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Survival of the ovine footrot pathogen *Dichelobacter nodosus* in different soils

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ABSTRACT

Dichelobacter nodosus (*D. nodosus*) is the causative agent of footrot in sheep; one of the most important health and welfare issues of sheep worldwide. For control programmes to be effective, it is essential that the transmission cycle of *D. nodosus* is understood and bacterial reservoirs in the environment are better defined. This study evaluated the survival of *D. nodosus* in different soils using soil microcosms. Cultivation independent and dependent methods were used to detect *D. nodosus* over 40 days from seeding in soil. A *D. nodosus* specific probe was used for quantification by qPCR and viability was assessed by cell permeability to an intercalating dye, PMA, and by culture. Survival varied dramatically depending on soil type, matric potential (MP) and temperature. Our findings indicate that *D. nodosus* survival was higher at 5 °C compared with 25 °C in all soils and significantly longer at both temperatures in clay soil (>44% clay) compared with other soil types. Survival under all conditions was longer than 30 days for both culture independent and dependent methods, this is substantially longer than previous studies and, if this is an infectious dose, longer than the current recommendation of resting a field for 14 days to prevent onward infection.

1. Introduction

Ovine footrot is an infectious disease of sheep that is caused by an anaerobic bacterium *Dichelobacter nodosus*, which is the essential transmitting agent [23,44]. The disease is transmitted from sheep to sheep via pasture or bedding. It is characterized by interdigital inflammation, with or without separation of the keratinous hoof horn from the underlying dermis. Both conditions result in lameness and loss of body condition [41].

Footrot is the main cause of lameness in sheep in the UK [15,20] and accounts for serious economic losses in countries worldwide [35,40]. Recent reports indicate that footrot results in annual losses of between £24 and £80 million in the UK [29,41]. Its significant financial impact is due to a reduction in meat and wool production and the expenditure associated with treatment and prevention [13,35,40,41]. It is a painful condition and thus is an important and challenging welfare issue [11].

Footrot is seasonal in some areas of the world, particularly arid areas of Australia and India, where there are predictable periods of transmission [13]. In the UK, there is no seasonality for severe footrot, rather a series of mini-epidemics throughout the year [14] with increasing temperature and rainfall favouring spread of the disease [34]. Epidemics of interdigital dermatitis are also reported in spring [42].

Part of the strategy used in control and elimination programmes is empirical evidence that *D. nodosus* cannot survive for more than a few days off the feet of ruminants [1,4]. Survival away from the host is dependent on moist, mild conditions [12] and *D. nodosus* is reported to survive at an infectious dose for no longer than two weeks on pasture under optimal (warm and damp) conditions [4,43]. A recent study using qPCR demonstrated that *D. nodosus* can survive up to 14 days in soil microcosms at 5 °C and for a further 24 days if powdered hoof horn was added to soil; survival was markedly reduced at 15 °C however, the moisture content was not stated [7]. Nevertheless such work is important to move to an evidence-based approach to manage environmental contamination with *D. nodosus*.

To date, the environmental conditions and soil types where survival is greater, have not been elucidated and further work is warranted on *D. nodosus* survival outside the host, taking into account edaphic factors that result in dramatic differences in matric

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potential and water availability. Whilst a large number of studies have focused on *Escherichia coli* survival in soil, it is still uncertain how fluctuations in water availability affect survival [37]. Survival studies are uninformative without taking soil type into account if bulk soil is used in microcosms. For these reasons this study focused on the survival of *D. nodosus* in soil to investigate the impact of soil type, temperature and matric potential on longevity of viable *D. nodosus* in soil.

2. Material and methods

2.1. Cultivation of D. nodosus on solid and liquid media and its growth conditions

A virulent strain of *D. nodosus* (VCS1703A) was cultured on 2–4% Trypticase Arginine Serine Hoof agar (TASH) and Trypticase Arginine Serine (TAS) (Becton, Sparks Maryland, USA; Sigma–Aldrich, St. Louis, USA) and incubated at 37 °C for 3–4 days in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, UK) [33,36]. The cells were harvested in 2 ml phosphate buffer saline (PBS) solution to yield a final concentration of approximately 10^8 cells ml^{-1}.

