ENVIRONMENTAL SURVIVIAL OF OVINE JOHNE'S DISEASE IN PASTURE SPELLING

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Summary

Mycobacterium avium subsp. Paratuberculosis (MAP) is the agent responsible for Johne's disease, which is a significant biosecurity risk in the livestock industry as it is difficult to control and causes production and economic losses. It is recommended that spelling contaminated pasture for 6 months is sufficient to remove risks of infection. Prior studies have found that MAP is capable of surviving beyond this point depending on environmental conditions. These studies however, were conducted in controlled laboratory settings, replicating environmental effects on MAP survival. To assess these findings in a real world pasture setting, this experiment sampled faecal samples from 17 different locations on pastures, with varying shade and ground coverage, over a 6month pasture spelling period. Samples were assessed using a high throughput Johne's test with quantitative PCR analysis as well as faecal culture. Samples collected from completely shaded sites returned positive result through-out the investigation, with some months also seeing positive results from unshaded sites. The unshaded positives however occurred during periods of high rainfall, which may have affected detection of MAP DNA presence. The consistency of the positives from the shaded sites indicates that an increase in environmental temperatures plays a crucial role in reducing MAP presence, which is in agreement with prior studies. It can be concluded that evidence suggests current recommended control measure of pasture spelling to manage MAP contamination are effective if areas are exposed to adequate sunlight.

I. INTRODUCTION

Johne's disease is a chronic enteric infection caused by the pathogen *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Transmission of MAP occurs most commonly via the faecal oral route (Whittington et al. 2005) and is characterised by chronic wasting; loss of body condition and not responding to good feed, with scouring also seen in some cases and species (Begg & Whittington 2008). These clinical signs of infection however may not be demonstrated for years, while the host remains subclinically infected (Whittington et al. 2004). During this time the host may be shedding large amounts of the pathogen, causing wide spread contamination of pastures. In addition to the difficulty in detection of infection, MAP has proven to be long-lived outside the host (Whittington et al. 2004; Eppleston et al. 2014; Whittington et al. 2005). These characteristics of MAP make the organism difficult to control when outbreaks occur and can cause large economic impacts on businesses.

There are three strains of MAP that have been identified; S-strain (ovine), C-strain (bovine) and a Bison Strain (Stevenson 2010; Whittington et al. 2001). The S- strain and C-strain are considered to be of greatest agricultural significance in Australia due to sheep and cattle being the

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predominant species affected, although other species such as cervids have been known to be infected (Heuer et al. 2012; Machackova et al. 2004).

One of the current recommended measures for control for MAP contamination is pasture spelling, with 6 months being the recommended period to spell (Animal Biosercuirty Unit 2008). However, studies such as those by Eppleston et al. (2014); Gumber & Whittington (2009) and Lamont et al. (2012) have found evidence that the organism is capable of surviving long periods given the right environmental conditions. These studies observed that MAP may employ a range of strategies to improve environmental survival such as a possible dormancy responses to extreme conditions (Gumber & Whittington 2009b) and a spore-like morphotype which is associated with nutrient starvation (Lamont et al. 2012). The most relevant observation of studies investigating environmental survival of MAP relative to the recommended paddock spelling guidelines is that evidence suggests that MAP has the ability to survive past the 6 month period, under certain conditions (Whittington et al. 2004; Eppleston et al. 2014; Whittington et al. 2005). Although these studies suggest that the length of environmental survival is strongly correlated with climate conditions such as rainfall and exposure to ultra violet radiation, they are limited by experimental designs being simulations of real world effects (Lovell et al. 1944; Salgado et al. 2013; Whittington et al. 2005; Eppleston et al. 2014).

The findings from previous studies bring into question the effectiveness of pasture spelling as a control method and whether a longer period may be required, if it is to be used as an effective method of MAP control. Therefore, the aim of this study was to investigate the effect pasture spelling has on the environmental survival of MAP, over a 6 month period under natural pasture conditions.

