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The bacterial pedome associated with foot pathologies in sheep: a case study

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SUMMARY

Hoof lameness is considered to be a major health issue in sheep, and can impact on both animal welfare and production of livestock. However the causes, although generally assumed to have a microbiological basis, are poorly understood. The work presented here investigated the pedome (the bacterial community of the foot) of sheep which were seen to have one of the following conditions: foot rot; a toe granuloma; Ovine Interdigital Dermatitis (OID) / scald. These were compared relative to samples collected from the healthy feet of the same animals. Samples were collected from commercial lambs from two flocks of sheep (one Beulahs, one Suffolks) at times of routine husbandry work. All animals in the flocks and those which showed signs of lameness (7 per flock) were used for sample collection. Interdigital scrapes were collected from lame feet, together with controls (i.e. non-lame feet) from the same animals. Of the lame feet, 3 were classified as having foot rot, 10 had OID / scald and 1 had a toe granuloma. DNA was isolated from the interdigital scrapes and analysed by next generation sequencing following amplification of DNA by PCR. All foot rot samples showed unusual microbial communities: one having an elevated abundance of *Fusobacterium* spp.; another with an elevated level of a *Corynebacterium* sp.; and the third an increased level of a number of unidentified sequences. One of the OID samples also had a high abundance of *Fusobacterium* spp., and another had a similar pattern of unknown organisms to that seen in the example of the foot rot case. The toe granuloma case showed an elevated level of a *Mycoplasma* sp. Therefore the organisms described here are different from those previously identified in a similar investigation into this topic. However the other eight OID samples had patterns similar to those in controls. This suggests microbial communities associated with ovine foot rot are complex, and that there are bacteria associated with the condition which remain unknown.

KEY WORDS

Sheep; foot rot; bacteria; pedome.

INTRODUCTION

Lameness in sheep has been ranked as the second highest threat to animal welfare and health in sheep, with a prevalence of 10.4% within UK flocks[1]. Prolonged chronic lameness has been proven to have detrimental effects on reproductive performance, lamb growth rates, body weight and wool growth.

The actual cause of lameness can vary greatly, but in many cases the lameness is due to diseased feet, with a probable microbial source of infection in most cases. This prompted the first investigation of the ovine pedome[2] to examine the microbial community resident on the feet of healthy sheep, as well as those in infected animals. This earlier work, using *post mortem* samples identified differences in the microbial populations between infected and healthy animals. The data presented here investigate this further with a novel dataset and using samples from living sheep.

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MATERIALS AND METHODS

Samples were collected at times of routine husbandry work from all four feet of all sheep showing signs of lameness in two flocks of lambs in West Wales during early July; a Beulah flock and a Suffolk flock. All animals were aged 5-7 months at the time of sampling. Clean surgical blades (Swann Morton Ltd, Sheffield, England) were used to collect scrapes of discharge and superficial bacteria from the skin surface and hoof edge of the interdigital area and were deposited in a 20ml aseptic tube (Gosselin™, Borre, France), and stored at -20°C within 30 minutes of collection. A fresh blade was used on each foot. Feet were classified as being: healthy (i.e. feet did not show any of the following signs); showing signs of Ovine Interdigital Dermatitis (OID) or scald; showing foot rot; or containing a toe granuloma, based on the descriptions of Hodgkinson (2010)[3].

In each flock 7 animals showed symptoms of lameness; Beulahs (2 foot rot, 4 OID and 1 toe granuloma) and Suffolks (6 OID and 1 foot rot). A healthy sample was collected from the bilateral hoof from each animal to act as a control for each of the animals sampled. DNA was extracted from scrapes taken from feet where lameness had been observed, together with a scrape from a healthy foot from the corresponding animal.

Extraction was performed using QIAamp® DNA Mini Stool Kits (Qiagen Ltd.; West Sussex, England). The manufacturer's standard protocol was followed, with the exception of an increased initial incubation from 70°C to 95°C for 5 min, which the manufacturers suggest helps lyse Gram-positive bacteria. Purity and concentration of DNA was determined using a BioTek Epoch Spectrophotometer System using measurements at A_{260} and A_{280}.

PCR was performed as described previously [4] using the reverse primer 355R (5'-CTG CTG CCT CCC GTA GGA GT-3') and the forward primer 27F (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3')[5] with a sample-specific "bar code" 10mer at the 5' of the forward primer. PCR cocktails (25 µl) contained 1ng of DNA with both primers (100nM each), 200 M of each dNTP, the manufacturer's buffer (supplemented to 1.8mM $MgCl_2$) and 1.25U FastStart high fidelity enzyme (Bioline). Amplification used the following stages: 2 min hot start at 95°C; 21 cycles (30 sec at 94°C, 30 sec at 52°C, 30 sec at 64°C); and a final extension of 7 min at 64°C.

