Veterinary Microbiology 166 (2013) 664–669

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Culture-independent identification of bacteria associated with ovine 'broken mouth' periodontitis

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ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form 19 June 2013
Accepted 29 June 2013

Keywords:
Ovine
'Broken mouth' periodontitis
Bacteria
16S rRNA gene
Sequencing
Polymerase chain reaction

ABSTRACT

'Broken mouth' periodontitis (BMP) is a painful condition of sheep grazed on rough pasture and involves periodontal infection of the incisor teeth and progressive tooth loss. This can reduce the efficiency of grazing of sheep, which contributes to malnutrition, weight loss, systemic health problems, poor quality of life and early culling from flocks. Consequently, this condition is a major economic problem to sheep farmers. However, there are no treatment or control methods available. The aim of this study was to identify the bacteria associated with BMP and oral health in sheep. Swabs were collected from the gingival pockets of three sheep with BMP and from the gingival margin of three orally healthy (normal) sheep. Bacteria were identified using culture-independent (16S rRNA gene sequencing) methods. In the normal samples, 26 phylotypes were identified. The most prevalent species were *Enterobacter hormaechei* (21.3% of analysed clones) and *Hafnia alvei* (21.3%), with uncultured (4.4%) and novel (5.0%) phylotypes also being identified. For the BMP samples, 24 phylotypes were identified. The most prevalent species were *Mannheimia ruminalis* (28.4%) and *Moraxella caprae* (13.5%), with uncultured (2.6%) and novel (24.5%) phylotypes also being identified. In conclusion, a distinct microflora is associated with BMP and oral health in sheep and *M. ruminalis* may be involved in the aetiology of BMP.

1. Introduction

Sheep suffer from a naturally occurring form of periodontitis known as 'broken mouth' periodontitis (BMP). It is a condition of sheep grazed on rough pasture and involves periodontal infection of the incisor teeth, their subsequent loosening and progressive loss (Spence and Aitchison, 1986; Spence et al., 1988). Abattoir surveys in Britain have found incisor loosening or loss in 60–70% of cull ewes (Aitchison and Spence, 1984). The prevalence in any one flock varies between 5% and 70%, with over 50% of flocks in Britain being affected (West and Spence, 2000). The disease is similar to human periodontitis in many respects (Ismaiel et al., 1989). BMP, in addition to being a painful condition, reduces the efficiency of grazing of sheep, which contributes to malnutrition, weight loss and systemic health problems (Anderson and Bulgin, 1984; Baker and Britt, 1990).

The aetiology of BMP is unknown, although the involvement of one or more of a number of periodontopathic bacteria stimulating an immune response resulting in tissue destruction is thought to be the overriding mechanism (West and Spence, 2000). Several different bacteria have previously been isolated by culture-dependent methods from BMP samples, particularly *Porphyromonas gingivalis*, which was suggested to play a role in periodontal destruction (Frisken et al., 1986, 1987; McCourtie et al., 1990; Dreyer and Basson, 1992). However, no treatment or control methods are currently available for BMP. Control of identifiable infectious agents

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http://dx.doi.org/10.1016/j.vetmic.2013.06.034

would be very important in helping to prevent progression of BMP. Although BMP is thought to be caused by an infection of a mixture of bacteria species, our present knowledge is incomplete since we do not yet know the role that uncultivable bacteria may play in this important ovine disease.

The purpose of this study was to identify the bacteria associated with ovine BMP and oral health using culture-independent (bacterial 16S rRNA gene sequencing) methods. The main advantage of this approach over traditional culture methods is that, in addition to detecting cultivable bacteria, it can identify bacteria that are uncultivable or very fastidious in their growth requirements and can also detect novel species. Such species, that are difficult or impossible to detect by culture, may play a role in the aetiology of BMP.

2. Materials and methods

2.1. Sample collection and processing

Ethical approval was obtained from the Local Research Ethics Committee. Subgingival plaque was collected from the periodontal pockets of sheep with BMP using a sterile curette and plaque was collected from the gingival margins of periodontally healthy sheep using a sterile swab. All samples were placed into 1.0 mL sterile water and transported to the laboratory. Bacteria were dislodged by vortex mixing for 30 s.

