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# Differential inflammatory responses of bovine foot skin fibroblasts and keratinocytes to digital dermatitis treponemes

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ABSTRACT

Bovine digital dermatitis (BDD) is a serious infectious inflammatory lameness causing pain and suffering to many cattle worldwide and which has severe economic implications. This study set out to investigate relationships between the treponemes considered causal of BDD and the local inflammatory response of the bovine host. Here we describe, for the first time, the isolation of bovine foot skin keratinocytes and fibroblasts as separate cell lineages. These cell lines were then exposed to treponeme whole-cell sonicates, and the gene expression of selected host inflammatory mediators investigated using quantitative reverse transcriptase PCR. Several genes, including those encoding RANTES/CCL5, MMP12, TNFα, TGFβ and TIMP3 were significantly upregulated in fibroblasts exposed to whole-cell sonicates derived from BDD treponeme phylotypes. For each of the above genes there were similar fibroblast expression increases for all three BDD treponeme phylotypes tested, suggesting common virulence mechanisms. With bovine foot skin keratinocytes, we were unable to detect expression of RANTES/CCL5 and after incubation with BDD treponeme constituents we were unable to observe any significant changes in expression of inflammatory mediators tested. These contrasting results suggest fibroblasts rather than keratinocytes may be an important shared target of pathogenesis for BDD treponemes.


## 1. Introduction

Bovine digital dermatitis (BDD) is a severe disease causing lameness of dairy cattle which is now a global disease (Cruz et al., 2001). The lameness is the result of painful lesions on cattle hind feet immediately above the coronet between the heel bulbs (Blowey and Sharp, 1988; Cheli and Mortellaro, 1974). The lameness, and the resulting pain, reduces reproductive capability and milk yield and has been identified as having significant economic implications, with a recent study reporting that BDD costs a holding of 65 cows $1517 per year (Bruijnis et al., 2010). Spirochetal bacteria have been implicated as the likely primary aetiological agents of BDD. Analysis of bacterial 16S rRNA-encoding genes in German BDD lesions indicated involvement of five spirochete phylotypes belonging to the genus *Treponema* (Choi et al., 1997). Isolation studies has allowed for the characterisation of three of these treponeme phylotypes in the UK and USA, namely "*Treponema medium*-like", "*Treponema phagedenis*-like" and

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*Treponema pedis* (formerly *Treponema denticola*-like) BDD spirochetes (Evans et al., 2008, 2009a; Stamm et al., 2002; Walker et al., 1995). There have been frequent further reports worldwide that confirm the role of treponemes in the pathology of BDD lesions, with certain phylotypes more prevalent in certain countries (Brandt et al., 2011; Evans et al., 2009b; Klitgaard et al., 2008; Nordhoff et al., 2008). A recent infection model provides substantial evidence towards fulfilling Koch's postulates for the role of BDD treponemes in the disease (Gomez et al., 2011).

Understanding how BDD treponemes produce the observed pathological changes in BDD lesions of cattle is very important. The BDD treponemes are likely to possess virulence factors which directly cause host damage or alternatively may initiate host responses which contribute to pathogenesis (Edwards et al., 2003a). In human oral diseases, several studies describe how treponemes may contribute to aggravating the host response to indirectly cause cytotoxicity (Schroder et al., 2006). The nature of host innate and acquired immunity to treponemes in BDD and the level of protection that is available and functional remains poorly characterised. An in vitro study has shown that *T. phagedenis*-like BDD spirochetes may downregulate innate immune responses, specifically macrophage expression of pro-inflammatory genes, which could allow treponemal persistence in infected tissues (Zuerner et al., 2007). In terms of acquired immunity and its protective value, exposure to BDD treponemes results in an adaptive immune response, including significant antibody production but this does not appear to confer any apparent immune protection and the disease repeatedly recurs (Demirkan et al., 1999; Walker et al., 1997). Currently, there is no effective vaccine. Thus, it is timely to consider local immune and inflammatory responses in the infected (foot) tissues, specifically with regard to the triggering treponemal infection. Here, for the first time, we culture and isolate bovine foot keratinocytes and fibroblasts separately, challenge them with the BDD treponeme constituents and characterise the gene expression of important immune and inflammatory mediators produced by bovine host tissues.