2.2. Soil microcosms

Soil was grounded with mortar and pestle and sieved through 4 mm mesh to produce soil pores of more uniform size as reported previously [45]. Soil microcosms (50 ml Falcon tubes without aeration vents) containing 5 g of non-sterile Warwick soil (sandy loam) were set up in triplicate at −33 kPa, matric potential (MP defined as the suction required to extract water as soils dry out [3] calculated from previous wetting–drying curves of Warwick soil [9]; Fig. S1). The soil bulk density was maintained at 0.48 g/cm^3. *D. nodosus* was inoculated to the soil microcosms to a final concentration of 10^8 cells g^{-1} soil at −33 kPa. Microcosms were incubated aerobically at 25 °C in the dark and destructive sampling was performed at days 0, 1, 2, 3, 6, 10, 14, and 21. A sample of soil 0.5 g was taken for DNA extraction and 0.5 g for RNA extraction to which 1 ml of RNA*later* Stabilization Solution (Life Technologies Ltd, Paisley, UK) was added and stored at −80 °C.

Soil microcosms with different non-sterile soil types (20 g) including Warwick (sandy loam), Basilicata (sandy loam), Abanilla (sandy) and Stockton (clay) (soil characteristics Table 1; wetting–drying curves Fig. S1) were set up in triplicate, inoculated with *D. nodosus* culture (10^7 cells g^{-1} soil). The soil bulk density was maintained at 0.78 g/cm^3. Microcosms were incubated at 25 °C in the dark. Further, a second triplicate set of microcosms were incubated at 5 °C and destructive sampling of both sets was performed at days 0, 3, 6, 9, 12, 20, 30, and 40. Uninoculated soil was used as control to determine *D. nodosus* background amplification.

2.3. Extraction of DNA from soil

DNA was extracted from 0.5 g soil samples using the FastDNA Spin kit for Soil following the manufacturer's instructions (MP Biomedicals, UK). DNA was then eluted into 70 μl of DNase Free water. Sterile soil was included as blank.

Table 1
Characteristics of four soil types.

Soil type	pH	Sand	Silt	Clay	TOC	Moisture	USDA texture
Stockton	8.00	17.65	37.93	44.42	10.89	52.7	Clay
Abanilla	7.39	63.60	11.52	28.32	1.00	23.9	Sandy loam
Basilicata	8.16	60.00	11.76	27.12	1.39	32.1	Sandy loam
Warwick	8.00	63.00	18.40	11.70	6.2	18.0	Sandy loam

2.3.1. Extraction of RNA and synthesis of cDNA

RNA was extracted from Warwick soil samples collected at different time points using RNeasy Mini Kit (QIAGEN, Manchester, UK) and Griffith's method with some modifications. RNA integrity and concentration were measured using the Prokaryote Total RNA nano (Agilent Bioanalyser). DNase treatment of the total nucleic acid was performed using Turbo DNA free kit following the manufacturer's instructions. From DNA-free RNA, cDNA was synthesized using High capacity RNA-to-cDNA Kit (Applied Biosystems, Warrington, UK).

2.4. End point PCR and qPCR

PCR amplifications targeting *D. nodosus* 16S rRNA were performed using specific primers Cc and Ac [24] in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) as described previously [28]. *D. nodosus* DNA from the strain, VCS1703A was included as a positive control and sterile water as a negative control.

The qPCR of the DNA and cDNA was performed in an Applied Biosystems, 7500 Fast real-time detection system (Applied Biosystems, Warrington, UK). The PCR targeted the *rpoD* gene (RNA polymerase sigma 70 factor, single copy number in *D. nodosus* genome) [6]. All PCR reactions were performed in triplicate as previously reported [28]. The *rpoD* copy number was estimated based on the standard curve obtained from analysis of ten-fold serial dilutions of DNA extracted from *D. nodosus* strain VCS1703A. The results were analysed using 7500 Fast System SDS software (Applied Biosystems, Warrington, UK).

2.5. Soil desiccation curves

To select the appropriate microcosm set up, drying out of soil was determined for up to 17 days using Warwick soil. Four different microcosm types were set up using 20 g soil in 50 ml falcon tube and included closed, open, partially closed and closed with aeration vents (two holes punched in lid and two in sides above soil level). Soil was prepared as previously reported [45]. Soil samples were moistened to approx. −33 kPa. The microcosms were incubated at 25 °C in the dark and reduction in weight was recorded at each day until reductions in weights were constant.