Amplicon purification and sequencing were performed as described previously[4] with AMPure XP bead and E-Gel agarose gel electrophoresis clean-up steps before normalisation (120 ng/µl) and pooling of samples. Pooled samples underwent emulsion PCR (emPCR) in preparation for Ion Torrent sequencing.

Quality filtering of DNA reads was performed using the Ion Torrent PGM platform's standard settings. Short reads (<250 base pairs) were removed and individually barcoded sample files were merged for OTU clustering (≥97% identity) using the CD-HIT-OTU program[6]. Sequences were checked for the absence of chimera and normalisation of numbers of sequences was performed using the daisychopper.pl script[7,8].

Initial identification of OTUs was performed using the RDP Bayesian classifier[9,10]. Sequences with identity levels of ≥97% were defined as the same OTU with classification at the phylum level being ≥90% identity. Cochran's Q test (present/absent binary scores) was used to qualitatively compare different samples. Percentage abundance calculations were performed for classification at phylum level. Kruskal-Wallis one-way analysis of variance was used to compare statistical differences between OTU abundance between category types.

RESULTS

From the 28 samples (14 lame feet and 14 control feet) Ion Torrent sequencing identified 1579 distinct OTUs (based on >97% sequence identity values) from 11 bacterial phyla, one chloroplast sequence and 1 sequence which could not be assigned to a phylum even at the level of 85% identity. Those assigned to a phylum were further classified into 21 identifiable bacterial classes, together with 467 OTUs (32 Bacteroidetes; 1 Fibrobacteres; 237 Firmicutes; 12 Proteobacteria; 185 TM7) which although identified at the level of the phylum could not be assigned to previously identified classes (Table 1). Further sub-classification identified organisms from the following number of taxonomic levels which had been described previously: 29 orders; 51 families; and 50 genera.

The major phyla observed in all control samples were Bacteroidetes and Firmicutes, with the most abundant phylum being Firmicutes in 8 samples and Bacteroidetes 6 in the oth-

ers. No other phylum exceeded 10%, with the exception of Proteobacteria in 3 of the 14 samples. Moreover, the number of sequences which could not be classified to a known phylum never exceeded 9%.

In the samples from the lame feet, most samples (12 out of 14) also had high levels of Bacteroidetes and Firmicutes present. The two exceptions to this pattern (1 foot rot and 1 OID) both had the highest representation from the phylum Fusobacteria - 89% and 82% respectively - and were almost exclusively from the genus *Fusobacterium* (0.1% of the foot rot sample being an unknown organism from the family Fusobacteriaceae). Of the remaining 12 samples, 5 of them (1 foot rot and 4 OID) had Proteobacteria levels in excess of 10%, although at levels similar to those seen in the 3 control samples mentioned above. In the case of the 8 samples where Proteobacteria constituted >10% of the total population this was due primarily to a mixture of sequences from both the genera *Acinetobacter* and *Psychrobacter*, irrespective of the health status of the source foot.

Sequences from the phylum Actinobacteria never exceed 5.6%, other than in a single foot rot sample where it reached 22%. This value was due primarily to 14% of the sequences being from a single OTU from the genus *Corynebacterium*.

Sequences from the phylum Tenericutes never exceed 4.4% other than the single toe granuloma sample where it reached 16.7%. In this sample this was due to 14% of the sequences being from three OTUs from the genus *Mycoplasma*.

The third foot rot sample also showed an unusual pattern, with the highest abundance of sequences (15.3%) unassigned to any phylum. This was based on elevated levels of 5 different OTUs, which were only found above background levels in one other sample (an OID) which had higher representation of 4 out of 5 of them present.

The Cochrans Q test showed a statistical difference within the bacterial communities in samples from lame feet relative to controls (Q = 6803). Kruskal-Wallis analysis showed 45 OTUs to be statistically different in controls relative to those from lame samples.

DISCUSSION

All three samples collected from the lame feet of animals with foot rot showed unusual and different sequence patterns: an elevated level of a *Corynebacterium* sp.; an elevated level of a *Fusobacterium* sp. (also observed in one of the OID samples); and elevated levels of unidentified sequences. In addition, the single example of a toe granuloma showed an elevated level of a *Mycoplasma* sp. However, although some of the remaining OID samples showed increased levels of Proteobacteria, these were similar to levels measured in many of the controls samples.

