THE EFFECT OF TEMPERATURE AND WATER ON THE SURVIVAL AND VIRULENCE OF YERSINIA ENTEROCOLITICA AND YERSINIA PSUEDOTUBERCULOSIS

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Abstract

*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are enterobacteria pathogenic to humans and a wide variety of domestic and wild mammals and birds. These organisms have been associated with a weaner enteritis and mortality syndrome in Merino sheep occurring predominantly in the high rainfall, colder climate regions of SE Australia. *Yersinia enterocolitica* can be isolated from grazing livestock all year round, whereas shedding of *Y. pseudotuberculosis* occurs seasonally.

The aims of this study were to better determine the effect of desiccation and exposure to a range of temperatures. Temperature gradients trialled reflected those that could occur in the high rainfall regions in Southern Australia, where this syndrome is prevalent. The study also examined the effect of temperature and desiccation on the retention or resilience of a plasmid coding gene for virulence.

In brief, field strains of *Y. pseudotuberculosis* and *Y. enterocolitica* were inoculated into faecal pellets, deposited onto soil in boxes and incubated at different fixed and fluctuating temperatures for up to 40 days. The rate of recovery of organisms from faecal pellets was determined by assessing the amount of growth (density of colonies) on the media at 36 hours. *Yersinia pseudotuberculosis* was isolated at consistently higher rates compared to *Yersinia enterocolitica* when adjusting for day, water and temperature treatments, and repeated measurements (*P*<0.001).

The virulence factor examined was not affected by desiccation or temperature. This investigation is a first step towards a better understanding of the effect of climate on survival of *Y. enterocolitica* and *Y. pseudotuberculosis* that infect livestock.

Introduction

*Yersinia* spp. are potentially pathogenic to humans and a wide variety of domestic and wild mammals and birds. These organisms have been associated with a weaner enteritis and mortality syndrome in Merino sheep occurring predominantly in the high rainfall, colder climate regions of SE Australia. Yersiniosis in sheep is caused predominantly by *Yersinia pseudotuberculosis* serotype III and *Y. enterocolitica* biotype 5, serotype O:2,3 (Slee & Skilbeck 1992).

There are differences in the epidemiology of the species of *Yersinia* pathogenic to sheep, with *Yersinia enterocolitica* isolated from grazing livestock all year round, whereas shedding of *Y. pseudotuberculosis* occurs seasonally, mainly in cooler high rainfall seasons, and is rarely found at other times of the year. There is no clear understanding as to why these differences occur.

Materials and methods

A controlled concentration (optical density 1.0) of two field strains of virulent *Yersinia enterocolitica* and one strain of virulent *Yersinia pseudotuberculosis* were inoculated into sheep faecal pellets. These pellets were placed at evenly spaced intervals on top of soil within plastic boxes, with each soil box containing only one strain of *Yersinia* species and exposed to a particular set of temperature and moisture conditions.

Sampling was conducted on nine occasions over 40 days. Treatments consisted of watered or dry soil and four fixed and three fluctuating temperatures, totalling 8 treatments for fixed
temperatures, and 6 treatments for fluctuating temperatures, with each treatment consisting of 3 replicates.

The soil for the boxes was collected from a property in a high rainfall region of south eastern Australia with a history of weaner scours due to Yersiniosis but from an area of the property not grazed by sheep. This soil was used to reduce the likelihood of residual contamination with *Yersinia* sp. in the soil prior to adding contaminated faecal pellets. The soil used in each box was from the same farm to control for pH, organic matter and microbial populations (Guan and Holley 2003, Tashiro et al. 1991).

The *Yersinia* isolates were obtained from samples submitted to the Mackinnon Project for investigation of outbreaks of disease in weaner Merino sheep in 2013 and 2014. The organisms chosen for inclusion in this experiment were one confirmed pathogenic strain *Y. pseudotuberculosis*, and two field strains of *Y. enterocolitica* isolated from two different properties in Victoria. One of the *Y. enterocolitica* strains was isolated from sheep with clinical disease and the other from asymptomatic sheep. The field strain of *Y. pseudotuberculosis* and the strain of *Y. enterocolitica* that were known to cause disease were associated with severe disease in sheep including, enteritis and mortality and abortion respectively. The asymptomatic strain of *Y. enterocolitica* was retrospectively classified as pathogenic after the presence of the 70 kb virulence plasmid gene *yadA* was confirmed by PCR. Known concentrations of these strains were then inoculated into individual faecal pellets collected from sheep confirmed not to have Yersiniosis.

The fixed temperatures selected the for treatment boxes were 20°C, 40°C, 4°C and -20°C. Each *Yersinia* sp. had two water treatments in each temperature group; one treatment box was watered every 48 hours in order to maintain a moist environment (watered), the other treatment left with the faecal pellets and soil dry (standard).