2.6. Cultivation of D. nodosus from soil microcosms

Soil samples taken at different time points over 40 days from the inoculated microcosms were streaked (approximately 100 μg attached to the tip of the loop and soil suspension (1:10) in quarter strength Ringer's (100 μl)) on 2% TASH plates and incubated anaerobically at 37 °C for 3–4 days. The colonies on 2% TASH plates were sub cultured on 4% TASH and again incubated anaerobically at 37 °C for 3–4 days for isolating the pure colonies. The colonies obtained on the 4% TASH were confirmed as *D. nodosus* by Gram staining and a *D. nodosus* specific 16S rRNA gene colony PCR.

2.7. Live and dead cell microscopy and propidium monoazide treatment

The presence of live and dead cells in *D. nodosus* culture (cells were heat treated at 100 °C for 15 min to obtain dead cells while untreated served as live cells) was analysed using 20 mM DAPI (4′, 6-Diamidino-2-Phenylindole, Dihydrochloride) and PI (Propidium Iodide) incorporation assay. *D. nodosus* cells were washed and resuspended in 0.85% NaCl, to which a mixture of DAPI and PI was added and incubated for 15 min in the dark. The cells were observed under fluorescent microscope to confirm the dead cells.

Anaerobically cultured *D. nodosus* cells on 2% TASH were

harvested (10^6 cells ml^{-1}) and heat treated at 100 °C for 15 min to produce dead cells. Untreated (live cells) served as a control. Serial dilutions of *D. nodosus* live and dead cells starting from 10^6 ml^{-1} were treated with propidium monoazide (PMA; Biotium, Hayward, CA) by following the manufacturer's instructions with some modifications. PMA penetrates the compromised membranes of dead cells and intercalates with the DNA, inhibiting PCR amplification [30]. DNA was extracted from non-PMA treated live cells and PMA treated dead cells using DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK) following the manufacturer's instructions. Quantification of *D. nodosus* was determined by a qPCR assay targeting *rpoD* gene.

Soil samples taken at different time points from Warwick soil microcosms were PMA treated as described above with some modifications. DNA was extracted from PMA treated samples using the FastDNA Spin kit for Soil (MP Biomedicals, UK) by following the manufacturer's instructions. A qPCR assay targeting *rpoD* was performed to determine the number of live cells in the given sample.

2.8. Turnover of D. nodosus DNA in soil

Soil microcosms containing 1 g Warwick soil were inoculated with *D. nodosus* in the form of live cells, dead but intact (UV treated), dead (Heat killed) and free DNA at a final concentration of 10^7 cells and *rpoD* genome equivalents in case of free DNA. The microcosms were moistened to −33 kPa and incubated at 25 °C in the dark. Destructive sampling was performed in triplicate at days 0, 4, 7, 10, 14, 20, 25, 30, 40, 50 and 60. DNA was extracted from 0.5 g soil and *D. nodosus* was enumerated by a qPCR assay targeting *rpoD* gene.

2.9. Statistical analysis

The *rpoD* mean counts obtained from the qPCR were compared between test days for Warwick soil using two-sample T-Test in GraphPad Prism 5.0 to test for significant differences in the amount of *D. nodosus* detected at various time points. The exponential decay rates were calculated using the formula $P(t) = P_0 e^{-rt}$, where P (t) = Amount of *D. nodosus rpoD* genome equivalents at time t; P_0 = Initial amount of *D. nodosus rpoD* genome equivalents at time t = 0; r = the decay rate; t = time (number of periods). Models were built using MlwiN 2.1 [31] to investigate soil parameters including soil type, incubation time, temperature, pH, total organic carbon, moisture, sand, silt, clay in univariable and multivariable mixed effect models to determine whether all parameters together or individually predicted the survival of *D. nodosus* in soil.

3. Results

3.1. Persistence of metabolically active cells in the soil microcosms

Survival studies indicated that *D. nodosus* had a biphasic decline in soil with a rate of 0.53 day^{-1} up to day 6 and 0.12 day^{-1} from 7 to 14 days (Fig. 1A). Metabolically active cells were detected using cDNA from *rpoD* RNA and declined in a biphasic manner as observed for DNA genome equivalents at 0.517 day^{-1} from day 1–6 and 0.021 day^{-1} from day 6–21 which was significantly lower than the initial rate of decline (p = 0.0049); the decay rate was constant after day 6 (Fig. 1B).

