Three year PhD studentship - The role of the microbiome and circulating endothelial cells in the pathobiology of cutaneous renal glomerular vasculopathy (CRGV) - Summary

Introduction

In the 1980s, a new disease was described affecting greyhounds in the USA (in Alabama); the disease (Alabama Rot or CRGV) was subsequently reported in other countries affecting other dog breeds. However, the cause of this devastating disease still remains unknown. Clinically the disease presents as a vasculopathy (blood vessel disease) resulting in skin lesions (sores) and sudden-onset kidney failure. A high proportion of affected dogs die and those that recover may have compromised kidney function. A number of older studies have proposed that the cause may be bacterial.

The University of Surrey has been working in collaboration with clinicians from Anderson Moores Veterinary Specialists to better understand the cause of the disease. Initial findings have shown that the gut microflora (bacterial/bug population) of dogs affected by CRGV differs significantly from healthy dogs.

Aim of the research project

This PhD studentship aims to characterise the gut microflora, blood changes and other factors associated with the disease, in order to try and determine the cause, and hopefully in time, improve the ability to diagnose and treat the disease.

Collection of data and samples

The University of Surrey and Anderson Moores Veterinary Specialists (AMVS) already have a collection of faecal samples from affected dogs stored in their respective archives. However, AMVS will continue to collect samples from affected dogs (and healthy dogs) for the first 12 months of the project. The PhD student will start by analysing the archived samples and expand the study as new samples are collected. Ongoing sample (faeces and blood) collection will be done with owner consent. All studies will be approved through the University of Surrey ethics committee.

Scientific summary

Gut microbiome studies - To gain an understanding of the gut microflora changes associated with CRGV and identify bacterial species (or changes in abundance and diversity) that may be associated with the disease.

Next-generation sequencing allows all microorganisms in the gut to be identified (culturable and non-culturable). 16s bacterial community analysis allows the community diversity and abundance to be studied, whereby deep sequencing enables individual species and gene content to be studied. For the faecal microbiome studies planned for this study, faecal samples will be collected from healthy (50) and affected dogs (50) identified by Anderson Moores Veterinary Specialists. Where possible, *post-mortem* samples (duodenum, ileum, colon and caecum) will also be collected to determine if a localised affect is present.

DNA will be extracted using the PowerSoil®DNA isolation kit. DNA sequencing will be undertaken using Illumina MiSeq sequence reads will be processed according to the microbiome-helper pipeline (https://github.com/mlangill/microbiome_helper/) using the QIIME2 tool. Paired-end reads will be merged based on overlapping ends using PEAR (http://sco.h-its.org/exelixis/web/software/pear/), before filtering data for base-calling quality and amplicon length. Processed sequences will be classified using the pick open reference OTUs process implemented in QIIME2 against Greengenes 16S rRNA gene database (http://greengenes.secondgenome.com/) using a 97% similarity cut-off. The resulting distribution of OTUs across multiple samples will be further analysed using QIIME2 to summarize the distributions and explore alpha (number of different types of sequences in a sample) and beta (how different types are distributed among samples) diversity. Samples selected for further studies (30) will then be sequenced using a deep sequencing approach (provides species level information including gene content). For these studies, the Novaseq will be used.

Body fluid metabolite studies – To determine any metabolic changes in body fluids that may be associated with CRGV and that could be used as diagnostic markers.

Metabolic profiling (metabonomics/metabolomics) is a powerful analytical approach that simultaneously measures the low-molecular weight compounds in a biological sample, capturing the metabolic profile or phenotype. These metabolic signatures contain thousands of molecular components that reflect biochemical events occurring within the complex biological system. The ability of metabonomics to capture signals from environmental sources, such as diet and gut microbiome and their interactions with host metabolism, enables the "global" metabolic system to be characterised. The holistic nature of metabolic profiling provides an effective tool for illuminating novel biomarkers associated with disease onset and potentially disease susceptibility. In the proposed studies NMR will be used to produce metabolic signatures and identify unique correlations between affected dogs and controls genotype. Using 1H NMR spectroscopy and multivariate statistical analysis, including principal component analysis (PCA) and projection to latent structures (PLS) analysis, we shall identify metabolic variation between groups. The group at Surrey has extensive experience of working with R and MatLab necessary for analysis.

Faecal water, blood and urine will be collected from affected (50) and control (50) dogs during routine clinical work-ups and subjected to NMR analysis.

Clinical pathology – To determine if the presence of CEC's and or increased ANCA can be used as predictors of CRGV.

Clinical pathology facilitates the study of cell abundance and diversity together with biochemical parameters. In this project the student will be expected to review the literature on vasculopathy in humans to gain ideas and insights that might be applicable to CRGV.

Whilst humans are not affected by CRGV specifically, they do suffer from renal glomerular vasculitis (RGV), which bears some similarity with the renal pathology seen in CRGV. A common cause of human RGV is the production of anti-neutrophil cytoplasmic (auto) antibody (ANCA), often secondary to viral or bacterial infections. Patients with ANCA-associated small-vessel vasculitis have a high probability of increased ANCA in the serum and ANCA is a good indicator for diagnosing and monitoring ANCA-associated vasculitis. ANCA is easily detected using an indirect fluorescent antibody test (IFAT) in humans and has also been applied to dogs where it was found that ANCA positivity occurred in dogs with both immune-mediated inflammatory diseases and bacterial infection. Although the cause of canine CRGV is unknown, one suggestion is that it is caused by a toxin produced by the bacterium *Aeromonas hydrophila* following environmental exposure to the organism. *A. hydrophila* produces a cytolytic endotoxin that promotes chemotaxis of neutrophils. One hypothesis we have is that the pathology of CRGV is driven by ANCA, secondary to an infection, such as with *A. hydrophila*. We shall use the IFAT and ELISA to determine whether ANCA is associated with CRGV, and if so, whether ANCA titre is associated with clinical outcome.

