the expression of monocarboxylate transporters (MCTs) 1 and 4 and the ancillary protein CD147 in the intestinal tract of healthy equids and determine the cellular location of CD147 in the intestinal epithelium.

ANIMALS
12 healthy horses and ponies slaughtered for meat production or euthanized for reasons unrelated to gastrointestinal tract disease.

PROCEDURES
The entire gastrointestinal tract was removed from each equid within 45 minutes after slaughter or euthanasia. Tissue samples were obtained from the antimesenteric side of the duodenum, jejunum, ileum, middle part of the cecum, sternal flexure of the ventral colon, pelvic flexure, sternal flexure of the dorsal colon, and descending colon (small colon). Expressions of MCT1, MCT4, and the ancillary protein CD147 were examined in tissue samples from each of the 8 intestinal locations by means of quantitative PCR assay, immunoblotting, and immunohistochemical analyses.

RESULTS
Expression of MCT1 was most abundant in the cecum and colonic sites, whereas expression of MCT4 was predominantly in the proximal section of the intestine (small intestinal sites and cecum). Immunohistochemical analysis revealed that MCT1 and CD147 were present in the membranes of enterocytes (in crypts and villi).

CONCLUSIONS AND CLINICAL RELEVANCE
The anatomic distribution of MCT1 and MCT4 in the equine intestinal tract determined in this study together with the previous knowledge of the sites of substrate absorption indicated that MCT1 might predominantly contribute to the uptake of short-chain fatty acids in the large intestine and MCT4 might predominantly contribute to the uptake of lactate in the small intestine. (Am J Vet Res 2015;76:161–169)

Short-chain fatty acids are produced by the microbiotic fermentation of carbohydrates in the gastrointestinal tract of herbivores. In ponies, up to 70% of the intake of dietary soluble carbohydrate and fiber reach the cecum and colon, where most fermentation takes place.1 A large amount of fiber in the diet increases the total amount of SCFAs produced in the hindgut of ponies.2 Most of the luminal SCFAs produced are acetate, propionate, and butyrate.1 Short-chain fatty acids are not only essential for the health and function of the intestinal epithelium2 but also provide an energy source. In ponies fed a high roughage diet, SCFAs can account for > 30% of the total energy provision at rest.1

ABBREVIATIONS
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
MCT Monocarboxylate transporter
gPCR Quantitative PCR
SCFA Short-chain fatty acid
SMCT Sodium-dependent monocarboxylate transporter

Because the pH of the hindgut of equids is approximately 6 to 7,3 SCFAs are mainly ionized and require a transporter for cellular uptake. In other species, MCTs are thought to be responsible for transport of SCFAs into and out of intestinal epithelial cells together with passive diffusion, an anion exchanger,4 and SMCTs.5 Members of the MCT family were identified as lactate and pyruvate transporters originally in RBCs and later in muscle.4 Several studies6–10 have identified MCT isoforms also in equine RBCs and muscles. In the MCT family, isoforms MCT1, MCT2, and MCT4 are monocarboxylate or proton cotransporters that facilitate both lactate and SCFA transport.6

The expression of MCT isoforms has been studied in the gastrointestinal tract of monogastric animals and ruminants.10–17 Most research in monogastric animals has involved rats; as in equids, rats have a large cecum and colon where fermentation of fiber takes place. In rats, MCT1 seems to be the predominant MCT isoform in the intestines. It is expressed along the en-
tire intestinal tract, from the stomach to the descending colon, but especially in the large intestine. In rats and mice, the highest expression of MCT1 was found in the cecum, predominantly in the basolateral enterocyte membranes that line both intestinal villi and crypts. The distribution of MCT4 differs from that of MCT1; in rats, mRNA expression of MCT4 has been detected predominantly in the small intestine. On the other hand, in humans and cows, MCT4 is present in the colon and to a lesser degree in the ileum. The third MCT isoform known to transport SCFAs is MCT2, but it has only been detected in the gastric pits of the stomach and not in the small or large intestine of any of the species studied (rats, mice, humans, and pigs).

Because MCTs are not glycosylated, an ancillary protein is required for their correct localization and function on the cell membrane. In most species, the ancillary protein for MCT1 and MCT4 is CD147 (also known as basigin or extracellular matrix metalloproteinase inducer [EMMPRIN]), which forms an active protein complex together with the MCT. The CD147 protein is a glycoprotein that is expressed in a large number of tissues; in addition to its function as an ancillary protein, it also has numerous other functions in the body. Previously, CD147 expression has been detected in both the small and large intestines in pigs and ruminants.

