

Interim report - The role of the microbiome in the pathology of cutaneous and renal glomerular vasculopathy (CRGV) – a shotgun metagenomic sequencing approach

February 2024

Produced by – Jack Whitehouse

Hypothesis: Does the gut microbiota play a role in the disease process of CRGV?

Importance: Establishing differences in the bacterial species in the gut microbiota of dogs suffering from CRGV compared to dogs suffering from Acute Kidney Injury, and Medically healthy dogs would improve our chances of identifying bacterial species that may be responsible for CRGV, either as a direct cause or as a contributing factor.

Background

Cutaneous and Renal Glomerular Vasculopathy (CRGV) is an emerging disease affecting dogs in the UK. CRGV was first reported in Alabama, United States in the mid-1980s affecting racing greyhounds. The location of some of the cases resulted in the colloquial term 'Alabama Rot'. Of unknown aetiology, the disease has contributed to ~ 318¹ dogs dying of clinical abnormalities associated with CRGV in the UK alone. There is no known cause or proven link to an infectious agent and treatment is limited in most cases, thus leading to poor prognosis. Dogs develop cutaneous lesions on their extremities before variably developing acute kidney injury (AKI), typically four days after lesion presentation². During the development of AKI, subsequent clinical abnormalities may also occur (e.g., anaemia, thrombocytopenia) before the onset of clinical signs associated with death or euthanasia². The lack of understanding regarding a definitive diagnosis relies on the identification of pathology, such as thrombotic microangiopathy (TMA) in renal tissue during a *post-mortem* examination. TMA is a pathology characterised by inflammation and damage, including endothelial damage and thrombosis within the microvasculature². Little is known about the pathology and aetiology of CRGV and what is known does little to advance the treatment, diagnosis, and survival rates of affected dogs.

The faecal microbiota encompasses a diverse range of organisms including bacteria that perform a variety of functions influencing the overall health of the host, such as nutrient metabolism, immune regulation, and host defense³. Changes in the abundance of bacterial species have previously been associated with several diseases³. For example, inflammatory bowel disease in dogs was associated with reduced faecal bacterial diversity with increases in the taxa Gammaproteobacteria and Bacilli³. *Aeromonas hydrophilia* has been reported as a potential aetiological agent for CRGV⁴ because of the geographical distribution of cases across wet environments (e.g., wet flood meadows) and the ability of *A. hydrophilia* to cause skin lesions in a range of species including humans, and *Leptospira*-like kidney lesions in dogs^{4&6}. Shiga toxin-producing *Escherichia coli* which is associated with Haemolytic Uraemic Syndrome (HUS) in humans and shares key disease hallmarks with CRGV^{2&5} may also be a potential aetiological agent. However, to date, investigations into these two possible aetiological agents have been limited and their role in CRGV remains unclear.

Characterising the structure of the faecal microbiota during health and disease has previously paved the way for determining the function and role of species in the development of disease. In the proposed study a shotgun metagenomic sequencing approach will be employed. Shotgun metagenomic sequencing is a high-throughput microbial genomic sequencing method, providing an unbiased and sensitive approach to comprehensively reviewing all bacterial species present in a faecal sample⁷. This high-throughput method performs deep sub-sampling and detects very low abundance constituents of the microbial community that may be unique to CRGV-affected dogs.

Progress against the original deliverables

1. Recruit veterinary clinics for the collection of AKI faeces.
2. Creation of a whole genome library.
3. Assembly and processing of data.
4. Draft a manuscript for peer review.

Current Progress

1. Recruitment of veterinary clinics for the collection of AKI faeces

A recruitment campaign was launched through the University of Surrey, School of Veterinary Medicine Partnership Network, and 22 veterinary referral centres across England expressed interest in joining the study. Following University of Surrey ethical approval, six referral centres joined the study once ethical approval was obtained from their managing companies. The recruitment campaign lasted nine months, and regular bi-weekly check-ins were conducted with a veterinary surgeon. The benefit of routine contact with the veterinary practices was the stable and steady recruitment of AKI cases. 15 AKI faecal samples were collected over the nine months, with the majority of samples being collected within the last four months of the recruitment campaign. The AKI faecal samples were collected from each of the veterinary referral centres by a designated third-party medical courier before arriving at the University of Surrey.