Additionally, circulating endothelial cells (CECs) are a reliable marker of disease activity in a variety of vascular disorders. Damage to microvascular endothelial cells is a hallmark of thrombotic microangiopathy. Therefore, we will investigate whether circulating endothelial cells predict either development of acute kidney injury, or outcome in CRGV patients. Blood samples will be collected from affected and control animals during routine clinical work-ups and analysed by clinical pathologists. The human literature suggests that elevated numbers of CECs may be a reliable prognostic marker for vascular diseases, but this has not been tested in detail in dogs. In this project, we shall optimise the isolation and enumeration of CECs from dogs and then the number of CECs between control and affected dogs will be compared.

The University of Surrey's Clinical Veterinary Pathologist will provide guidance for this part of the project.

VPG (a veterinary clinical pathology diagnostics company) have agreed to support the project through the provision of canine blood samples.

Supervision

Professor Roberto La Ragione – Deputy Head of Vet School and Head of the department of Pathology and Infectious Diseases *BSc (Hons) MSc PhD FRSB CBiol FIBMS CSci AECVM FRCPath*

Roberto is an experienced veterinary microbiologist, with particular interest in host-microbe interactions and the role of natural microflora in health and disease. Roberto has published over 150 peer-reviewed publications in veterinary microbiology and pathology. Roberto has successfully supervised >25 PhD students, many linked to external partners, including veterinary practices. Roberto will be the primary supervisor and oversee the microbiology component of the study.

Professor Mark Chambers – Professor of Microbiology and Disease Intervention *BSc (Hons) PhD (Cantab)*

Mark is trained in cell and molecular pathology and has spent 25 years as a research microbiologist understanding host-pathogen interactions. Most of his work is interdisciplinary, in particular with analytical chemists and physicists. Mark has published 102 publications in immunology, microbiology and pathology. Mark is currently co-supervising 3 PhD students, linked to external partners. Mark will be co-supervisor.

Mr David Walker – Head of Medicine, Anderson Moores Veterinary Specialists *BVetMed(Hons) DipACVIM DipECVIM-CA FRCVS RCVS, American and EBVS® European Veterinary Specialist in Small Animal Internal Medicine*

David is an experienced specialist internal medicine clinician with 15 years' experience in academic and private referral practice. He has led the investigation into CRGV in the UK since first recognised at Anderson Moores Veterinary Specialists (AMVS) in 2012. David will be the primary point of contact for clinical input. The majority of faecal samples collected so far are currently held at AMVS.

Mrs Laura Holm – medicine clinician, Anderson Moores Veterinary Specialists *BVM&S CertSAM MRCVS RCVS Advanced Practitioner in Small Animal Medicine*

Laura is an experienced advanced practitioner in medicine who has worked with David on CRGV for 7 years. Laura will support David in the roles mentioned above.

Project Partners and Collaborators

Anderson Moores Veterinary Specialists - Rationale for collaboration

Anderson Moores are one of the largest private companion animal referral practices in the UK. They have extensive experience of working with industry and academia and have an established working relationship with the School of Veterinary Medicine at the University of Surrey. Anderson Moores have worked with the University on the aetiology of CRGV for 3 years. They have built up the largest set of clinical samples relating to the disease in the UK and access to these is fundamental to the success of the project.

Mr David Walker and Mrs Laura Holm are recognised experts on CRGV and have extensive experience of dealing with clinical cases of CRGV in the UK, including recent cases. Mr Walker and Mrs Holm have published a number of articles on CRGV along with other authors. Moreover, David and Laura have good contacts with the dog owner community and have provided scientific input to the Alabama Rot Research Fund Charity. All project proposals are assessed by an independent veterinary specialist.

The student will benefit from interacting with an external partner and working with supervisors with different expertise. The partners at AMVS will play an active role in the project with regular meetings held alternatively at the University and Anderson Moores. The student will be encouraged to work closely with the clinical team to understand the challenges associated with collecting clinical samples and clinical research.

Project Costing

Fully funded PhD, stipend of £15,009 per year x 3 years = £45,027
Estimates for Bench fees, consumables and travel:

Year 1 - £16k
Research materials and consumables to support external bioinformatics course - £1k
Sample collection and general consumables - £1k
DNA extraction kit - £1k
Software licences - £1k
Antibodies - £1k
Sequencing £5k
NMR Analysis - £2k
Clinical pathology - £1k
FACS analysis £2k
Travel and subsistence (Sample collection and courses) - £1k

Year 2 - £15k
Sample collection and general consumables - £1k
DNA extraction kit - £1k
Antibodies £1k
Sequencing £4k
NMR Analysis - £2k
Clinical pathology - £1.5k
Conference attendance £1.5k
FACS analysis £2k
Travel and subsistence (Sample collection and courses) - £1k

Year 3 - £10k
Miscellaneous laboratory consumables £5k
Publication cost - £2k
Travel and subsistence (Sample collection and courses) - £1k
Conference attendance £1.5k
Thesis printing and binding £0.5k

Funding

University of Surrey - contribution towards fees and stipend - **£50k**
Alabama Rot Research Fund - contribution towards fees and stipend - **£50k**
Anderson Moores Veterinary Specialists (contribution in kind via time input on the project) - **£15k**