Among domestic species, equids are unique because of their large body size and capacity to produce substantial amounts of SCFAs through hindgut fermentation. To our knowledge, there is no published information on MCT or CD147 expression in the intestines of equids. The purpose of the study reported here was to characterize the expression of MCT1, MCT4, and CD147 in the intestinal tract of healthy horses and ponies and to determine the cellular location of CD147 in the intestinal epithelium. The hypothesis was that MCT1 and MCT4 as well as their ancillary protein CD147 are present in the equine intestinal tract and that the amount of these transporters is related to what is previously known regarding the amount of SCFA absorption at different intestinal sites in equids.

Materials and Methods

Animals

Twelve equids (6 stallions, 3 geldings, and 3 mares) were used in the study. The mean age of the animals was 14 years (range, 4 to 31 years). Breeds included Standardbred (n = 6), pony (3), Finnhorse (2), and warmblood (1). The horses and ponies were either slaughtered for meat (human consumption) and the gastrointestinal tract was donated for research (8 horses and 2 ponies) or euthanized for clinical reasons unrelated to the gastrointestinal tract and the carcass was donated for research (1 horse and 1 pony). The Helsinki University Viikki Campus Ethics Committee approved the study, and slaughterhouse Vainion Teurastamo gave permission for collection and use of samples from slaughtered horses and ponies.

The slaughtered equids were first stunned with a penetrating captive bolt and thereafter exsanguinated. The euthanized equids were first sedated with detomidine hydrochloride (20 µg/kg, IV) and anesthesia was induced with a mixture of diazepam (80 µg/kg, IV) and ketamine hydrochloride (2.2 mg/kg, IV). An injectable solution of embutramide, mebezonium iodide, and tetracaine hydrochloride was administered (to effect, IV) after a surgical level of anesthesia was achieved.

Tissue Samples

The entire gastrointestinal tract was removed from the 9 horses and 3 ponies through a midline abdominal incision, and tissue samples were obtained from 8 gastrointestinal tract sites within 45 minutes after slaughter or euthanasia. Samples were collected from the antimesenteric side at each location. Tissue samples were collected from 3 small intestine sites: 30 cm distal to the pylorus (duodenum), 3 m proximal to the ileocecal opening (jejunum), and 10 cm proximal to the ileocecal opening (ileum). Tissue samples were collected from the lateral aspect of each of 5 large intestine sites: the middle part of the cecum, sternal flexure of the ventral colon, pelvic flexure, sternal flexure of the dorsal colon, and small colon. Pieces of the intestinal wall samples measuring 0.8 X 1.5 cm (2 samples/site) were cut and gently washed in saline (0.9% NaCl) solution. The first sample cut from each gastrointestinal tract site was frozen in liquid nitrogen and stored at -80°C until western blot analysis and qPCR assay were performed. The second sample cut was placed in phosphate-buffered 4% paraformaldehyde for 24 hours at room temperature (22°C) for immunohistochemical analysis.

Preparation of Cell Membranes

From each of the tissue samples allotted to undergo western blot analysis and qPCR assay, approximately 1 g of frozen mucosa was homogenized and placed in ice-cold buffer (0.3M sucrose, 2mM ethylene glycol tetraacetic acid, and 10mM Tris-HCl; pH, 7.2) containing 0.5% (wt/vol) bovine serum albumin, 0.14mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (50 µL of protease inhibitor/g of tissue). The tissue suspension and a medium for density gradient centrifugation were mixed and centrifuged for 10 minutes at 15,900 X g, and the membranes (upper phase) were washed 3 times in the same buffer without bovine serum albumin (centrifugation for 40 minutes at 48,000 X g). The final pellet was resuspended in the homogenization buffer without bovine serum albumin and protease inhibitors, and the protein concentration was determined with a bicinchoninic acid-based reagent (for the colorimetric detection and quantitation of total protein concentration). Membrane preparations were stored at -80°C until western blotting was performed.
WESTERN BLOTTING

Duplicate 10-µg samples of membrane proteins from samples obtained from each of the 8 intestinal locations were separated by 10% SDS PAGE and transferred onto nitrocellulose filters. Loading of equal amounts of protein was confirmed with Ponceau-S stain. Filters were blocked for 1 hour with 10% dry milk in Tris-buffered saline solution with 0.1% Tween, followed by incubation with primary antibody in the same buffer overnight (approx 18 hours) at 4°C. Polyclonal primary antibodies were produced in rabbits against C-terminal peptide of equine MCT1, MCT2, MCT4, and CD147, and each was affinity purified.  

Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control protein and was blocked with an antibody against mouse GAPDH.  

For each antibody, a band of appropriate molecular size was detected in the filters. The specificity of antibody was confirmed with peptide blocking and determining the molecular size of each band with commercial antibodies. Immunoblots were incubated with horseradish peroxidase–conjugated anti-rabbit antibody (1:2,000) for 1 hour at 21°C and bands were detected with a chemiluminescence reagent.  

Images were obtained from the filters, and image data were analyzed.  

Two control samples of pooled intestinal membranes (isolated from tissues samples obtained from all intestinal locations in 1 pony) were loaded onto each gel (1 loaded sample was 5 µg, and the other was 15 µg). The intensities of the bands were measured in relation to the intensities of these controls. The amount of each protein studied was normalized against the amount of GAPDH. All the proteins of the intestinal samples were measured from the same filter in a predetermined order as follows: MCT4, GAPDH, MCT1, and CD147. The filters were stripped after application of each antibody by shaking them in a stripping buffer containing 65mM Tris, 2% SDS, and 100mM 2-mercaptoethanol for 30 minutes at 37°C. After stripping, the filters were washed (25mM Tris, 192mM glycine, and 20% methanol) several times (2 washes of 30 minutes’ duration and 4 washes of 15 minutes’ duration) and blocked for 2 hours in the 10% dry milk buffer used previously before addition of another antibody.

IMMUNOHISTOCHEMICAL ANALYSIS

Each of the tissue samples allotted to undergo immunohistochemical analysis was embedded in paraffin and cut in 6-µm sections, which were placed on slides. After deparaffinization, the slides were pretreated with peroxidase for 5 minutes, after which they were washed with distilled water. For epitope retrieval, the slides were heated to 95°C for 4 minutes in buffer solution. After washing with distilled water, the slides were blocked for 30 minutes at room temperature. The primary antibodies against MCT1, MCT4, and CD147 at dilutions of 1:200, 1:50, and 1:200, respectively, were added and the slides were incubated for 1 hour at room temperature. Negative controls were incubated without primary antibody. The slides were washed twice with PBS solution containing 1% Tween and incubated with the secondary antibody for 1 hour and then washed. Thereafter, 3,3′-diaminobenzidine was added to the slides for 1 to 2 minutes, followed by washing in water, counterstaining with hematoxylin, dehydration, and mounting. Images were obtained with a microscope equipped with a camera. Positive controls for MCT1 and CD147 were prepared from muscle sections. The muscle sample was obtained from the center of the middle gluteal muscle at a depth of 6 cm from 1 of the Standardbreds used in the study. The slide preparation was performed according to Mykkänen et al.

qPCR ASSAY

For each sample from each intestinal location, RNA was extracted from 15 to 25 mg of tissue by use of a kit according to manufacturer’s instructions. Subsequently, 300 ng of total RNA was converted to cDNA, and qPCR assay was performed on 10 ng of the original RNA sample with a qPCR system. The amplification conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds and 40 cycles at 60°C for 30 seconds. The hydrolysis probe and primer sequences were the same as used previously. Equine 18S rRNA was used as an endogenous control gene to determine relative gene expression. Efficiencies of amplification varied from 98.2% to 99.9%, and the R² value of the calibration curves was 0.966 to 0.996. Differences in the expression levels among samples were determined by use of the comparative cycle threshold (ΔΔCt) method. The cycle threshold value of a single tissue sample (from the sternal flexure of the ventral colon of 1 horse) was set as a value of 1, and the other values were calculated in relation to this sample.