II. MATERIALS AND METHODS

a. Sample Collection

Four sampling sites were selected in each of the four paddocks with a positive control, giving total of 17 samples (Fig.1). The positive controls used in this experiment, were faeces from known infected sheep, with infection performed using standardized protocol (unpublished data). The four paddocks selected had previously experienced varying levels of MAP exposure ranging from high exposure to transient (Table.1). Sampling occurred once a month over a 6month period. The four areas selected with in the paddocks were marked using semi-permanent metal star pickets with site numbers marked. The samples were collected from a 1-meter radius around the star picket, which was marked out using measuring tape and paint around the area. The marked areas sampled from were selected based on varying ground coverage and environmental exposure, such as shaded and non-shaded.

Each collection aimed to attain 4 – 5 random pellets from each site, which were stored in a serum vial. These were then brought to the laboratory to have their moisture content recorded and stored in a -80^{0}C freezer for analysis when sampling is completed.

After the first sampling in September 2015, additional samples were added onto the paddock Q8. This was due to sheep being reintroduced to the paddock during the experiment. Sample numbers were then increased from 17 to 21.

Weather data for the duration of the study was also collected from the bureau of meteorology. This data was obtained from Camden airport which is located nearby the sampling areas (Fig.5 & 6).

b. Faecal Pooling Method

Stored samples from the 21 sites were weighed in the serum vial, before adding 3ml of sterile 0.85% saline. These were then mixed using a rocking platform and a vertical turn table for a

minimum of 2 hours to allow rehydration. The faecal material was then broken up using a wooden stick inside the serum vials to produce a well-blended pooled sample.

After each sample had been broken into a blended consistency, 1.5g of the pooled samples was weighed into 15ml tubes. These were then stored in a -80°c freezer for later culture. The remaining samples in the serum vials where stored separately in -80°c freezer for High through-put Johne's PCR analysis (HT-J) (Fig.2).

c. Faecal Culture

10mls of sterile 0.85% saline was added to each sample 1.5g pooled faecal sample in the 15ml tube. Negative and positive control faecal samples were performed to confirm the standard method (INFDIS SOP 04-15.1) with the addition of a further set of controls for the detection of growth (Plain et al. 2014; Whittington et al. 2013).

d. High-throughput Johne's Test (HTJ)

Prior to the experiment, 0.3ml of 0.1mm zirconia/silica beads were added to 2ml screw cap tubes. The pooled faecal samples were thawed and 0.3g (+/- 0.3g) of faecal samples and 10ml of sterile saline was added into 15ml saline tubes. Each sample was then shaken vigorously for 5 minutes by hand, allowed to settle for 30 minutes and 3-5ml from the top of the samples (not including faecal sediment) was transferred to a 15ml centrifuge tube. Samples were centrifuged (900 *x g* for 30 minutes at RT)

The supernatant was discarded, before adding 600µl of lysis/binding solution (Table.2). Samples were then mixed using a sterile pipette, transferred into the prepared 2ml screw cap bead tube and mechanically lysed using a Tissue Lyser II (1:40m/sec at the frequency of 30.0 1/s), twice. Following this, samples were centrifuged (16000 *x g* for 3min at RT) and 500µl of supernatant was transferred into a 1.5ml screw cap tube stored at 4°c overnight.

Reagents were prepared as per instructions from the Biosprint 96 One-for-All Vet kit (Table.3) and aliquoted into the plates as per (Table.4), before being covered to prevent any contamination.

The refrigerated samples were allowed to reach RT before being centrifuged (16000 *x g* for 3min), 400µl of sample was then added to the Proteinase K in the Lysate plate as per the plate plan (Table.4). Bead mix was then vortexed for 30 seconds before adding 300µl per well to the same plate.

Plates were then placed in the MagMax-96 as per the plate loading protocol and program 'BS96 Vet 100' run.

Once completed, the eluted DNA samples were transferred into a 96 well PCR plate. A separate 1:5 dilutions of DNA was prepared in buffer AVE. Samples not being analysed were stored at -20°C before analysis.

e. Quantitative PCR

PCR plates were loaded into the Stratagene MX3000P® qPCR machines, as per operating procedure. Denaturing and annealing run cycle parameters were set via MXPro® a qPCR software with the results being exported into text files. Both HTJ test samples and the faecal culture samples were analysed using these machines.

