## 1. Title of Study:

Study Name: Identification and investigation of feline *Trichomonas foetus* surface antigens as a target for diagnosis and treatment

## 2. List ALL Principal Investigator(s) Information:

<table>
<thead>
<tr>
<th>Number</th>
<th>Name &amp; Title</th>
<th>Institution</th>
<th>Email</th>
<th>Mailing Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>M. Katherine Tolbert, DVM, PhD, DACVIM</td>
<td>The University of Tennessee College of Veterinary Medicine</td>
<td>Email:</td>
<td>Mailing Address:</td>
</tr>
<tr>
<td>b.</td>
<td>Emily N. Gould, DVM, MS candidate (Comparative &amp; Experimental Medicine)</td>
<td>The University of Tennessee College of Veterinary Medicine</td>
<td>Email:</td>
<td>Mailing Address:</td>
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## 3. Agency/Institution Information (where grant would be payable):

<table>
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<tr>
<th>Agency Name</th>
<th>Mailing Address</th>
<th>EIN Number (US Applicants)</th>
<th>Check Made Payable to</th>
<th>Grant Administrator Name</th>
<th>Grant Administrator Email</th>
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## 4. Amount Requested:

$16,077.76

### Signatures:

Signature of the principal investigator and appropriate grant administrator:

- **Signature:** Katherine Tolbert
- **Signature:** Jane Burns

Typing your name above constitutes electronic signature.
II. Scientific Summary Abstract

*Tritrichomonas foetus* (Tf) is a flagellated protozoal parasite that infects the distal ileum and proximal colon of domestic cats. Tf is a prevalent cause of severe, chronic diarrhea in cats worldwide. Only one drug is available to treat feline Tf and this drug is associated with increasing resistance and the risk of neurotoxicity. Tf infection also induces reproductive failure in cattle. Despite a difference in organ tropism between Tf genotypes, our lab and others have shown evidence for conserved virulence factors between feline and bovine Tf. Two surface antigens (1.15, 1.17) on bovine Tf participate in adhesion and cytotoxicity towards the urogenital epithelium. Inhibition of these antigens ameliorates bovine Tf-induced cytotoxicity. We have demonstrated that cytotoxicity of feline Tf is dependent upon adhesion to the intestinal epithelium. In preliminary studies, we identified the presence of surface antigen 1.15 on an isolate of feline Tf. Thus, identification of 1.15, 1.17 surface antigens on all feline Tf isolates and inhibition of adhesion by antibody opsonization may result in the identification of novel diagnostic and treatment strategies, respectively, for feline trichomonosis. The aims of the proposed study are: 1) to determine if all feline TF isolates tested express surface antigens 1.15 and 1.17 using indirect enzyme-linked immunosorbent assay and immunoblot studies, and 2) to evaluate the role of these antigens in mediating feline Tf intestinal cytopathogenicity using a validated co-culture model system. We believe the results of these studies will identify novel targets for development of diagnostic and/or therapeutic strategies for feline trichomonosis.

III. Lay-language abstract

*Tritrichomonas foetus* (Tf) is a protozoal parasite that is a prevalent cause of chronic diarrhea in domestic cats globally. The reported prevalence rate is as high as 30% in densely housed environments. No rapid, bedside assays are available to diagnose this infection. Moreover, only one drug is available to treat feline Tf and this drug is associated with increasing treatment failure and unacceptable side effects. As feline Tf closely resembles other intestinal infections of cats, it can be challenging for veterinarians to rapidly diagnose and treat Tf infection. Tf also causes abortions in cattle. Our group has previously demonstrated that cat and cattle Tf share common strategies for infecting their hosts. Two surface markers (1.15, 1.17) on bovine Tf participate in establishment of infection and induction of clinical signs. Thus, the aims of this study are to evaluate the expression of 1.15 and 1.17 in feline Tf and to determine if these markers play a role in injury of the intestine. All of our investigations can be performed on the benchtop using feline Tf organisms and a previously validated co-culture model system. Therefore, no cats will be harmed as a result of these studies and no live animal studies will be necessary. A vaccine against 1.17 is commercially available and effective against bovine Tf. Therefore, the results of these studies may lead to the development of a novel therapy and/or diagnostic strategy for cats infected with feline Tf.