STATISTICAL ANALYSIS

A Kolmogorov-Smirnov test and Q-Q plots were used to assess the normality of the data for the variables. For almost all of the variables, the data were not normally distributed; therefore, nonparametric tests were used. The differences in the expression of MCT1, MCT4, and CD147 among the 8 sample locations were tested with a related samples Friedman 2-way ANOVA by ranks. The differences between the amounts of proteins in combined small (duodenum, jejunum, and ileum) and large (cecum, ventral colon, pelvic flexure, dorsal colon, and small colon) intestine samples were tested with the Wilcoxon signed rank test, when possible, or otherwise with the Mann-Whitney U test. The significance values were corrected with Bonferroni correction for pairwise comparisons. Correlations between the amount of all different mRNAs and all different proteins and correlations between mRNAs and respective proteins as well as their correlation with the age of equids were examined by means of Spearman correlation. Differences were considered significant at a value of P < 0.05.
Results

WESTERN BLOT

Monocarboxylate transporter 1, MCT4, and CD147 were detected in the immunoblots derived from all equine intestinal locations (Figure 1). Monocarboxylate transporter 2 could not be detected in samples from any intestinal location but was found to be present in the gastric pits, as in other species (data not shown). The small intestine (means for duodenum, jejunum, and ileum combined) had significantly ($P < 0.001$) less MCT1 expression than did the large intestine (means for cecum, ventral colon, pelvic flexure, dorsal colon, and small colon.

![Figure 1](image_url)
The expression of MCT4 was higher ($P < 0.01$) in the proximal part of the intestine (means for duodenum, jejunum, ileum, and cecum combined) than in the distal part of the intestine (means for ventral colon, pelvic flexure, dorsal colon, and small colon combined). The differences in the expression of MCT1 and MCT4 between the small and large intestine locations remained similar when calculated as raw values without normalization against GAPDH. The expression of CD147 and GAPDH did not differ between the small and large intestine locations. The amount of MCT1 ($\rho = 0.31; P < 0.01$) and of MCT4 ($\rho = 0.24; P < 0.05$) each correlated with the amount of CD147. None of the protein expressions was correlated with age of the equids.

IMMUNOHISTOCHEMICAL ANALYSIS

Results for the negative and positive immunohistochemical controls were as expected (Figure 2). Expressions of MCT1 and CD147 were detected in the membranes of enterocytes in samples from all equine intestinal locations and in both crypts and villi (Figure 3). For MCT1, the stain was more intense on the basolateral membrane, compared with stain intensity on the apical membrane. The anti-MCT4 antibody failed to stain any of the intestinal sections.

qPCR ASSAY

The amount of mRNA for MCT1 was more abundant ($P < 0.01$) in the large intestine (means of cecum, ventral colon, pelvic flexure, dorsal colon, and small colon combined) than in the small intestine (means for duodenum, jejunum and ileum combined; Figure 4). The amount of mRNA correlated with the expression of MCT1 protein ($\rho = 0.33; P < 0.05$). No positive correlation was found between the amount of MCT4 or CD147 mRNA and expression of the respective proteins. None of the mRNA amounts was correlated with age.

Discussion

To our knowledge, the present study is the first to characterize the expressions of MCT1 and MCT4 and their ancillary protein CD147 in tissues of the equine intestinal tract. Similar to findings for other species studied, MCT1 was detected along the entire intestinal tract, with the level of expression increasing toward the distal part of the small intestine and was most abundant in the large intestine.15–18 According to Argenzio et al,2 the concentration and absorption of SCFAs in ponies is highest in the cecum and ventral colon, which in the present study were the sites with the highest MCT1 expression. In addition, as SCFA con-
centrations decreased in the dorsal colon and further decreased in the small colon, a decrease in the expression of MCT1 was detected (Figure 1). This concomitant anatomic distribution of the transporter with its substrate is compatible with the suggestion that MCT1 contributes to SCFA absorption in the equine hindgut. In rats, MCT1 accounts for approximately 51% of butyrate absorption, 37% of propionate absorption, and 19% of acetate absorption. Both absorption and passive diffusion rates are known to be influenced by the luminal pH. However, in the present study, the function of MCT1 in the intestines was not demonstrated. Moreover, another family of transporters capable of SCFA transport, the SMCTs, has been identified. The physiologic role of SMCTs as transporters in the gastrointestinal tract has not been determined, but they may also contribute to SCFA absorption in equids.