III. RESULTS

a. High-Throughout Johne's PCR Analysis to Detect MAP DNA

During the first month of the experiment, no positive results were detected apart from the positive controls placed at sample site 5.1. In the following month (October) samples 4.4, which were placed in the completely shaded area (paddock Q4) showed a positive result for MAP DNA. The samples collected form this site continued to return positive results throughout the remainder of the experiment (October – February). The amount of MAP DNA detected in these samples appeared to be decreasing over time, but shows a large spike during the January month (Fig.4).

As well as the shaded sampling area, samples collected from paddock Q8 returned positive results (sampling sites 3.1, 3.1A, 3.2, 3.2A, 3.3, 3.4). However, these were limited to the second half of the experiment (December – February) (Fig.2 & Fig.3).

Samples that returned positive results during this investigation appear to be mainly limited to the paddocks of high MAP exposure; Paddock Q4 and Paddock Q8 (Table.1) (Fig.3 & 4). Apart from one positive at site 2.1 during December, no MAP DNA was detected on paddocks D8, which experienced transient exposure and paddock D9, which had some known exposure.

b. Weather Data

Data collected from the Bureau of Meteorology shows that monthly average rainfall began to slightly increase during December 2015, when compared to the first two months of the experiment. This was followed by a dramatic increase in January 2016, reaching 254mm, before a sharp decline in February 2016 to 20.6mm (Fig.5).

Monthly mean temperature was seen to steadily increase through November 2015 – December 2015. After these months, mean temperatures began to level out for the duration of the experiment with the lowest between these last 3 months being 21.3°C in December 2015 and the highest 21.5°C in both January 2016 and February 2016 (Fig.6).

c. Faecal Culture

Culture analysis of the samples returned few culture positive results. In total only two of the samples gave a culture positive reading. The positives, detected were the positive control sampled at the beginning of the experiment from site 5.1 (January) and positive control samples from site 5.1 at the end of the experiment (results not shown).

IV. DISCUSSION

A major aim of this investigation was to conduct an examination of MAP environmental survival in a real world pasture setting. To achieve this, the experimental design was based around the likelihood of a positive sample in a 1 metre radius, with each sampling area having varying levels of ground coverage and shade. There were certain limitations when structuring the experimental design that accommodates for a real world setting. For instance, the contamination of pastures was conducted by allowing grazing of flocks exposed to MAP. Therefore, excluding the positive control, MAP contamination of these sampling sites was not confirmed before the experiment nor was it quantified.

Sampling sites may have also been affected by the movement of wildlife throughout the experiment, as wild wallabies were noticed in the quarantined areas. Studies have shown that macropods such as kangaroos pose a low risk of becoming infected (Clarke 1997; Cleland et al. 2010). However, they may still act as passive vectors, spreading contaminated faeces. Cervids such as deer are known the be reservoirs to MAP and may contribute to environmental contamination. Although deer fencing is set up at these sites, this is not completely effective (Cleland et al. 2010).

The positive results for MAP DNA yielded from the investigation indicate that those samples that were provided with the most shade, appeared to give a consistent positive result (samples from site 4.4). The first sampling month (September) did not show any positives apart from the positive control. This may be due to the random selection of samples, in which samples with no MAP previous to the experiment may have been selected. However, the consistency of the positives results for samples collected at the 100% shaded areas is suggestive that sunlight exposure plays a role in MAP DNA degradation.

Interestingly samples from the low ground cover, unshaded area (paddock Q8), returned positive results towards the later months of the study. This may be attributed to an increase in rainfall seen during the Dec – Jan months (Fig.5). The analytical methods used in this investigation (qPCR) relies on the detection of MAP DNA. It is possible that the increased rainfall, which was exacerbated by greater ground coverage, allowed for rehydration of previously desiccated MAP DNA in the faecal, allowing for an increase of detectable MAP DNA (A. Purdie., Personal communication.2016). In addition to this, an increase in the quantity of MAP DNA of samples collected from 4.4, were also detected (Fig.2).