IV. No continuation study
V. Study Proposal

BACKGROUND AND SIGNIFICANCE: Tritrichomonas foetus (Tf) is a protozoal pathogen responsible for chronic, large bowel diarrhea in domestic felids and abortions and early embryonic death in cattle. Feline Tf is highly prevalent and globally distributed with infections recognized in North America, Europe and Australia among others. [2-7] Feline trichomonosis presents a preventative, therapeutic, and diagnostic challenge to veterinarians. Firstly, there are no vaccines for prevention of feline trichomonosis. The only strategy for minimizing spread of Tf infection is to reduce close crowding in cats with high suspicion of infection. Although prolonged periods of clinical remission may occur, cats may remain subclinical, chronic carriers resulting in continued spread of the pathogen. [8] Thus, shelters and other high density housing environments may not recognize an infected cat before an outbreak occurs. The development of a vaccine would decrease the prevalence of disease in susceptible cats.

Secondly, there are limited effective and safe treatment strategies for feline Tf infection. [9,10] Ronidazole, the only treatment identified to be effective in some cats with trichomonosis, has been associated with neurotoxicity and development of drug resistance. [11-14] Thus, there is a compelling need for the development of novel therapies for the treatment of feline trichomonosis.

Thirdly, identification of feline Tf infection can also prove to be a diagnostic challenge. Direct smear light microscopy is the most widely available and inexpensive assay for the diagnosis of Tf. However, direct smear has a low sensitivity (positive in only 14% of naturally infected cats [15]) and misdiagnosis may be common given that feline Tf is often present as a co-infection with Giardia. [3, 15-17] Misdiagnosis can result in delay of therapy and/or risk of side effects in cats who receive inappropriate treatment. The In-Pouch™ TF-Feline test (Bio-Med Diagnostics) has an improved sensitivity (50%) compared to light microscopy [18], however, like light microscopy, pouch culture requires viable trichomonads (cannot use refrigerated fecal samples) and can result in bacterial overgrowth if too much fecal matter is applied to the culture. False positives in the presence of other trichomonads (e.g. Pentatrichomonas hominis) may also result in misdiagnosis. [19] PCR is the gold standard assay for diagnosis of feline Tf [20], however results may take up to 1-2 weeks and may not be a viable financial option for some owners and shelters. Thus, the development of a rapid and inexpensive bedside assay would facilitate early diagnosis and treatment.

Bovine Tf infects the reproductive tract in cattle, however despite this difference in tropism, bovine and feline Tf are genetically similar and utilize comparable virulence factors in their pathogenesis [21-24] We demonstrated that feline Tf, just like bovine Tf, express cysteine proteases that promote adhesion-dependent cytotoxicity towards the intestinal epithelium. [22] However, inhibition of Tf cysteine protease activity does not result in complete inhibition of epithelial injury. Thus, as would be expected with any eukaryotic parasite, additional virulence factors likely play a role. We believe that feline Tf shares additional mechanisms of pathogenicity with bovine Tf. Our lab is dedicated to the identification of bovine Tf virulence factors that are shared by feline Tf, and therefore serve as novel targets for the development of preventative, treatment and diagnostic strategies in feline trichomonosis.

We have demonstrated, similar to bovine Tf, that cytotoxicity of feline Tf depends on adhesion to the intestinal epithelium. [22] Thus, feline Tf surface antigens may serve as the perfect target for the development of a diagnostic and treatment strategy. Hodgson et al [25] identified two surface antigens of bovine Tf (1.15, 1.17) that when inhibited by monoclonal antibodies (MAbs 1.15, 1.17) significantly inhibited trichomonad adhesion to bovine vaginal epithelium. Later studies demonstrated conservation of both antigens amongst 37 bovine isolates. [1] Tf1.17 (also known as lipophosphoglycan (LPG) complex protein [26]), is recognized as a major surface antigen with expression of 10^6 1.17/LPG complex/bovine Tf organism. [27] Heifers vaccinated with Tf1.17 developed a robust local immunoglobulin response [28], cleared infections more rapidly [29-32], and had decreased severity of endometritis as assessed by histopathology than non-vaccinated cattle. [29] Additionally, vaccination of bulls with Tf1.17 protected against trichomonad colonization of preputial and penile epithelium. [33] Inhibition of adhesion by antibody opsonization of surface antigens such as 1.15 and 1.17 may result in prevention of infection and/or amelioration of clinical signs in cats with trichomonosis. Based on preliminary
studies, we believe that one or both of these bovine Tf surface proteins may be conserved in feline Tf isolates and thus would represent a novel target for the development of a diagnostic and/or treatment strategy.

