Investigating intrafamilial transmission of enterotoxigenic **Bacteroides fragilis** in New Zealand sheep

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Introduction

In 2020, colorectal cancer (CRC) was the third leading cause of cancer mortality globally for males as well as females (Siegel *et al.* 2023). CRC has been projected by The World health Organisation to reach 3.2 million annually by 2040 (Global Cancer Observatory, 2025; https://gco.iarc.fr/en). Only about 5% of CRC cases are known to be of hereditary origin, the rest being labelled as 'sporadic', caused by environmental risk factors or carcinogens associated with changes in the gut microbiome. As specific gut bacterial pathogens are thought to function as drivers of both CRC and dysbiosis (Tjalsma *et al.* 2012), it is important to understand how healthy persons become carriers with these bacteria because of the potential risk factor. One such driver bacteria is the enterotoxigenic form of *Bacteroides fragilis* (ETBF), which secretes an extracellular 20-kDa metalloprotease toxin [BFT]). The *B. fragilis* is an anaerobic, gram-negative bacteria which is a member of one of the two major phyla (Firmicutes and Bacteroides) found in the colon of both humans and sheep. However, *B. fragilis* only represents approximately 0.6% of the culturable bacteria in the mammalian colon (Moore 1974). As it is known that Bacteroides species colonise the neonatal gut of humans in the first weeks of life, it is paramount to understand if ETBF also becomes part of the gut flora at an early age.

As many sheep and livestock are naturally infected with ETBF (Sears 2009) an objective of this study was to determine if there was a likely vertical transmission of ETBF from dam to lamb or any evidence of persistence of ETBF. In order to properly address whether these objectives could be achieved – several things would need to be verified. (1) Is the animal suited as a model? (2) Is there a reliable method to monitor ETBF in sheep faeces? (3) Can we measure the difference between transient ETBF and colonisation with ETBF? (4) Can we measure the identity of the ETBF found between a dam and her offspring to the strain level? I will examine each of these points with reference to the present study.

Is the sheep a suitable model for transmission?

Sheep are not often used for models of human gut metabolism because sheep are polygastric foregut fermenters and humans are monogastric hindgut fermenters. More often pigs, primates or rats/mice are utilised for human GI tract models. This study is about ETBF vertical transmission or carrier status in a 'naturally' infected sheep. Sheep have short reproductive cycle; lambs are kept with their dams in the neonatal period and the pedigree trees are well documented on most breeding farms. Ergo, in spite of the difference to the human digestive tract, sheep are potentially the next best model to a controlled human transmission study when compared to other animal model options.

Is there a reliable method to monitor ETBF in sheep?

Most human or animal studies transmission studies utilise bacterial from faecal material, blood or other tissues, associated with some form of clinical disease. In clinical disease, the microbiological specimen of interest is

present in abundance, facilitating isolation and genetic sequencing for single nucleotide polymorphism (SNP). As veterinarians are well aware from work with Mycobacterial infections in ruminants, isolation is not an easy task in subclinical diseases of the colon which contains trillions of different microbes reducing the chances of isolating ETBF on selective media to almost zero. These media preferentially grow most *Bacteroides* species with no discernible differences between ETBF and others. Otago colleagues have shown that using the using the right primers with the qPCR testing can determine the presence of ETBF to the detection level of 10^2 colony forming units (CFU) per gram of human faeces (Keenan 2016). The qPCR from faecal can only be used to determine ETBF subtype (subtypes 1-3), but this is only of limited use for transmission studies. Likewise, Next generation sequencing (NGS) with 16S amplicons or metagenomic methods are up to now only able to determine genes present such as *B. fragilis* to species level from a mixed faecal sample.

Can we measure the difference between transient ETBF and colonisation with ETBF?

Essentially, the answer here is no, because we do not know the shedding patterns of ETBF by carrier sheep and we have no verifiable gold standard for carrier status even post-mortem. However, this question is related to the next question below. If we could verify the same strain type over time, then a colonised animal shedding an identical or nearly identical strain over time would suggest colonisation.