Although the viability of the MAP presence detected by the qPCR cannot be determined. The results still indicate that the amount of shade from direct sunlight heavily impacts on the longevity of the presence of MAP on contaminated pasture. Previous studies that investigated the effect environmental conditions have on MAP support these findings, as they found similar results (Eppleston et al. 2014; Whittington et al. 2004; Whittington et al. 2005). A study conducted by Eppleston et al., (2014) to investigating the survival of MAP in contrasting climate zones across NSW, found samples exposed to the direct sunlight demonstrated a 9 to 14-fold greater hazard for death, compared to the shaded samples. Additionally, the study by Whittington et al., (2004) showed that a significantly reduced viability of MAP during the higher sunlight exposure, observing that 55-week survival of MAP under 100% shade cover was reduced to 10 – 32 weeks under 70% shade. It should, be noted that Whittington et al., 2004 makes the distinction between the effect of ultraviolent radiation (UV) and infrared radiation and its effect on MAP survival rates. Whittington et al (2004) states that the UV would not be able to penetrate the faecal pellets in the study, being able to only effect organisms on the outside of the pellet, and therefore little reduction in MAP would occur.

Excluding the spike in MAP DNA collected from 4.4, findings are similar to those seen in the Whittington et al., 2004 & Eppleston et al., (2014) studies. The samples collected from the fully shaded area, indicated longer environmental stability. In addition to this, it can be seen that although the presence of MAP is extended in these environments, it is still finite. This is indicated by a correlation between the amount of MAP DNA declining as the temperature steadily increases (Fig.2 & Fig.6). It can also be seen that the spike in the 4.4 samples, occurs when average daily temperature, begins to no longer increase (Fig.2 & Fig.6).

Culture analysis was conducted to provide a clearer indication of viable MAP presence in the samples. Interestingly though, very few culture positives results were observed, with only positive control 5.1 at start of the experiment (September) and end (February) giving a positive result. Previous studies, however (Eppleston et al. 2014; Whittington et al. 2004) have observed culture negative results later becoming positive, indicating evidence of dormancy. Further culture analysis would be required to determine if a dormancy response has occurred in these samples.

Findings appear to be in support of previous studies which found significant evidence that shelter from environmental conditions that increase temperature, can extend environmental survival. It is unclear if the spike in positive samples and MAP DNA observed towards the end of the investigation was due to an increase in rainfall. In previous studies, moisture from rainfall appeared

to have no effect on promoting survival. Additionally, the increase in MAP DNA detected, does not equate to a definite increase or even presence of viable MAP organisms.

The findings from this and previous studies indicate that pasture spelling can be an effective control measure for MAP contamination. Evidence suggests however that areas which are shaded and grazed on, such as those with considerable ground cover can reduce the effectiveness of pasture spelling. It is therefore recommended that these areas are cleared to allow environmental exposure when undertaking pasture spelling as a control measure.

V. REFERENCES

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Appendix

Table.1 Exposure of MAP to Paddocks Sampled from and Ground Coverage Prior to and after Sampling.

Sampling Paddocks and Site	MAP Exposure	Time when Sheep were removed	Paddock Ground Coverage Prior to Experiment	Paddock Ground Coverage End of Experiment
Paddock D8 1.1-1.4	Transient Exposure	March 2015	Little ground cover Rocky and dry ground	Moderate ground cover Some areas still rocky and dry but knee height grass
Paddock D9 2.1-2.4	Some Known Exposure	March 2015	Lush green short grass	Long dense grass waist height
Paddock Q4 3.1-3.4 &3.1A-3.4A	High exposure	March 2015	Lush green short grass large weeds, in some sampling areas Very open, no shade	Long grass about knee height.
Paddock Q8 4.1-4.4 & 5.1	High Exposure	March 2015	Significant ground coverage of grass and weeds but still very short	Significant ground coverage of grass and weeds about shoulder height

Table.2 Reagent Amounts used for Buffer RLT, as per laboratory protocol

Reagent	1x Samples	40x Samples	80x Samples
Isopropanol	300µl	12ml	24ml
MagAttract Suspension G	25 µl	1ml	2ml