**Research Hypothesis and Specific Aims**

We hypothesize that the expression of bovine Tf surface antigens 1.15 and 1.17 are conserved in feline Tf and therefore one or both of these antigens will serve as novel targets for the development of novel preventative, treatment and/or diagnostic strategies for feline trichomonosis.

The specific aims of this proposal are 1) to identify if bovine Tf surface antigens 1.15 and/or 1.17 are present on all feline Tf isolates tested; and, 2) to evaluate the role of surface antigens 1.15 and 1.17 in feline Tf intestinal cytotoxicity.

In preliminary studies, we have developed indirect enzyme-linked immunosorbent and dot blot assays to evaluate for the presence of surface antigens 1.15, 1.17 in feline Tf isolates. Early results suggest the presence of at least one of these antigens (1.15) on the surface of feline Tf (Figures 1 and 2).

![Figure 2. Presence of bovine Tf surface antigen 1.15 on feline Tf isolates.](image)

Representative dot blot of cellular protein lysates from feline trichomonad isolates for the presence of surface antigen 1.15. Lane 1, Positive control (1.15 antibody); Lane 2, Negative control (40µg IPEC-J2 protein lysate); Lanes 3-5, 40µg each of protein lysates from Tf isolates from 3 different domestic cats.

**Experimental Design: Feline Tf Isolates**

For all studies we will use 6 isolates of feline Tf (to account for strain variability) collected from naturally infected cats geographically distributed throughout the USA. An isolate of feline *Pentatrichomonas hominis* (Ph) and bovine Tf will be used in all assays as negative and positive controls, respectively. All isolates will be cultivated as previously described. [34] **Intestinal epithelial monolayers:** To model the intestinal epithelium, we will use non-transformed cultures of porcine intestinal epithelial cells (IPEC-J2) that will be seeded on polystyrene plates and allowed to reach confluence before infection. IPEC-J2 will be used for all studies pending the development of a feline intestinal epithelial cell line. Cultures with un-inoculated media (negative control) and Tf incubated with antibody diluent (isotype control) will be used for all assays. Additional controls may be included as warranted. For all immunoassays we will use monoclonal antibodies (MAbs) targeting bovine surface antigens 1.15 and 1.17 that were kindly provided by Dr. Bibhuti Singh (Upstate Medical University).

1. **Enzyme-linked immunosorbent assay (ELISA):** An indirect ELISA will be performed as previously described with some modifications. [1, 25] Tf will be harvested at mid-logarithmic phase, centrifuged at 15000 rpm for 5 minutes and suspended in 5% formalin in phosphate buffered saline (PBS pH 7.4) at 10^7 organisms/ml. Isolates will be washed in PBS prior to use. Organisms will be fixed to 96 well microtiter plates (Fisher Scientific) at 5 x 10^4/well and allowed to adhere overnight at room temperature (RT). Wells will be washed using a plate washer (ELx405, Bio-Tek Instruments, Inc) and blocked with 100 µl PBS with 0.05% Tween 20 and 2% Gelatin (PBS-TW-G) for 1 hr at 37°C. Plates will be washed with PBS and 0.05% Tween 20 (PBS-TW). 100 µl of MAbs Tf1.15 or Tf1.17 diluted to 1:500 in 2% milk will be applied wells. Plates will be incubated for 1 hr at 37°C and washed with PBS-TW. 100 µl of HRP-conjugated goat anti-mouse IgG and IgM (Kirkegaard and Perry Laboratories Inc) diluted to 1:500 will be applied wells, incubated for 1 hr at 37°C and washed with PBS-TW. 100 µl of TMB HRP substrate (ThermoScientific) will be applied and incubated at 37°C until maximal color change is perceived. The plate will be read on an ELISA reader (ELx800, Bio-Tek) using Gen5 Data Analysis Software (Bio-Tek) at 490 nm wavelength with blanked and negative control samples. Blanked samples, containing no antigen to correct for plate background, and samples lacking primary antibody to determine cut off points, will be used in all assays.