3.2. Impact of aeration and survival of D. nodosus by soil type and temperature

For the first 10 days, moisture declined most rapidly in the open microcosms at 1.5% followed by ventilated, (1.4%) partially closed,

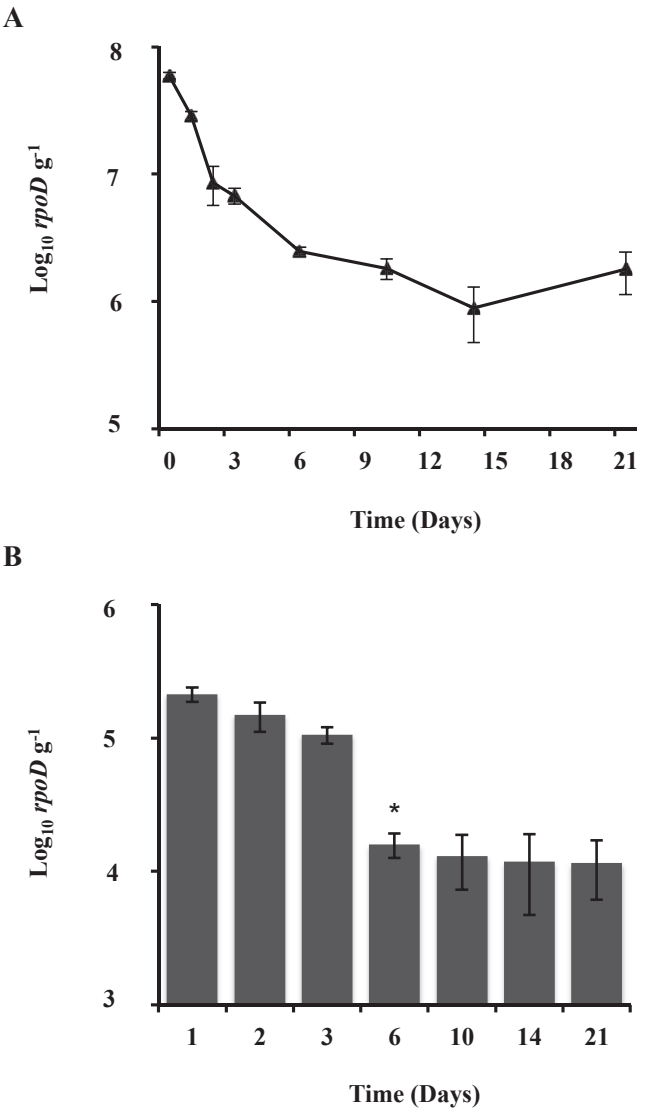

Fig. 1. Molecular detection and quantification of *D. nodosus* in soil. **(A)** Quantification of *D. nodosus* by qPCR targeting *rpoD* gene **(B)** Detection of metabolically active cells using cDNA qPCR derived from RNA. Bars represent mean ± SD; n = 3; (*p = 0.0049).

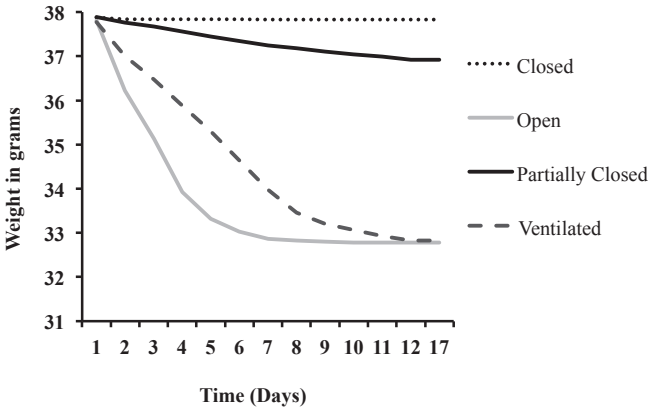

Fig. 2. Soil desiccation curved. Drying out of soil under different microcosm types, closed; partially closed; ventilated; open. Drying out of soil was determined using 20 g of Warwick soil using four different microcosm setups. The decline in moisture was determined at each time point until the complete drying out had occurred.