In the present study, both the amount of MCT mRNA and the expression of MCT protein varied markedly among equids. Some of this variation could be attributable to the heterogeneity of the tissue sources because the equids represented several breeds and included ponies, and their ages varied from 4 to 31 years. Age is known to affect MCT expression in equine muscle. However, in the present study, there was no correlation between age and MCT expression in the equine intestinal tract. A possible explanation might be the unique physiologic adaptive response of muscle tissue to both age and training. The previous diet of the equids used in the present study was unknown. Examination of stomach contents during the sample collection procedure revealed that the study equids were fed either predominantly or exclusively hay or haylage and very little or no concentrate or grain. In ponies fed a diet with high roughage content, the distribution of SCFAs in the cecum and ventral colon is approximately 70% acetate, 20% propionate, and 10% butyrate. The distribution of SCFAs changes in the colon from the proximal to the distal portion; the percentages of acetate, propionate, and butyrate in the small colon are 80%, 15%, and 5%, respectively. However, the amount of starch fed to equids can markedly affect the microbiota in the intestines, amount and type of SCFAs produced, and possibly the expression of MCTs. On the other hand, by means of immunostaining, Iwanaga et al demonstrated the presence of SMCT1 in the apical membranes of colonic enterocytes. Therefore, it has been hypothesized that SMCT1 is responsible for the uptake of SCFAs from the intestinal lumen, whereas MCT1 transports SCFAs out of the enterocyte toward the bloodstream. However, some controversy exists,
because immunoblots of luminal vesicles from pigs and humans have more intense expression of MCT1, compared with that in immunoblots of basolateral vesicles. A possible explanation for this is a species difference, because the volume, site, and composition of SCFAs and therefore also the required capacity to transport these molecules is known to vary considerably among monogastric species.1

Results of previous immunohistochemical studies15,17,18 have indicated that MCT1 is present most abundantly on the membranes of crypt enterocytes and to a lesser degree on membranes of enterocytes at the tips of villi in the species studied. It has been suggested that the localization of MCT1 is basolateral in the immature cells of the crypt and villus base and that the localization gradually changes toward a more lateral and also apical distribution in the fully mature cells of the villus tips.17,18 However, findings of the present study did not support this observation, given that a distinct difference between the enterocytes lining the crypts and villi could not be determined (Figure 3).

In immunoblots prepared from the equine intestinal tissue samples in the present study, MCT4 was found to be present in all parts of the intestinal tract. The highest amounts were in the proximal part of the intestine (means of duodenum, jejunum, ileum, and cecum combined). In pigs, the small intestine has a higher expression of MCT4, compared with findings for the colon locations.16 In mice, the small intestine also has the highest amount of MCT4 mRNA, compared with that found in other intestinal locations.19 However, some controversy exists, because Iwanaga et al15 did not detect any MCT4 mRNA or MCT4 protein along the entire intestinal tract in mice. On the other hand, Gill et al10 reported that humans have the most intense signal for MCT4 in the large intestine, with less intense signal in the ileum and none in the jejunum. In horses, especially those fed a diet with a low roughage content, lactic acid is formed in the stomach by endogenous mucosa-associated bacteria.2,34,35 The amount of lactic acid decreases along the intestinal tract from 20 to 30 mM in the duodenum to only 2 to 3 mM in the cecum.2 Both MCT1 and MCT4 are efficient lactate transporters, and their expression in horses has been described in several reports of exercise physiology studies.8,9,36 The MCT4 Michaelis constant (Km) for lactate is 30 mM, which indicates high potential capacity to transport lactate.37 Monocarboxylate transporter 4 is also known to prefer lactate over other substrates.37 In the present study, the highest MCT4 expression was evident in the intestinal section where lactic acid concentration decreases most. Therefore, the results of present study have suggested that the role of MCT4 in the small intestine is related to lactate transport. On the other hand, the MCT1 Km for lactate is 3.5 mM, which indicates minor contribution to lactate transport in the small intestine, but possibly a more important role in the large intestine.38

In the equine large intestine, feeding has a marked influence on the lactate content in the intestinal lumen. When horses are fed a diet with a high starch content, the amount of lactic acid produced in the ce-

Figure 4—Box-and-whisker plots of amounts of MCT1 (A), MCT4 (B), and CD147 (C) mRNA (relative to amounts in a single sample of ventral colon tissue from 1 horse set as a value of 1) determined by qPCR assay in tissue samples obtained from various locations of the intestinal tract in the 12 equids in Figure 1. Differences in the expression levels among samples were determined by use of the comparative cycle threshold (ΔΔCt) method.† Brackets indicate a significant (P < 0.01) difference in median values between the small and large intestine locations.
cum and colon rapidly increases. Unlike humans, the amount of bacteria that can use lactate as a substrate for SCFA production in the intestinal tract of horses is limited. As a result, lactate accumulates in the lummen and the pH of luminal contents becomes markedly reduced, making horses susceptible to gastrointestinal tract disturbance and laminitis. The limited expression of the high capacity transporter, MCT4, in the equine large intestine might contribute to lactate accumulation.