2.2. DNA extraction

Bacterial DNA was prepared from each sample by treatment with 1% SDS and proteinase K (100 ug/mL) at 60 °C for 1 h, followed by boiling for 10 min. DNA was stored at −20 °C until required.

2.3. PCR amplification of bacterial 16S rRNA genes

Bacterial 16S rRNA genes were amplified by PCR with the universal primers 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi et al., 1998). PCR was carried using approximately 100 ng of extracted DNA as a template in a 50 μL reaction containing 1× GoTaq® PCR buffer (Promega, Southampton, UK) 1.25 U GoTaq® polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (New England Biolabs, Hitchin, UK), and 0.2 μM of each primer. PCR was conducted as follows: initial denaturation step of 5 min at 94 °C; 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and primer extension at 72 °C for 1.5 min; primer extension step at 72 °C for 10 min. Stringent procedures were employed to prevent contamination (Dolieslager et al., 2011).

2.4. Cloning and PCR amplification of 16S rRNA PCR products

PCR products were cloned into plasmid pSC-A-amp/kan using the StrataClone PCR Cloning Kit (Agilent Technologies, Stockport, UK) according to the manufacturer's instructions. At least 50 clones from each clone library were randomly selected and the 16S rRNA gene insert from each clone was re-amplified by PCR with the primer pair 5′-CCCTCGAGGTCGACGGTATC-3′ (M13SIF) and 5′-CTCTAGAACTAGTGGATCCC-3′ (M13SIR). The M13SIF binding site is located 61 base pairs downstream of the M13 reverse primer binding site and the M13SIR binding site is located 57 base pairs upstream of the M13 −20 primer binding site in the pSC-A-amp/kan plasmid.

2.5. Restriction enzyme analysis

Re-amplified 16S rRNA gene inserts were subjected to restriction enzyme analysis. Approximately 0.5 μg of each PCR product was separately digested in a total volume of 20 μL with 2.0 U of each of the restriction enzymes *Rsa*I and *Mnl*I (New England Biolabs) at 37 °C for 2 h and restriction fragments were visualised by agarose gel electrophoresis. For each library, clones were initially assigned to restriction fragment length polymorphism (RFLP) groups based upon their *Rsa*I profiles. More precise discrimination was achieved following digestion of clones with *Mnl*I, and clones with identical profiles for both enzymes were finally grouped together in distinct RFLP groups.

2.6. DNA sequencing

Sequencing of the 16S rRNA gene insert of a single representative clone from each RFLP group was conducted. Sequencing reactions were performed using the SequiTherm EXCEL™ II DNA Sequencing Kit (Cambio, Cambridge, UK) and IRD800-labelled 357f sequencing primer (5′-CTCCTACGGGAGGCAGCAG-3′) with the following cycling parameters: (i) initial denaturation at 95 °C for 30 s; (ii) 10 s at 95 °C, 30 s at 57 °C and 30 s at 70 °C, for 20 cycles and (iii) 10 s at 95 °C and 30 s at 70 °C for 15 cycles. Formamide loading dye (6 μL) was added to each reaction mixture after thermal cycling and 1.5 μL of each reaction mixture was run on a LI-COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech, Milton Keynes, UK).

2.7. DNA sequence analysis

Sequence data were compiled using LI-COR Base ImagIR 4.0 software, converted to FASTA format and compared with bacterial 16S rRNA gene sequences from the EMBL and GenBank sequence databases using the advanced gapped BLAST program, version 2.1 (Altschul et al., 1997), which was run through the National Centre for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov). Clone sequences showing at least 98% identity with a known sequence from the database were considered to be the same species as the matching sequence with the highest score. Sequences with less than 98% identity were regarded as potentially novel phylotypes.

3. Results

16S rRNA PCR analysis confirmed that all of the three normal and three BMP samples were positive for the presence of bacteria.