(0.2%) and closed microcosms, (0.01%) (Fig. 2); after 10 days rates were constant. Therefore, the microcosm with aeration vents was chosen for further experiments.

We detected *D. nodosus* 16S rDNA by end point PCR in all soil types at each time point during the 40-day study at both 5 °C and 25 °C. *D. nodosus* colonies were observed in culture over 40 days and confirmed by staining and colony PCR with the exception of Warwick soil at 25 °C on day 40 (Table S1).

At day 0, *D. nodosus* was present at 10^7 *rpoD* genome equivalents in all soil types and declined to below 10^5 *rpoD* genome equivalents over 40 days at 5 °C and 25 °C in Warwick, Basilicata and Abanilla soils (Fig. 3). There was a biphasic rate of decay in *D. nodosus* populations in the four soil types (Table 2); Stockton (heavy clay) had a significantly slower rate of decay in both phases compared with other soil types. In the univariable mixed effects model, the decay in survival over time of genome equivalents of *D. nodosus* was a cubic function of day (days^1, ^2 and ^3) demonstrating a non-linear decay over time. The %TOC, %moisture, %clay, %silt, were significantly associated with increased survival time, %sand was associated with a decreased survival time and pH was not associated with survival time when each of these variables were fitted in turn (Table S2). In the multivariable model, once survival over time was fitted, *D. nodosus* survived for significantly longer at 5 °C compared with 25 °C and in Stockton (heavy clay) soil compared with other soil types (Table 3). No other variables were significant because soil types were highly associated to %TOC, moisture, %clay, %silt (Table 1).

Table 2
Rate of decline per day of *D. nodosus* in four different soil types at two temperatures over two phases. 'D' denotes time interval in days for rate calculated.

Soil type	Decay rates at 25 °C (DNA decrease day^{-1})		Decay rates at 5 °C (DNA decrease day^{-1})	
	(D0–D9)	(D9–D40)	D0–D30	D30–D40
Stockton (Clay)	0.61	0.78	0.60	0.28
Abanilla (more sandy)	0.95	0.49	0.52	0.94
Basilicata (Silt)	0.96	0.67	0.88	0.96
Warwick (sandy loam)	0.96	0.56	0.84	0.94

Table 3
Mixed effects model of factors associated with mean *D. nodosus* genome equivalents adjusted for replicate and day, 192 sample.

	Mean	S.E.	Lower 95% CI	Upper 95% CI
Intercept	6.987	0.129	6.734	7.240
Days^1	−0.116	0.025	−0.165	−0.067
Days^2	0.005	0.002	0.001	0.009
Days^3	0.000	0.000	0.000	0.000
Temp 5 °C	Baseline			
Temp 25 °C	−0.647	0.082	−0.808	−0.486
Clay soil	Baseline			
Soil type_1	−0.35	0.116	−0.577	−0.123
Soil type_2	−0.431	0.116	−0.658	−0.204
Soil type_3	−0.254	0.116	−0.481	−0.027