To investigate if a change in temperature influenced survival, *Yersinia* sp, were also subject to fluctuating temperatures (20 to 4°C, 4 to 20°C and 20 to 40°C). Soil boxes were moved between fixed temperature environments (e.g. fridge to room temperature) at 48 hour intervals.

At sample collection, faecal pellets were removed from their soil box treatments using sterile forceps. Individual pellets were then macerated with sterile water to produce a standard consistency and plated onto selective media (CIN agar). Growth was scored and recorded after 36 hours incubating at 30°C for 48 hours. Density of colony growth was scored from 0 to 4, where 0 was no growth and 4 maximum growth.

Where colonies morphologically consistent with *Yersinia* sp. were detected, a multiplex species PCR was applied to those isolates. DNA was chosen from each treatment in week one and from the week before colony morphology differed and subsequent weeks after morphology changed.

To determine pathogenic strains from non-pathogenic strains, isolates were screened for the presence of the virulence plasmid, by detection of the *Yersinia* adherence protein gene (*yadA*). YadA is partly responsible for resistance to phagocytosis and involved in the resistance of *Yersinia* sp. to the antimicrobial activity of polypeptides from human granulocytes (Cornelis et al. 1998). A simplex PCR, to assess virulence associated with the presence of the *yadA* gene, was applied to samples confirmed as positive in the multiplex species PCR. The samples tested were selected to demonstrate that the plasmid was present at day 0 and then to determine if and when it was lost by testing samples from the last point at which growth was positively identified as *Yersinia* sp. Only one replicate of DNA was tested for most samples.

**Statistical analysis**

The model used was a mixed effects ordinal logistic regression in Stata Version 13. This model was used as the culture score data was categorical and recorded over time. Associations were made between treatments based on the odds ratio and the 95% confidence intervals. One model compared *Y. enterocolitica* to *Y. pseudotuberculosis* over all treatments. Another compared all temperature and water treatments for *Y. enterocolitica* only and lastly treatments for *Y. pseudotuberculosis* only.
Figure 1: Growth scores for watered treatments of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* incubated at fixed temperature of 40°C and fluctuating temperatures of 20°C to 40°C. Plots jittered on x and y axis.
Results

Survival between Yersinia species for all treatments

At day 0, all CIN plates for both fixed and fluctuating temperatures had high growth scores (3 or 4). Higher scores were observed for Y. pseudotuberculosis compared to Y. enterocolitica in all treatments, after adjusting for day, water and temperature and repeated measurements (P<0.001).

Over all treatments, the effect of water on the survival of either species of Yersinia was not statistically significant. The relative effect of water on survival for Y. pseudotuberculosis and Y. enterocolitica was 58% (P=0.54) and 20% (P=0.29) respectively. Yersinia pseudotuberculosis survival at 20°C was strongly associated with water treatment (P<0.001), whereas Y. enterocolitica survival was not.

The most pronounced difference between species occurred when the organisms were exposed to temperatures fluctuating between -20°C and 4°C. Under these conditions survival of Y. pseudotuberculosis was greater than Y. enterocolitica over all water treatments when adjusted for day and repeated measurements (P<0.001).

Compared to all other treatments the fixed 40°C and fluctuating 20°C to 40°C temperature treatments had the greatest rate of decline in survival of organisms, with the addition of water having no effect on the survival of either species. Yersinia pseudotuberculosis was recovered for 5 days more when fluctuating to 40°C compared to the fixed treatment at 40°C (Figure 1).

Fixed temperatures of 4°C and -20°C, and temperature fluctuations from 4°C to 20°C (both with and without water) supported the greatest survival of all Yersinia species. Yersinia pseudotuberculosis survived until the end of the study (day 40) when fluctuated between -20°C and 4°C and when watered at 20°C.

Detection of Yersinia sp. and the virulence plasmid (yadA gene)

DNA collected from Yersinia sp. at the last sample point of the study confirmed that Yersinia isolates did not lose the virulence plasmid gene yadA. At day 33 only one replicate was able to be tested for virulence for the standard treatments of the two strains of Y. enterocolitica at the fluctuation between 4°C and 20°C.

Discussion

Results from this study show that Yersinia enterocolitica and Yersinia pseudotuberculosis do not survive well in faecal pellets at temperatures over 20°C. Neither species survived more than 7 days when incubated at 40°C, nor when subjected to temperature fluctuations between 40°C and 20°C, even when watered. This suggests Yersinia sp. are unlikely to survive for over a week in faeces on soil if ambient temperature remains above 20°C.

These results were unexpected because it has been previously reported that Y. pseudotuberculosis had an optimal growth temperature of 22°C, and a maximum temperature tolerance to 33.7°C (Bhaduri & Phillips, 2011), whilst Yersinia species could multiply at temperatures reaching 43°C (Moxley 2013). However, the current study design inoculated organisms into faecal pellets placed onto soil, which was in contrast to many previous studies which used nutrient rich broth or media. It is possible that such nutrient rich environments may have facilitated the longer survival. Alternatively, in nutrient broths there may have been less competition for available nutrient compared to the potentially less nutrient rich environment of a faecal pellet in which many other microorganisms are present.