2. **Indirect immunofluorescence:** Immunofluorescence will be used to qualitatively evaluate for the presence of 1.15 and 1.17 on feline Tf isolates. Trichomonads will be centrifuged at 1500 rpm for 5 minutes, washed in PBS and fixed with 4% paraformaldehyde for 20 minutes at 4°C. Cells will be washed in PBS and applied to Plus slides (Fisher Scientific) at 5 x 10^6 Tf/ml using a Cytospin (Shandon Cytospin® 2 Cenrifuge). Slides will be rinsed briefly in PBSplus and permeabilized with Triton X-100 (Sigma Aldrich) for 10 minutes at RT. Slides will be rinsed with PBS prior to 1 hr incubation in block buffer (PBS Plus, 5% goat serum, 2% BSA). Slides will be incubated in primary MAb (1.15 or 1.17) diluted 1:100 in block for 3 hours in a humidified chamber. Following incubation, slides will be rinsed thrice in PBS for 5 minutes each. Cy5 goat anti-mouse IgG and IgM (Jackson Immuno Research) diluted 1:500 in block will then be applied and incubated for 1 hour at RT. Slides will be washed thrice with PBS for 5 minutes each. A DAPI counterstain (Vectashield, Vector
Crystal violet cytotoxicity assays:

Timeline

VI. cytotoxicity assays.

immunofluorescence assays. Analysis of SigmaStat (Jandell Scientific). Descriptive data will be generated from immunodot analysis and indirect normality, as well as analyzed with either parametric or non-parametric statistics where appropriate using SigmaStat (Jandell Scientific). Descriptive data will be generated from immunodot analysis and indirect immunofluorescence assays. Analysis of variance will be used to evaluate for differences between groups in cytotoxicity assays.

Anticipated Results and Potential Pitfalls:

Upon completion of these assays, we anticipate to have identified at least one bovine Tf surface antigen shared by feline Tf isolates that will serve as a novel target for the development of innovative preventative, therapeutic and/or diagnostic strategies. Given that previous studies in bovine Tf led to development of a vaccine successful in ameliorating clinical signs of in cattle, we feel results from the proposed studies may lead to development of a feline vaccine. The ideal model for these studies would be feline intestinal epithelial cells, however this cell line does not exist. Therefore, these studies will utilize porcine intestinal epithelial cells (IPEC). However, the IPEC-J2 cell line is a suitable alternative as the porcine trichomonad, Trichomonas suis, is genetically similar to Tf and exerts the same tropism for the gastrointestinal tract. Moreover, we have validated that this co-culture model system mimics in vivo Tf epithelial cytopathogenicity. [22, 34]

Data Analysis:

For all studies, a minimum of 6 replicates will be assessed for each isolate and antibody combination. Each study will be performed three times. For ELISA, the average of blanked samples will be subtracted from each reading to account for background, and a positive will be defined as two standard deviations above the mean of each negative control. For each isolate, outliers will be defined as values two standard deviations above or below the mean of the sample. Raw data will be interpreted first for variance and normality, as well as analyzed with either parametric or non-parametric statistics where appropriate using SigmaStat (Jandell Scientific). Descriptive data will be generated from immunodot analysis and indirect immunofluorescence assays. Analysis of variance will be used to evaluate for differences between groups in cytotoxicity assays.

VI. Timeline

All specific aims may be performed concurrently. Studies can be completed within one year.
VII. **Itemized Budget:**

**Enzyme -Linked Immunosorbent Assays**
Gelatin block buffer, PBS buffer, 96 well flat bottom plates, secondary antibody (goat anti-mouse HRP IgG+IgM), TMB Substrate, multi-channel pipette tips $1452.00

**Immunofluorescence Assays**
Fluorescent secondary antibody (goat anti-mouse HRP IgG+IgM, Jackson Immuno Research), goat serum (Jackson Immuno Research), Triton X-100, CaCl2 dihydrate, cover slips, coplin jars, Vectashield with DAPI (Vector Laboratories), pap pens, PLUS slides $716.00