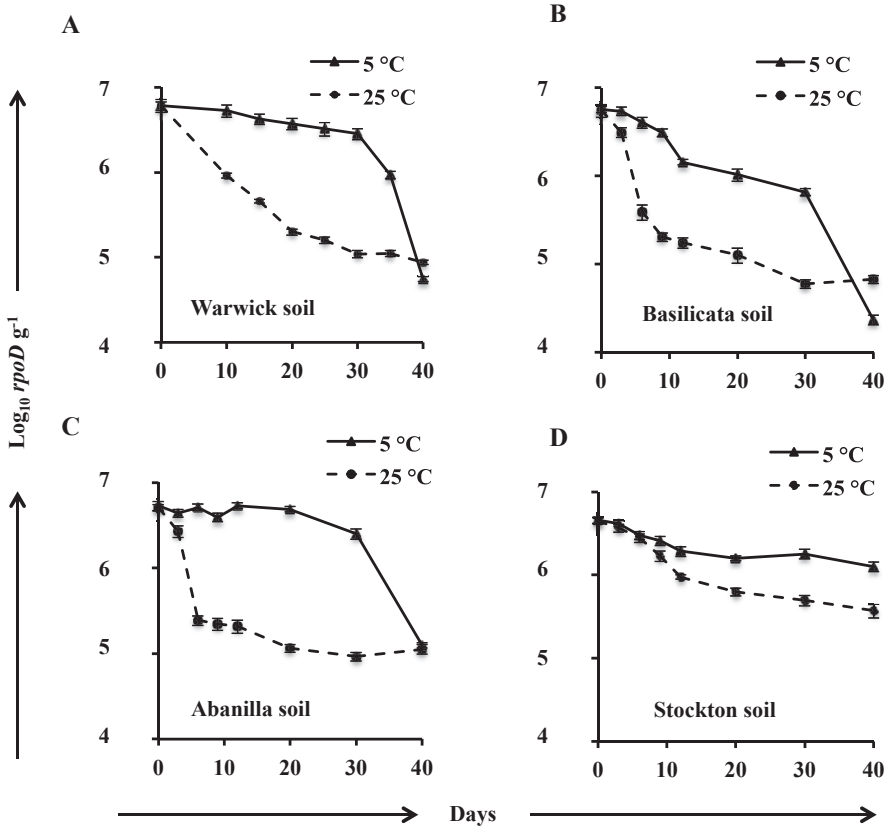

Fig. 3. Absolute quantification of *rpoD* gene from DNA over 40 days (Error bars represent ± SD). **(A)** Quantification of *rpoD* gene from Warwick soil. At day 0, *D. nodosus* was present at 10^7 copies and declined to 10^5 over 40 days. **(B)** Quantification of *rpoD* gene from Basilicata soil. **(C)** Quantification of *rpoD* gene from Abanilla soil. **(D)** Quantification of *rpoD* gene from the Stockton soil. The DNA was extracted using fast DNA spin kit for soil and elution was done in 70 μl of the elution buffer.

3.3. Direct evidence for survival of viable cells in Warwick soil microcosms

The heat-treated cells were dead as observed by PI staining (Fig. 4A). PMA treatment of dead cells resulted in a significant reduction in amplification (Fig. 4B) with background amplification of 10^2 cells, which was constant throughout the dilution series. These results confirm that PMA detected dead cells. At day 30, *D. nodosus* was present in Warwick soil at 10^5 *rpoD* genome equivalents as detected by qPCR but PMA treatment led to significant reduction ($p = 0.0002$) to just above the detection limit of 10^2 *rpoD* genome equivalents. There was no significant difference in the number of live cells observed at 5 °C and 25 °C at day 30 (Fig. 4C).

3.4. Turnover of D. nodosus DNA in soil

DNA turnover in Warwick soil was fastest for free DNA (over 10 days) followed by UV and heat-treated cells. The live and intact UV treated cell survivals were not significantly different (Fig. 4D and Table 4). Rates of decline were biphasic for all with change in rate as follows: day 4 for heat-treated; day 7 for free DNA; day 10 for live cells and day 14 for UV treated cells (Fig. 4D).

Table 4
Rates of decay per day of *D. nodosus* DNA from various sources at 25 °C over two time periods.

Cell type	Decay rates (DNA decrease day^{-1})	
Live	D0–D10	D10–D60
	0.521	0.007
UV treated (dead intact)	D0–D14	D14–D60
	0.415	0.007
Heat treated (dead)	D0–D4	D4–D60
	1.502	0.013
Free DNA	D0–D7	D7-D60
	1.335	0.003

4. Discussion

This is the first study to demonstrate that viable *D. nodosus* is detectable for 40 days off host in microcosms with aeration vents and that survival rate is affected by moisture, temperature and soil type. This is a step change in our understanding of survival of *D. nodosus* off host. A previous study of transmission of *D. nodosus* from floor to sheep indicated a 10-day off host survival of an infectious dose, defined by causing disease [43] and a microcosm

