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Systematic literature review: Association between soil and clinical expression of Johne's disease

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Abstract

Prima facie there is little doubt that soil plays an important role in the epidemiology of Johne's Disease (JD), given its role in JD transmission. However, the aim of this review was to assess whether there was an association between soil type (in particular soil pH) and the occurrence of JD in sheep or cattle. A comprehensive and systematic literature review of the role of soil type in clinical expression of JD was conducted. Over nearly a century, a large amount of literature exploring the association between JD/*Mycobacterium avium* ssp. *paratuberculosis* (Mptb) and soil has been published. However objective evidence to assess the association between soil and JD is limited.

Various features of soil affect the survival, retention and movement of Mptb in soil. These many influential soil features interact in complex ways and vary markedly across the national and international range of JD. This indicates that it may be very difficult to generalise the role of soil in JD epidemiology. Not surprisingly therefore, associations identified were not consistent and were often contradictory, with some papers reporting an association between soil features and JD/infection with Mptb and others finding no association.

Several hypotheses were mentioned in the literature that sought to explain observed or perceived associations. Some have little support whilst others may be plausible but are supported by just one observational study.

Further study is required to assess the role of soil type in clinical expression of JD, since the evidence at this stage is inconclusive. However, the need for further study must be balanced against the practicality of applying any future research results.

Executive summary

1. *Prima facie* there is little doubt that soil plays an important role in the epidemiology of JD, given its role in JD transmission. That is, there is good evidence that Mptb can persist in soil for many weeks or months after a shedding animal defecates. Mptb contaminated soil can lead to contaminated leachates, run-off and plants. These contaminated products (including soil directly) can then be ingested by grazing animals, potentially leading to faecal oral transmission of Mptb and the establishment of JD. However, the aim of this review was to assess whether there was an association between soil type (in particular soil pH) and the occurrence of JD in sheep or cattle.
2. A comprehensive and systematic literature review of the role of soil type in clinical expression of Johne's disease (JD) was conducted. The objectives were to: (1) Identify associations between soil type (and in particular soil pH) and the occurrence of JD in sheep or cattle; and, (2) if a likely relationship is identified, evaluate and describe the hypothesised pathways and mechanisms underpinning these possible relationships.
3. A list of key search words was generated that would encompass JD and its association with soil or pH. Two scientific and popular databases were searched (Web of knowledge, Scopus and Google scholar). A large amount of literature exploring the association between JD/*Mycobacterium avium* ssp. *paratuberculosis* (Mptb) and soil has been published over the past century. Papers that provided the highest degree of evidence about the role of soil and JD can be split into two categories; soil and Mptb microbiology/experimental papers and epidemiological observational studies (cross sectional surveys). Randomised controlled trials have not been conducted. These would provide the best level of evidence to allow assessment of an association. Thus, objective evidence to assess the association between soil and JD is limited.
4. Experimental papers revealed that various features of soil and environment affect the survival, retention and movement of Mptb in soil (including clay and organic matter, pH, electrolytes and soil structure). Grazing animals can consume Mptb from contaminated soil (and water and plant material) and hence it is plausible that soil features can affect infectious doses consumed and hence potentially development of JD. Importantly though, these influential soil features interact in complex ways to affect Mptb. Additionally, soil characteristics vary markedly across the global range of JD. This indicates that it may be very difficult or impossible to generalise the role of soil in JD.
5. Cross sectional surveys have specifically measured associations between soil features and JD. These studies vary widely in quality, methodology and findings. Importantly, associations identified were not consistent and were often contradictory, with some papers finding an association between soil features and JD/infection with Mptb while others found no association. This inconsistency likely reflects a variable effect of soil on JD or differences in study methodologies (including in treatment of confounding factors) (or both). The two best designed pieces of research each found different associations between soil features and Mptb or JD. These are discussed below.
 - A study of Bovine JD in Michigan dairy cattle found associations between soil pH, liming, soil iron and infection with Mptb (Johnson-Ifeorunlu and Kaneene, 1999). Importantly, the associations identified were relatively weak and much less important

than many other associations identified between management and biosecurity practices and infection. The value of the study may have been limited by a failure to consider some confounders. Generalising the results from Michigan to soils and climates in Australia may be difficult.