Table. 3 Reagent amounts for Lysis/Binding Solution

Reagent	1x Samples	43x Samples	62x Samples
Buffer RLT	597 µl	25.7ml	37.0ml
Carrier RNA	2.8 µl	120.4 µl	173.6 µl
Total	600 µl/tube	600 µl/tube	600 µl/tube

Table.4 Amounts of Buffers and Reagents aliquoted into plate wells

Plate Name	Plate Type	Reagent	Volume per Well
Elution	Standard 96	Buffer AVE	75 µL
Wash 3	S-Block	Buffer RPE	500 µL
Wash 2	S-Block	Buffer RPE	500 µL
Wash 1	S-Block	AW1	700 µL
Lysate	S-Block	Proteinase K	40 µL

Figure.1: Map Camden University of Sydney paddocks outlining the areas where sampling was conducted

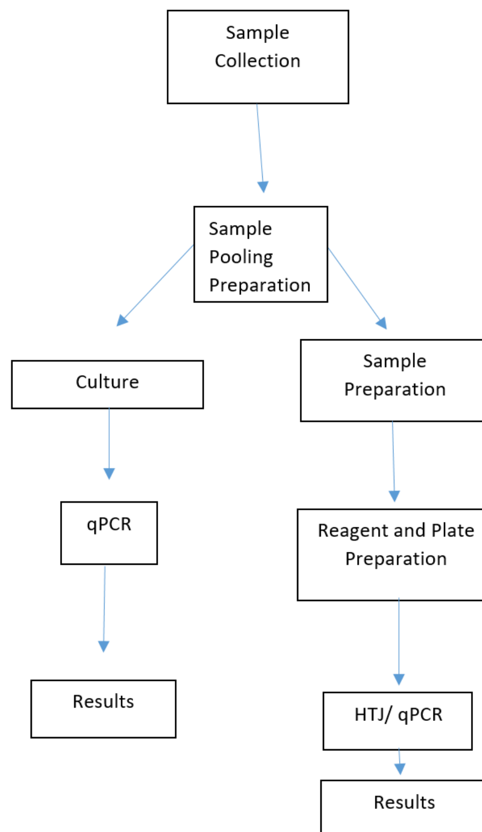

Figure.2 Method order for sample preparation for HTJ/qPCR and culture analysis

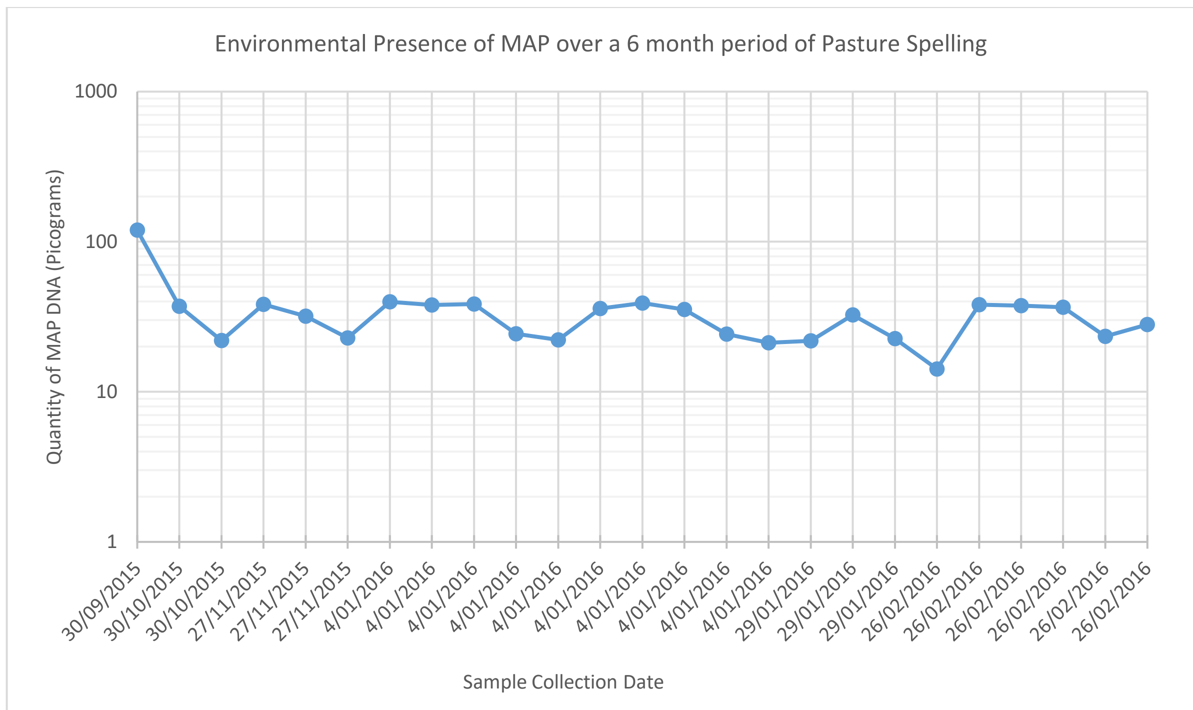

Figure.3 The MAP positive samples shown on a logarithmic scale against sampling months. The positives seen in the first month are the 5.1 positive controls.

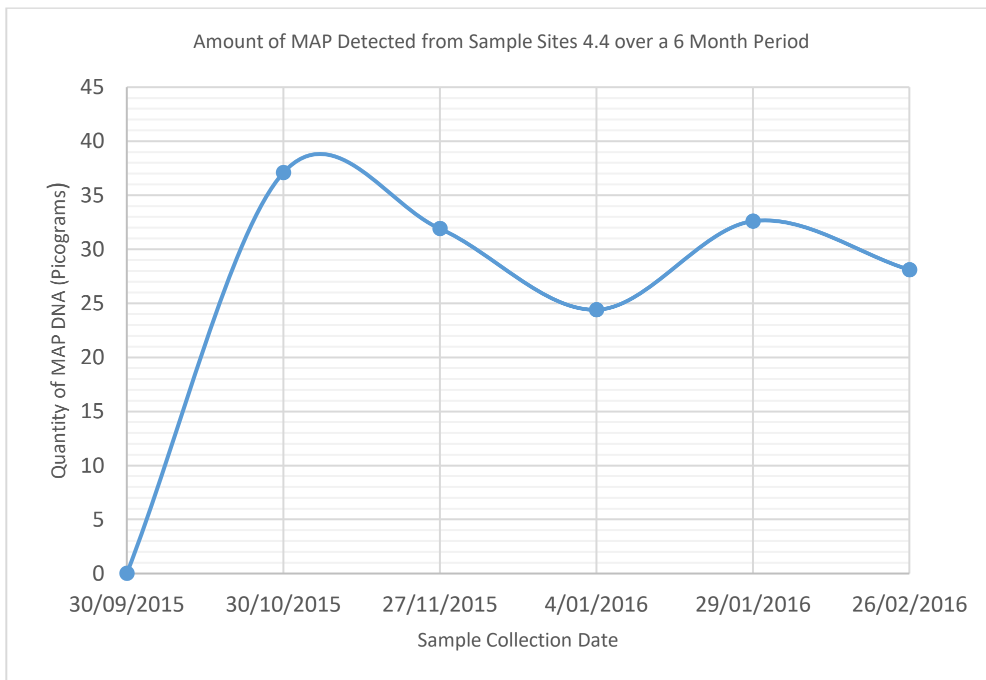

Figure.4 Only positives from sample sites 4.4 are being shown in this plot. A large spike in the quantity of detectable MAP DNA can be seen on the month of January. Previous to this the amount detect appeared to be falling.