A previous study demonstrated that survival of Yersinia enterocolitica in soil and water was greater at 4°C, compared to at 20°C, because other enterobacteria were unable to survive at temperatures below 4°C (Tashiro et al. 1991). Conversely, Yersinia sp. are poor competitors with other psychotropic bacteria at temperatures above 4°C (Stern, Pierson, & Kotula, 1980). Thus, at higher temperatures, the rapid clearance of Y. enterocolitica and Y. pseudotuberculosis may be partly explained by other bacteria being better able to survive and compete for available nutrients within the faecal pellet.

Previous studies have shown that infection with Yersinia pseudotuberculosis is quite seasonal, occurring mainly in cool, wet months, whereas infection with Y. enterocolitica...
occurs throughout the year (Slee & Skilbeck, 1992). The current study demonstrated that, when incubated at 20°C and watered, *Y. enterocolitica* and *Y. pseudotuberculosis* survived for 19 and 40 days, respectively. The increased environmental survival of *Y. pseudotuberculosis* makes it potentially less reliant on persisting in the gastrointestinal environment of a host animal for effective transmission. However, the opposite was observed for *Y. enterocolitica*, and so it is potentially more reliant on surviving in the host. This suggests that *Y. enterocolitica* may need to cycle through a number of host animals in a flock in order to build sufficient environmental contamination to initiate transmission to new hosts, and this is consistent with the shedding of *Y. enterocolitica* throughout the year.

Water enhanced the odds of survival of *Y. pseudotuberculosis* when it was incubated at 20°C. This highlights that additional moisture, in the form of rainfall or increased retention of moisture in soil and vegetation, may assist its environmental survival and is consistent with the winter outbreaks of Yersiniosis due to this bacterium. This is also supported by the observation that in temperate regions, greater numbers of *Y. enterocolitica* were found in soil from deciduous forests compared with soil from grasslands (Botzler, 1987). *Y. pseudotuberculosis* survived for 40 days when watered and incubated at 20°C, confirming that moist environments favour environmental survival of this *Yersinia* species. It also highlights the difficulty in decontaminating pasture following an outbreak of yersiniosis the previous winter, especially if frequent rainfall occurs during the summer or an early autumn break makes conditions more favourable for survival and transmission.

Temperature fluctuations were included in the study design to test the hypothesis that if the bacteria were not exposed to continually hostile conditions (i.e. extremes of fixed temperatures) they would survive for longer. The results suggest that pathogenic *Yersinia* may be able survive in faeces throughout much of the year in temperate climates.

The temperature fluctuation of 20°C to 4°C was chosen to be representative of winter and early spring in south east Australia, a region that experiences severe seasonal outbreaks of yersiniosis. The results confirm that pathogenic *Yersinia* has the capacity to survive for at least 40 days in faecal material under simulated ‘natural’ conditions in this area, with the potential to survive much longer under field conditions. This makes the implementation of management procedures to decrease transmission quite a challenge, with less decontamination of pasture compared to other areas with less summer rainfall and hotter temperatures.

Exposure to high temperatures (37°C) can lead to the loss of the 70 kb virulence plasmid carried by *Yersinia*, whereas it persists between 1 and 31°C (Goverde, Kusters et al. 1994). Consequently, it was important to investigate if exposure to high temperatures, and subsequent desiccation, had damaged the virulence plasmid in the isolates used in the current study. Contrary to expectations, the yadA gene within the virulence plasmid was not lost. However, although the plasmid was still present it may not necessarily be functional and capable of causing disease, and so this aspect deserves further investigation.

Shade from grass, rocks and trees, and damp areas around dams and sheep camps, could allow increased survival of *Yersinia* bacteria. These factors need to be considered in future studies, especially when investigating attempts at decontamination or management strategies that aim to decrease transmission and the expression of disease. For example, to reduce transmission of infection and the likelihood of disease outbreaks it may be advisable to avoid grazing susceptible animals, such as weaned lambs, on pastures grazed by affected sheep the previous season. It is unlikely that pasture can be made ‘free’ of *Yersinia* species, but systems to decontaminate pasture could also be devised. In general these will be consistent with current recommendations for internal parasite control in this region, such as ‘Smart Grazing’ (Niven et al, 2002), or reducing the transmission of other diseases transmitted by the faecal oral route, most notably ovine Johne’s disease. Preparation of lower risk pastures should use mature animals that are likely to shed fewer *Yersinia* (Slee & Skilbeck, 1992), with pastures grazed to a shorter length enabling increased mortality of organisms due to increased exposure to ultraviolet light and desiccation.
References