F. necrophorum is often associated with foot rot and a *Fusobacterium* sp. was observed at high (>80%) levels in one of the foot rot samples and one of the OID samples. The level of abundance in these two samples may be a reflection of our sampling method, only taking from around the infected area. However, to our knowledge, *Corynebacterium* spp. or *Mycoplasma* spp. have not previously been implicated in lameness studies. Likewise the sequences which could not be identified at the phylum level are unknown in lameness studies. It is also unclear the extent these organisms play in terms

Table 1 - Percentage abundance of each phylum / class within the individual samples.

Percentage abundance of each taxonomic category. Groups: FR = Foot Rot (FR1–FR4); Gr = granuloma (Gr1–Gr5); S = ODI (Scald) (S1–S9); C = Control (C1–C9).

Phylum / Class	FR1	FR2	FR3	FR4	Gr1	Gr2	Gr3	Gr4	Gr5	S1	S2	S3	S4	S5	S6	S7	S8	S9	C1	C2	C3	C4	C5	C6	C7	C8	C9
ACTINOBACTERIA																											
Actinomycetales	0.3	21.6*	0.6	1.3	3.1	0.3	0.5	1.8	0.5	0.5	0.6	0.7	0.9	0.2	0.1	0.5	5.5	0.5	0.5	8.1	0.1	5.3	0.6	0.6	0.1	2.1	1.9
Coriobacteriales	0.09	0.2	-	-	0.5	-	-	0.2	-	-	-	-	0.09	0.07	-	-	0.2	-	-	0.15	-	-	-	-	-	0.3	0.8
BACTEROIDETES																											
Bacteroidia	5.1	12.1	17.8	37.1	25.0	7.8	25.0	25.7	14.9	23.6	12.8	20.5	19.7	26.2	10.6	37.6	10.3	20.8	14.9	15.6	17.4	9.9	9.0	19.7	11.1	8.6	34.3
Flavobacteria	-	-	0.2	-	-	-	0.04	-	-	0.7	0.1	0.07	0.04	0.04	0.6	-	0.08	0.1	0.9	-	0.3	-	-	0.03	-	0.3	0.2
Sphingobacteria	-	0.04	0.3	0.01	0.09	0.1	0.7	0.1	0.9	0.4	0.3	0.4	0.1	0.1	0.4	0.2	0.2	0.4	-	0.8	0.1	0.1	-	0.3	3.9	0.5	-
Unknown Bacteroidetes	0.4	16.8	10.1	13.9	20.5	1.4	22.9	32.5	14.0	5.5	20.0	9.8	27.6	32.5	12.6	9.1	10.6	21.7	15.2	20.8	19.0	40.0	40.5	26.2	19.4	23.1	25.8
Chloroplast	0.09	0.04	-	0.5	0.6	-	0.2	0.2	-	0.05	0.03	-	0.07	-	0.6	-	0.4	0.03	0.5	0.4	0.2	0.02	0.05	-	0.9	0.3	-
Fibrobacteres																											
Fibrobacteria	-	-	0.02	-	-	-	-	-	-	0.05	0.3	0.1	0.03	-	-	-	-	-	-	-	0.06	1.2	0.8	0.1	-	-	-
Unknown Fibrobacteres	-	-	0.02	-	0.09	-	-	-	-	-	-	-	-	0.2	0.2	-	0.30	-	-	-	0.03	-	-	-	-	-	-
Firmicutes																											
Bacilli	0.3	1.1	3.2	0.2	2.4	-	0.06	0.5	2.6	1.4	5.1	4.2	0.6	0.4	0.5	0.04	0.5	-	0.3	-	0.3	1.1	0.5	1.9	3.5	11.8	1.2
Clostridia	2.1	28.3	25.8	16.5	25.5	4.2	33.5	15.5	27.6	20.7	31.5	17.7	22.0	22.8	55.9	31.2	44.3	38.0	44.1	41.8	40.6	35.9	38.9	25.9	19.1	15.3	12.7
Erysipelotrichia	-	0.7	0.4	0.04	1.5	0.4	0.06	0.4	1.0	0.2	0.3	0.7	0.5	0.2	0.9	0.08	3.0	0.2	1.6	0.6	0.7	0.1	0.05	0.5	0.5	0.8	0.2
Negativicutes	-	1.8	2.5	0.2	1.9	0.6	0.3	1.3	2.5	1.0	2.4	2.2	0.9	1.5	2.7	1.8	4.0	1.9	3.7	0.8	2.3	-	0.05	1.8	1.6	0.5	0.8
Unknown Firmicutes	0.4	7.9	3.0	2.4	10.6	1.0	9.5	7.3	7.0	3.4	4.2	3.9	4.5	4.7	8.2	6.4	7.6	8.8	6.5	10.3	8.3	5.8	6.6	5.1	4.6	5.4	7.9