In the study by Gill et al., MCT4 was found in the basolateral membrane vesicles of both the small and large intestine and MCT1 was present in the apical membrane vesicles, thereby prompting proposal of a theory of MCT1-mediated SCFA uptake in the cells and MCT4-mediated transport from the cells. However, as in the present study, Gill et al. were unable to successfully perform immunohistochemical analysis of MCT4. In the ruminant small intestine, MCT4 was localized at the brush border and the basolateral membrane of the epithelial cells lining the villi but was also found in the apical membranes of the crypt cells. In the present study, immunohistochemical staining of MCT4 failed despite attempts made to analyze cryosections and paraformaldehyde-fixed sections of several tissues and use of horse-specific and other antibodies. Therefore, the location of MCT4 in the equine intestinal tract remains to be determined.

The ancillary protein CD147 was found in tissue samples from all intestinal locations evaluated in the present study. Immunohistochemical analysis revealed equivalent staining of all enterocyte membranes along the crypts and villi in a pattern resembling that of the expression pattern of MCT1. A similar finding has previously been reported for the caprine large intestine. Although CD147 has many functions in cells, a positive correlation was found for the amount of this protein and both MCT1 expression and MCT4 expression in the present study, suggesting that the primary function of CD147 in the intestinal tract is to form a transporter complex together with MCTs, which can potentially facilitate SCFA transport.

A correlation between the amounts of MCT1 mRNA and MCT1 protein has also been detected in several other species and tissues. In humans, butyrate increases both MCT1 transcription as well as the stability of the transcript, leading to increased protein expression of MCT1 in the gastrointestinal tract. A correlation between the amount of MCT1 mRNA and MCT1 protein expression was found, and it is therefore possible that MCT1 is regulated by substrate concentrations via similar mechanisms in horses and in humans. However, in the present study, there was no correlation between the amount of MCT4 mRNA or CD147 mRNA and CD147 protein expression. This might be a consequence of posttranscriptional regulation of protein expression. Bonen found MCT4 mRNA in rat hearts, but MCT4 protein could not be detected, which is indicative of a posttranscriptional regulation mechanism that has not yet been defined.

More recently, CD147 has been investigated in the field of cancer research and an upregulating transcription factor as well as a small interfering CD147-targeting RNA have been reported to influence CD147 protein expression.

In the present study, MCT1 was found to be the most abundantly expressed MCT isofrom in the equine cecum and colon; MCT4 was detected in the small intestine and cecum. The distribution of MCT1 indicated that it is responsible for MCT-mediated SCFA uptake in the equine large intestine, whereas MCT4 may have a role in lactate transport in the small intestine. The amount and distribution of SCFAs have an important effect on energy provision and gastrointestinal tract health. Therefore, further research, including SCFA analysis of the intestinal content, in a homogenous group of equids is warranted to better understand the effect of diet on expression of MCTs and SMCTs and to elucidate their role in SCFA transport in horses and ponies.

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Footnotes
a. Domosedan, Oriola, Espoo, Finland.
b. Ketaminol, Intervet, Boxmeer, Netherlands.
c. Intervet, Boxmeer, Netherlands.
d. Sigma-Aldrich, St Louis, Mo.
e. Pierce, Rockford, Ill.
g. DAKO, Glostrup, Denmark.
h. Supersignal West Dura, Pierce, Rockford, Ill.
i. LAS-3000 CD-camera, Fujifilm Life Science, Düsseldorf, Germany.
j. AIDA, Raytest, Straubenhardt, Germany.
k. Biocare Medical, Walnut Creek, Calif.
l. Rodent Decloaker Buffer, Biocare Medical, Walnut Creek, Calif.
m. Background Punisher, Biocare Medical, Walnut Creek, Calif.
n. Rabbit-on-Farma HRP-Polymer, Biocare Medical, Walnut Creek, Calif.
o. Pertex, Histolab Products, Gothenberg, Sweden.
p. Leica DM4000 microscope, Leica Microsystems, Bensheim, Germany.
q. Olympus DP70 camera, Olympus, Hamburg, Germany.
r. GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich, St Louis, Mo.
u. DyNAmo Flash Probe qPCR Kit, Finzymes, Espoo, Finland.
v. Mx3000P qPCR system and MxPro qPCR software, Stratagene, La Jolla, Calif.

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