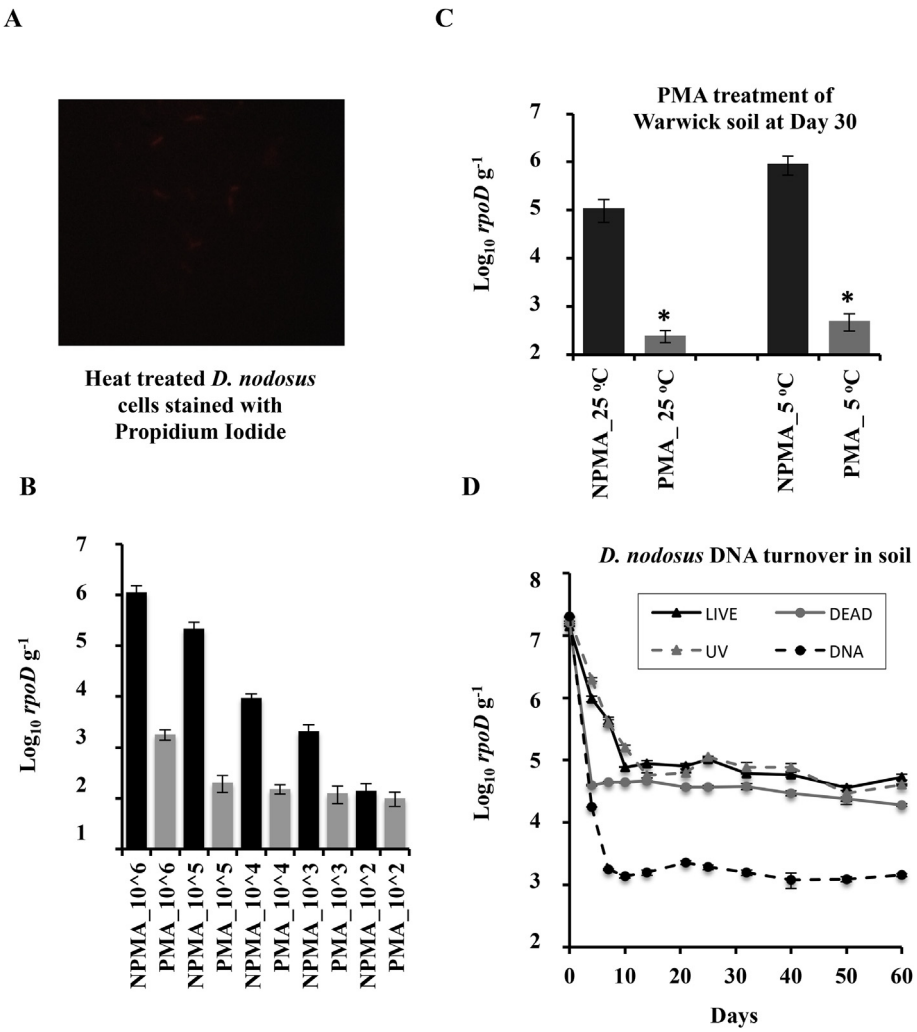

Fig. 4. Viability of *D. nodosus* using propidium monoazide method. **(A)** Live-dead staining with PI, red indicating dead cells. **(B)** Quantification of *rpoD* gene of the PMA treated *D. nodosus* cells at various dilutions. PMA denoted PMA treated and non-treated is NPMA. **(C)** Comparison of non-PMA and PMA treated Warwick soil sampled on day 30 incubated at 5 °C and 25 °C. Error bars represent mean ± SD; n = 3; (*p = 0.0002). **(D)** Turnover of *D. nodosus* DNA in soil *rpoD* genome equivalents; soil inoculated with live, dead, UV treated, and free DNA (Error bars represent ± SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

study reported detection of *D. nodosus* for 14 days [7]. In the current study, populations of *D. nodosus* ~10^6 cells were detected by qPCR in desiccated, cool, clay soil after 30 days (Fig. 3). Given that approximately 10^2 cells were viable at day 30 in Warwick (non-clay) soil where qPCR was 10^4, it is likely that $>10^4$ cells were viable in clay soil at day 30 because of the overall higher load. In addition, the analysis of mRNA in the current study supported the observation of a significant viable *D. nodosus* population of 1.1×10^4 at 21 days; this was in microcosms with constant moisture content. Although it is not possible to determine whether 10^4 is an infectious dose, it is a similar load to that detected on healthy and footrot affected feet by [44] and highlights that certain soils, in cool damp conditions, might be a source of an infectious dose of *D. nodosus* for more than 30 days.