Can we measure the identity of the ETBF found between a dam and her offspring to the strain level?

Essentially, the answer is also not here, However, this research then examined whether a smaller portion of the ETBF gene, which included the pathogenicity island, had sufficient heterogeneity to be useful for differentiation or sources without a pure culture.

Methodology

In 2019, with two physically separate sheep flocks (Romney breeding flock) and Coopworth breeding flock) both ewes and lambs were sampled at weaning by taking a faecal sample with a clean glove from the rectum, placing the sample in a cryotube and snap-freezing with dry ice. Samples were transported on dry ice to the University lab where they were frozen at -80C until further processing. Follow-up samples were taken in the Coopworth flock in the same manner from the lambs six months later and again in in both ewes and lambs at weaning in 2020 and 2021. Paired ewe-lamb dyads were chosen for DNA extraction of faecal samples. A total of 148 ewes and 330 lambs were sampled over the three-year period

DNA was extracted using the Qiagen QIAmp fast DNA stool mini kit and DNA quality was tested using the Nanodrop 8000 (Thermofisher, Germany). Testing for ETBF was performed with SYBR green chemistry and a qPCR as previously described (Keenan 2016). PCR amplicons were purified, then a 1ng/100bp sample of the amplicon was prepped and sequenced on an Applied Biosystems 3730xl DNA Analyzer. The sequences were checked against the *bft1, bft2* and *bft3* genomes to determine ETBF subtype.

The odds ratio of an ETBF positive offspring (case) due to exposure from her positive dam was compared to the odds of a positive lamb (cases) from a more limited exposure (negative dam) in the same flock (Altman, 1991) and the P value for the test of significance was calculated as described elsewhere (Sheshkin, 2004). The Chi-square distribution for 4x2 tables were used to examine the significance of subtype distribution within pairs (dam-lamb dyad or time sequence with one animal

A specific locus (CTn86, per Buckwold 2007) unique to the ETBF gene, was first examined utilising published ETBF genes available from the US National Institutes of Health (NIH) gene database. The CTn86 area was extracted and these sequences were compared to determine that there was sufficient heterogeneity. Several faecal samples from qPCR positive animals were selectively enriched in a Bacteroides broth. The microbial DNA was then extracted and purified. DNA samples were barcoded per animal. A specific locus (CTn86) unique to the ETBF gene, was extracted and sequenced on the MinION (Oxford nanopore technology, Oxford, UK) utilising adaptive sampling techniques (De Groot 2024).

Results and discussion

Ewe-lamb dyads from two flocks were at weaning in the two flocks showed 29% (33/113) of ewes and 38% (42/113) of lambs positive for ETBF. The relative risk of a positive lamb at weaning given that the mother was positive (exposure) was 2.94 (CI 1.86 to 4.61). Similarly, when examining Chi square tables of different subtypes within positive dyads the distribution of subtypes suggests that there was clustering of ewes with their dams per subtype was significant ($P<0.05$). It is noted that odds ratios to determine relative risk in a common occurrence can sometimes be misleading. Furthermore, twin pairs were examined as dyads, each with the same mother while a more intricate model could correct for the lack of independence. At any rate, these results could be due to environmental influences as well as vertical transmission.

As a proof of concept, faecal samples from three qPCR positive sheep were selectively cultured and the DNA sequenced utilising MINION adaptive sampling. Only in one of three qPCR positive samples were we able to extract and identify the CTn86 area of the *bft* gene utilising adaptive sampling. The other two positive samples showed low growth of *B. fragilis* strain types relative to other Bacteroides strains such that insufficient ETBF was isolated to determine the sequencing of the CTn86 region.

This research highlights the difficulties in actually proving transmission without pure isolates for SNP genotyping. The author will discuss future options and the relevance of ETBF research into neonatal transmission and carrier status.

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