**Dot blot assays**
Nitrocellulose membranes, BCA assay kit, secondary antibody (goat anti-mouse HRP IgG+IgM), trisbuffered saline 0.05% Tween 20 (TBST), Starting block buffer (Thermo Scientific), chemiluminescent substrate $600.00

**Crystal violet assays and cell culture supplies**

**Media and additives**
- Advanced DMEM/F12 (Fisher) $125.37 for 6-pack of 500 ml bottles x 5 packs $626.85
- HBSS (w/o Ca, Mg; Fisher) $86.61 for 6-pack of 500ml bottles x 4 packs $346.44
- dH2O $80.70 for 6 pack of 500 ml bottles x 4 packs $322.80
- Fetal Bovine Serum $256.45 for 500 ml x 4 $1025.80
- Equine serum $98.60 for 500ml x 5 $493.00
- Insulin/Transferrin/Selenium (Fischer) $92.88 per 5 ml x 2 vials $185.76
- Epidermal growth factor (BD) $155.40 for 100 μg x 2 $310.80
- Pen/Strep (Fisher) $31.75 for 100 ml x 6 $190.50
- Amphotericin (Fisher) case of 8 (20 ml) $283.71
- Trichomonad culture additives: casein peptone, yeast extract, maltose, cysteine, L-ascorbic acid, penicillin $1585.00

**Culture disposables**
- Chamber slides 4 well pack $161.00 x 4 $644.00
- Culture supplies: 15 and 50 cc conicals, sterile vials, serological pipettes, aspirating pipets, T75 and T25 tissue culture flasks, transwell polycarbonate plates, polystyrene plates $3,407.50
- Pipet tips $125.70, $125.70, $160.60 per case of 960 x 2 (Fisher) $824.00
- Media filter systems (250ml @ $104.30/case; 500 ml @ $186.60/case; Corning) x 4 $1163.60
- Misc – Crystal violet, PFA, ethanol, methanol PBS, SDS, gloves, paper supplies $1500.00

**Shipping charges** $400.00

**Total budget request** $16,077.76

**Personnel:**

M. Katherine Tolbert  Role: Principal Investigator  25% effort
Dr. Tolbert will be responsible for helping to conduct all assays. She will help to direct the study design, data analysis, and interpretation and publication.

Emily Gould  Role: Co-principal Investigator 75% effort
Dr. Gould, a master’s student under the direction of Dr. Tolbert, will be responsible for conducting all assays. These studies will serve as the backbone of her thesis project.
XIII. Animal Justification Statement not applicable

IX. References


A. Positions and Honors.
2011 Graduate Student of the Month (North Carolina State University)
2011 Digestive Disease Week Travel Award Recipient
2011 College of Veterinary Medicine Forum Presentation Winner (North Carolina State University)
2010 Best Oral Abstract Presentation ACVIM Forum, Anaheim, CA, (Comp Gastro Society)
2007-10 Resident in Small Animal Medicine (North Carolina State University)
2006-7 Intern in Small Animal Medicine and Surgery, (UGA College of Veterinary Medicine)
2006 Forehand Clinical Excellence Veterinary Scholarship (UGA College of Veterinary Medicine)
2006 Cum Laude Graduate (UGA College of Veterinary Medicine)
2002 Research Specialist, (Dr. Amy Lee, Emory University, Neuropharmacology Department
2001 Magna Cum Laude Graduate (Berry College)
2001 Who’s Who Recipient (Berry College)
2001 TranSouth Athletic Conference Scholar Athlete (Berry College)
2001 NAIA Academic All-America (Berry College)
2001 Omicron Delta Kappa Honoree (Berry College)
2001 Alpha Zeta Honoree (Berry College)
2000 TranSouth Athletic Conference Scholar Athlete (Berry College)
2000 NAIA Academic All-American (Berry College)

Memberships
American Gastroenterological Association
Comparative Gastroenterology Society
American College of Veterinary Internal Medicine

B. Selected Peer-Reviewed Publications and Abstracts (most relevant to study proposal)
Abstracts from Scientific Conferences  (* indicates presenter)


3. **Tolbert MK**, Stauffer SH, Gookin JL. Serine and cysteine proteases of feline *T. foetus* promote survival and adhesion to intestinal epithelial cells. 31st Annual Forum of the ACVIM, Seattle, WA.