## 2. Materials and methods

### 2.1. Isolation and culture of keratinocytes and fibroblasts

Samples of healthy bovine foot tissue from the plantar aspect of rear feet proximal to the interdigital cleft between the heel bulbs were collected post mortem from Holstein Friesian cattle sent to slaughter. For isolating both keratinocytes and fibroblasts a method developed to isolate canine keratinocytes was used initially (Kohler et al., 2001). Briefly, full thickness skin (3 $cm^3$) biopsies were taken and placed in a container with William's Medium E (WME, Invitrogen, Paisley, UK) containing 300 μg/ml neomycin, 150 μg/ml gentamycin, and 7.5 μg/ml amphotericin B (all antibiotics from Sigma, Gillingham, UK) and transported back to the laboratory. Biopsies were then cut into smaller pieces (0.5 $cm^3$) and incubated overnight at 4 °C in WME plus 10 mg/ml Dispase II (Sigma). Forceps were used to separate the epidermis from dermis and a further incubation was carried out with 5× Trypsin/EDTA (0.25% (w/v)/0.1% (w/v); Invitrogen) at room temperature for 30 min. The released dermal cells were then supplemented with WME containing 10% (v/v) foetal calf serum (FCS) to inactivate trypsin, passed through a cell strainer, counted and seeded into complete WME (one third concentration of the antibiotics listed above plus 2 mM glutamine, 20% (v/v) foetal calf serum (FCS), 0.1 nM cholera toxin (Sigma), 10 ng/ml human recombinant epidermal growth factor (Sigma)). Cloning rings (10 × 10 mm, 250 μl, Sigma) were used to attempt initial cell line isolation by detaching cells with 5× Trypsin/EDTA at 37 °C incubation for 5 min and inoculating each ring into individual 2 ml wells of six well tissue culture plates and incubating at 37 °C until confluence was achieved (4–7 days) and then further screening those cells exhibiting the morphological features of keratinocytes. Further isolation used an approach to separate keratinocytes and fibroblasts based on their different binding affinities for tissue culture plate surfaces (Yano and Okochi, 2005). Briefly, fibroblasts were isolated from keratinocytes by washing the adherent mixed-cell monolayer cultures with Hank's balanced salt solution (HBSS, Invitrogen) in triplicate and then detaching the fibroblasts using 0.5× Trypsin/EDTA (0.025% (w/v)/0.01% (w/v); Invitrogen) for 5 min at 37 °C and inoculating them into complete WME (without cholera toxin) with weekly passage. This selective process was then repeated at $2 \times 10^4$ cells/ml initial incubation concentration. Keratinocytes remaining attached to the flask after fibroblast removal were then washed in triplicate with HBSS and themselves released from the surface using 5× Trypsin/EDTA with 37 °C incubation for 5 min. Weekly inoculation of keratinocytes (for cell number expansion) was at $4 \times 10^4$ cells/ml, included the above fibroblast removal step for the first three passages and used complete WME plus 30 μg/ml bovine pituitary extract, 0.4 μg/ml hydrocortisone and 5 μg/ml human insulin (all aforementioned supplements purchased from Sigma).

### 2.2. Exposure of fibroblasts and keratinocytes to BDD treponeme sonicates

The BDD spirochetes T19 (*T. medium*-like), T320A (*T. phagedenis*-like) and T3552B (*T. pedis*), representatives of each of three phylotypes highly associated with BDD lesions (Evans et al., 2009b), were grown as previously described (Evans et al., 2008). Each treponeme phylotype was grown to late exponential phase in 70 ml of culture, pelleted by centrifugation at 4500 × *g* at 8 °C for 20 min, followed by two phosphate buffered saline (PBS, pH 7.2; Invitrogen) washes and bacteria then resuspended in 6 ml of PBS. The bacteria suspension was sonicated (Rapidis 150 sonicator, Ultrasonic Limited, Aldershot, UK) for 5 min consisting of continuous 10 s alternating cycles of sonication and rest with the suspension kept on ice (4 °C). Cell disruption of sonicates was verified by phase contrast microscopy. Sonicates were then stored at −20 °C until use. Sonicates were analysed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Dhawi et al., 2005) and protein