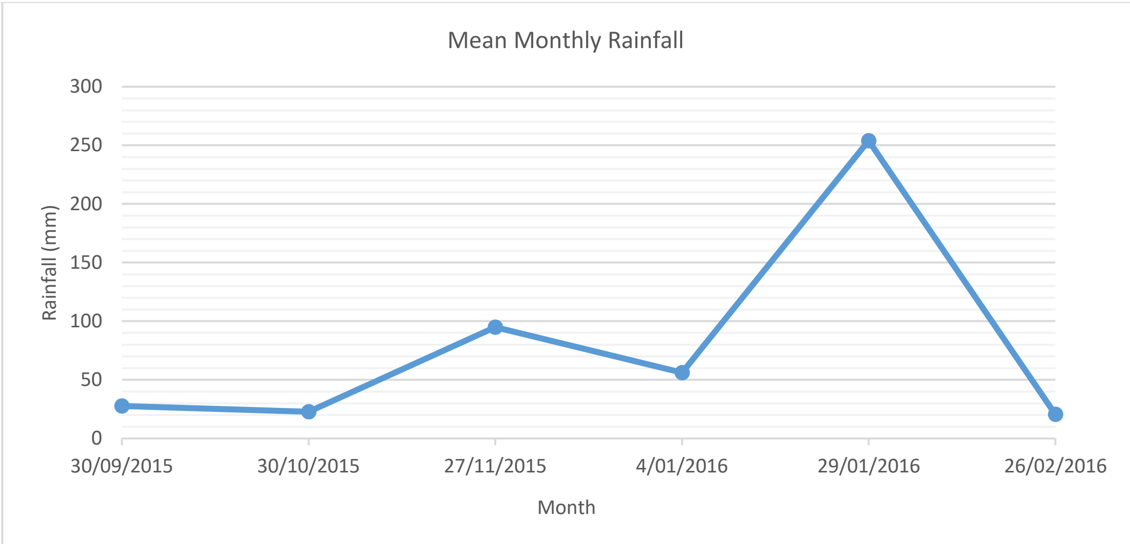

Figure.5 The average rainfall throughout the duration of the investigation

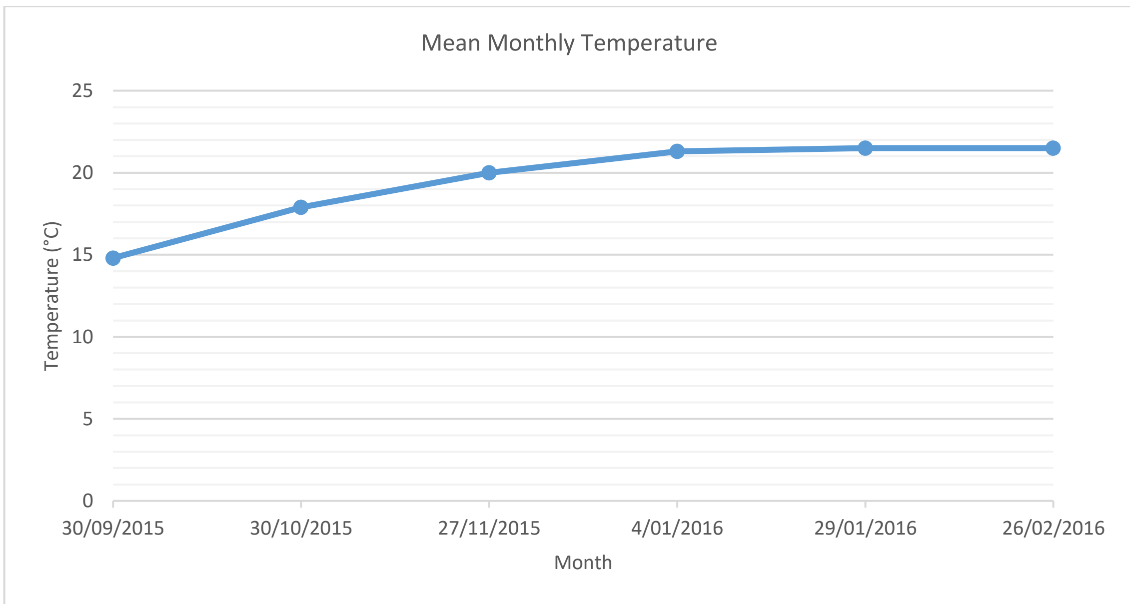

Figure.6 The average temperature throughout the duration of the investigation