7. **Tolbert MK**, Gookin JL. “*Tritrichomonas foetus* adheres to intestinal epithelium via receptor-ligand and cysteine protease dependent mechanisms.” *Digestive Disease Week*. San Diego, CA.

8. **Tolbert MK**, Gookin JL. “The adherence characteristics of *Tritrichomonas foetus* to the intestinal epithelium.” *Center for Gastrointestinal Biology and Disease Research Competition*. Raleigh, NC.


C. Current Research Support

1. **Morris Animal Foundation (MAF)**, **Tolbert MK (PI)** (D14FE-302) Cysteine Proteases: Novel Molecular Targets for Pharmacologic Control of Feline Trichomonosiosis-$48,322$ (12/1/13-12/1/14).


3. **Center of Excellence in Livestock Diseases and Human Health (COE)**, **Tolbert MK (PI)**. Modeling the intestinal epithelium through recapitulation of the intestinal stem cell niche-$14,900$. (2013-2014).
## BIOGRAPHICAL SKETCH

<table>
<thead>
<tr>
<th>Name</th>
<th>Position Title</th>
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<tbody>
<tr>
<td>Gould, Emily Nissa</td>
<td>Comparative &amp; Experimental Medicine Masters Student – University of Tennessee</td>
</tr>
<tr>
<td></td>
<td>College of Veterinary Medicine</td>
</tr>
</tbody>
</table>

### Occupation

Doctor of Veterinary Medicine

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### EDUCATION/TRAINING

<table>
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<tr>
<td>University of Tennessee College of Veterinary Medicine,</td>
<td>M.S.</td>
<td>2016</td>
<td>Comparative &amp; Experimental</td>
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<tr>
<td>Knoxville, TN</td>
<td></td>
<td></td>
<td>Medicine</td>
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<tr>
<td>University of California at Davis, Davis, CA</td>
<td>B.S.</td>
<td>2006</td>
<td>Animal Biology</td>
</tr>
<tr>
<td>University of California at Davis, Davis, CA</td>
<td>D.V.M.</td>
<td>2012</td>
<td>Veterinary Medicine</td>
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### A. Positions and Honors

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<tr>
<td>2013-14</td>
<td>Specialty Intern in Small Animal Internal Medicine (Animal Specialty &amp; Emergency Center, Los Angeles, CA)</td>
</tr>
<tr>
<td>2012-13</td>
<td>Intern in Small Animal Medicine and Surgery (Veterinary Medical and Surgical Group, Ventura, CA)</td>
</tr>
<tr>
<td>2012</td>
<td>Doctorate of Veterinary Medicine (UC Davis College of Veterinary Medicine)</td>
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<tr>
<td>2012</td>
<td>Oliver Chocolate Chip Cookie Scholarship Recipient (UC Davis College of Veterinary Medicine)</td>
</tr>
<tr>
<td>2006</td>
<td>Research Assistant (Dr. Leslie Lyons, University of California at Davis, Center for Comparative Animal Health, Laboratory of Feline Genetics)</td>
</tr>
<tr>
<td>2006</td>
<td>Who’s Who Recipient (University of California at Davis)</td>
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<tr>
<td>2006</td>
<td>High Honors Recipient, Bachelors of Science in Animal Biology (University of California at Davis)</td>
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Memberships

American Gastroenterological Association (AGA)
Comparative Gastroenterology Society (CGS)
American Veterinary Medical Association (AVMA)

B. Selected Peer-Reviewed Publications and Contributions to Projects

Peer Reviewed Publications

8. Carolyn A. Erdman, Emily N. Gould, Leslie A. Lyons. Chromosomal localization of an inherited craniofacial defect in cats. Department of Population Health & Reproduction, School of Veterinary Medicine, University of California, Davis. Created linkage map as a part of senior practicum and NIH funded research project.

C. Didactic teaching

Knoxville, TN

VMD 887 Feline medicine elective (1 hr)
Application Based Learning Exercise (ABLE; 1 hr)

D. Continuing Education Contributions

Ventura, CA