2. Creation of a whole genome library

The consumables for the Illumina whole genome library were ordered, which then took 2–3 months to fully arrive. To ensure the successful creation of an Illumina library⁹ for shotgun metagenomic sequencing, a bead tagmentation approach was utilised. The approach required DNA from the samples to be normalised to 5 ng/μL, then ‘tagmentation’ of the metagenomic DNA was performed. Tagmentation of the metagenomic DNA with bead-linked transposomes to DNA fragments ensured unique dual index codes could be attached to each sample’s DNA. These unique dual index codes were to ensure each sample could be identified in the downstream *in silico* analysis. A quality control step was implemented, and the DNA concentrations were determined using a Qubit, before the samples were pooled together at a 10–50 ng/μL. Finally, quantification of the final Illumina library was performed using a Bioanalyzer (Agilent, California, United States), which ensured the library was viable for shotgun metagenomic sequencing at a depth of 50 million reads per sample. In total it took 16 hours to process and create the Illumina library. The library was dispatched to a third-party sequencing provider using a medical courier.

3. Assembly and processing of data

The data have been received and are undergoing analysis at the University of Surrey using the SqueezeMeta bioinformatic pipeline¹⁰. The expected data outputs include taxonomic assignment, functional annotation (e.g., what are the microorganisms doing?), and microbial community structures of the faecal microbiota of CRGV-affected dogs compared to AKI and medically healthy dogs.

4. Draft manuscript

Once the data have been assembled and processed, a concise and robust bioinformatic report will be produced for ARRF, and a manuscript for publication will be drafted and submitted to a high impact-journal.

Supporting references

1. Alabama Rot – Anderson Moores Veterinary Specialists (2024). [Online]. Available from: <https://www.alabama-rot.co.uk/>.
2. Walker, J. J. A., *et al.* (2021) ‘Clinicopathological features of cutaneous and renal glomerular vasculopathy in 178 dogs’, *Vet Rec*, 189 (4). doi: 10.1002/vetr.72.
3. Pilla, R., *et al.* (2019) ‘The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease’, *Front Vet Sci*, 14 (6), pp. 498. doi:10.3389/fvets.2019.00498.
4. Macdonald, F. A. (2015) ‘Role of *Aeromonas hydrophila* in CRGV in Dogs’, *Vet Rec*, 177 (23), pp. 602. doi:10.1136/vr.h6667.
5. Mayer, C. L., *et al.* (2012) ‘Shiga Toxins and the pathophysiology of haemolytic uremic syndrome in humans and animals’, *Toxins*, 8 (11). doi: 10.3390/toxins4111261.
6. Stevens, K. B., *et al.* (2018) ‘Spatiotemporal patterns and agroecological risk factors for cutaneous and renal glomerular vasculopathy (Alabama Rot) in dogs in the UK’, *Vet Rec*, 183 (16), pp. 502. doi: 10.1136/vr.104892.
7. Quince, C., *et al.* (2017) ‘Shotgun metagenomics, from sampling to analysis’, *Nat Biotechnol*, 35 (9), pp. 833 – 44. doi: 10.1038/nbt.3935.

8. Hassler, B. H., *et al.* (2022) 'Phylogenies of the 16S rRNA gene and its hypervariable regions lack concordance with core genome phylogenies', *Microbiome*, 10 (1). doi: 10.1186/s40168-022-01295-y.
9. Shotgun Metagenomic Illumina Library Preparation – Quadram Institute (2023). [Online]. Available from: <https://quadram.ac.uk/6-shotgun-metagenomic-illumina-library-preparation/>.
10. Tamames, J., and Puente-Sanchez, F. (2018) 'SqueezeMeta, A Highly Portable, Fully Automatic Metagenomic Analyse Pipeline', *Frontiers in Microbiology*, 9. doi: [10.3389/fmicb.2018.03349](https://doi.org/10.3389/fmicb.2018.03349).