Non-sterile soil microcosms with four contrasting soil types were used that allowed soil to undergo drying to mimic the environmental conditions, survival in a range of MP's in soil can significantly impact on survival [32]. The wetting and drying curves of the four soils were determined so that the correct volume of water was added to standardize MP at −33 kPa average field moisture content for the UK. During trial desiccations studies the MP varied from −33 kPa to −10,000 kPA in heavy clay soil of Stockton and from −33 kPa to −37 kPa in Basilicata, a sandy loam (Fig. S2). This reflects the suction required to extract water as these soils dry out [3]. Previous work focused on *D. nodosus* survival in soil but did not consider changes in MP nor soil type and reported survival up to 14 days at 5 °C and 7 days at 15 °C respectively but water content was kept constant and MP was not determined in the soil used [7].

Studies on the survival of bacterial pathogens in soil have used viable cell counts [25,26,38] and molecular assays [18]. In the current study we used both, since *D. nodosus* does not form distinct colonies due to twitching motility: qPCR was used to enumerate cells and then RNA extraction and an in situ viability dye (PMA) were used provide a robust measure of viability. There was a significantly lower viable count than total count in the current study but still moderate loads of viable cells. Very few studies have used PMA on starved cells in soil therefore further work is needed to investigate cell integrity during survival.

The most significant reduction in *D. nodosus* DNA occurred in the first four days after inoculation with free DNA when water was present, in contrast, *D. nodosus* DNA persisted in soil for up to 60 days when the microcosm had dried out and prevented further degradation of DNA after the first week. Similar trends in turnover of free DNA and degradation were reported for *Mycobacterium bovis* DNA [45]. Their study also demonstrated that dead cells degraded quickly and that DNA did not persist at normal field moisture content for Warwick soil [45]. Thus it is likely that *D. nodosus* DNA detected in the current study is from living or, at worst, recently dead cells.

The biphasic decline in *D. nodosus* load in all soils related to water content indicates the critical drying out MP of each soil. In the case of Stockton this was −1033 kPa at 25 °C but only −58 kPA in Abanilla. It is plausible that bacterial cells are so small that they do not respond in the same way to large changes in MP. Stockton soil showed the least decline in percentage water loss and survival mirrored changes in water content.

qPCR provided clear evidence of different t_d by soil type with slowest t_d in heavy clay soil (Stockton). Edaphic factors are critically influential as clay soils can hold more water than sandy soils and dry out more slowly, however, in this experiment the MP increased dramatically in clay soil as drying out resulted in greatly increased suction. This did not impact on *D. nodosus* survival, in contrast to the impact of MP on plants [2], and therefore strongly suggests that bacteria can survive within clay lattices as they dry out but retain water with *D. nodosus* surviving in a clay soil with MP of ~−10,000 kPa. It is likely that the clay content improved survival of culturable cells in Stockton despite the high suction required to access water in a dry clay soil. Gram-negative bacteria studies in soil have demonstrated that clay content [25]; water availability [32]; organic carbon [39] and strain type [26] could all markedly impact survival, with a general trend of heavy, wetter clay soils with higher organic carbon aiding survival. Other features of clay include buffering capacity and better nutrient availability [5,10,16]. The decrease in particle size due to increased clay content increases the surface area of the soil and may result in protection from parasites, predators, toxins, UV and antibiotics [17,19,21]. The type of clay also influences survival and more work is needed on this. Selected studies have implicated the role of the natural community as a key driver in the survival of pathogens in soil where for certain soils the community had a negative effect on survival [27]. Following our analysis of the physicochemical properties affecting *D. nodosus* survival, work is needed to address the microbial community impacts but these are likely to be soil specific.

5. Conclusions

From these findings, we conclude that for the gram-negative pathogen *D. nodosus*, a temperature of 5 °C compared with 25 °C, and clay soil compared with other soil types, allowed survival of greater loads of *D. nodosus* over time than the previously reported studies. The results from field trials in Australia and from farmers' empirical experience in the UK suggest that sheep are not at risk from *D. nodosus* infection once fields have been rested for 14 days. The current study has indicated that *D. nodosus* can survive in soil with viable cells detected at day 40, and tolerate ultra-low soil MPs. Further work is warranted to determine how clay impacts pathogen survival in soil and whether surviving cells are infectious, and at what dose, and work investigating presence of cells from field samples would provide information on infection load in rested fields.

Conflicts of interest statement

None of the authors have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2015.12.010.

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