Fusobacteria																											
Fusobacteria	88.8*	-	3.3	0.01	0.9	81.9*	1.4	0.2	-	4.0	0.1	3.0	-	0.3	-	0.4	1.9	-	-	0.02	-	-	-	-	-	-	1.0
Proteobacteria																											
Gemmatimonadetes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09	-	-	-	-	0.01	-	-	-	0.3	-	-	-
Alphaproteobacteria	0.09	0.04	0.1	0.04	0.5	0.1	0.06	0.06	0.09	0.09	0.02	0.3	0.4	0.01	0.6	0.3	0.2	0.03	0.3	0.2	0.09	0.07	-	0.01	-	0.3	-
Betaproteobacteria	-	-	0.04	0.01	0.09	0.06	-	-	-	0.05	0.2	0.3	-	0.4	1.0	0.2	0.08	0.03	0.3	0.4	0.09	0.02	-	0.03	0.9	1.6	1.5
Deltaproteobacteria	-	0.1	0.02	-	-	-	0.02	-	-	-	0.05	-	0.03	0.03	-	-	0.08	0.09	-	-	0.06	-	-	0.03	-	-	-
Epsilonproteobacteria	1.4	2.5	1.2	4.8	0.5	0.6	0.02	2.0	0.5	6.8	3.1	4.0	0.8	1.1	0.8	4.0	0.8	0.3	0.6	-	1.9	0.2	0.2	3.2	0.7	0.5	0.6
Gammaproteobacteria	-	0.5	12.5	0.8	0.09	-	0.06	0.3	21.3	22.6	8.6	22.4	10.6	3.3	0.6	0.2	1.9	0.6	0.5	0.2	0.2	0.09	2.6	8.2	23.0	21.4	4.2
Unknown Proteobacteria	0.2	0.4	0.02	0.01	0.8	-	0.4	-	1.0	0.6	0.3	0.1	0.3	0.08	0.08	0.09	0.4	0.5	0.6	1.0	0.8	0.07	0.1	0.4	0.2	0.5	-
Spirochaetes																											
Spirochaetes	-	-	-	-	-	-	-	-	0.09	-	0.3	1.7	-	0.06	-	-	-	-	-	-	-	-	-	-	-	-	0.2
SR1																											
SR1_genera_incertae_sedis	-	-	0.2	0.03	0.5	-	0.08	2.8	-	0.3	0.3	-	0.08	0.1	0.08	0.7	-	0.06	-	-	0.06	-	-	0.01	-	-	0.4
Tenericutes																											
Mollicutes	0.9	0.1	0.2	16.7*	0.3	1.0	4.4	0.3	0.2	3.6	1.5	1.1	0.2	0.2	0.08	4.1	0.2	0.03	-	-	0.06	-	0.02	0.01	0.7	1.1	0.2
TM7																											
TM7_genera_incertae_sedis	-	-	-	0.2	-	-	-	0.02	0.02	0.05	-	-	0.08	0.08	0.2	-	-	0.06	0.06	-	-	-	-	0.03	-	-	-
Unknown TM7	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown phylum	-	5.7	15.3*	5.3	4.0	0.6	2.2	4.8	5.9	4.5	8.0	6.4	5.8	5.7	6.2	5.5	1.7	6.3	4.9	7.1	7.0	2.8	3.0	6.5	9.0	5.4	6.2

All values are expressed to one decimal place with the exception of those which were present at less than 0.1% of the total for that sample. "Gr" denotes granuloma. Sequences which were not detected within a particular sample are denoted as "-". Values which are significantly increased (P<0.05) in samples, relative to control values, are indicated "*".

of causing the initial problem, or if they are opportunistic secondary colonisers.

Firmicutes comprised the major phylum in a previous study by Calvo-Bado[2] and again they were highly abundant in most of the samples in the current work. However, it is interesting to note that we did not identify the organisms highlighted in the previous work in these hoof samples. This demonstrates that microbial infection of the ovine hoof and its association with foot rot, toe granuloma and OID, remains poorly explored and merits further investigation.

ACKNOWLEDGEMENTS

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