- A study of Australian sheep found an association between soil organic matter and Ovine JD (Dhand, 2008; Dhand et al., 2009a). This detailed and high quality study appeared to adequately control confounding variables. One potential limitation was that the study focused on mostly acidic soils. No association between pH and JD was observed.
6. Many other studies were conducted and are discussed in this report. Importantly, several found an association, but also found that JD or Mptb still occurred in 'lower risk soils', albeit at lower prevalence. Many studies dealt poorly with confounders (e.g. animal density or other management factors) and used relatively poor data to represent soil type. Several studies found no association between soil type and JD or infection with Mptb.
 7. Several hypotheses were mentioned in the literature that sought to explain observed or perceived associations.

These include (paraphrased for brevity):

- i. The survivability of Mptb is increased in soils of low pH (<6.5) because iron (an essential but potentially growth limiting mineral for mycobacteria) availability is higher (Johnson-Ifeorunlu and Kaneene, 1999).

This hypothesis was flawed as it implies that Mptb replicates in the soil even though it is known to be an obligate parasite. This hypothesis could only be sustained if pH (and iron solubility) had an effect on either initiating or breaking Mptb dormancy or Mptb survival whilst in a dormant state. There was no published evidence found to assess this.

- ii. Poor soil quality and improved pastures leads to micronutrient deficiencies in sheep which allows clinical expression of JD (Lugton, 2004b).

This review drew on the Michigan dairy cattle paper (Johnson-Ifeorunlu and Kaneene, 1999). However, the hypothesis was supported by an ad hoc collection of papers that were somewhat subjectively discussed to support the hypothesis. The hypothesis was also at odds with some published research that did not find an association between deficiencies of micro-nutrients in sheep and OJD (Lugton, 2004a). Thus the hypothesis appeared to have limited support in the literature. Although this does not refute the hypothesis, there is simply not enough evidence one way or the other.

- iii. Higher soil organic carbon leads to greater plant growth and shading, greater water retention or greater nutrient availability to Mptb which leads to greater Mptb loads in soil and greater transmission and expression of JD (Dhand, 2008; Dhand et al., 2009a).

Each of these three hypotheses is possible, although other hypotheses may also explain the association (see immediately below).

8. An alternative and unifying 'hypothesis' for all the associations observed is that complex interactions of soil and environmental features can lead to variable retention and survival of Mptb in upper soil strata. This would affect upper soil level contamination and could lead to different infectious doses of Mptb being ingested by grazing ruminants - depending on soil and environmental characteristics. This, in turn, could affect clinical expression of disease (Dhand, 2008; Dhand et al., 2009a).

A mechanism such as this would be observed during observational studies as an inconstant association between soil characteristics and JD/Mptb (in terms of size and direction). Inconstant associations have been observed across many cross sectional surveys, supporting this conjecture.

9. Further study is required to assess the role of soil type in clinical expression of JD.

The optimum study design in terms of objective evidence would be large randomised controlled field trials. These would be expensive, of long duration (sufficient to allow for disease expression) and may be fraught with financial and social issues. A cheaper and more manageable study design (but one providing a lesser level of evidence) would be a further observational study that utilises a large spatial and temporal data set. The advantages of such an approach include that data may already exist, that spatial and temporal autocorrelation could be adequately dealt with, that regional production characteristics (e.g. density) could be incorporated and that the study would be relatively inexpensive. Dealing with spatial and temporal autocorrelation would be important as JD is a slowly progressing epidemic, and failure to assess this could falsely implicate soil as a risk factor, when the real risk factor is proximity to other infected animals (in space and time) or animal movements. However, collecting suitable data may be difficult or impossible, limiting the ability of such research to identify a genuine association. Additionally, assuming an association was detected, practical applications for control may be limited.

10. In conclusion:

- Evidence to assess the association between JD and soil features is limited in terms of quality and quantity.
- The available evidence revealed inconsistent associations between soil characteristics and JD or infection with Mptb. The most relevant Australian study in sheep was also the highest quality. It revealed that soil organic matter was associated with JD but there was no association with soil pH.
- Various hypotheses describing mechanisms of these associations have been suggested. Hypotheses such as that organic matter affects JD had some evidence in support. The hypothesis that micronutrient deficiencies have a role in JD could not be supported or refuted with the available evidence. A unifying hypothesis across many observed associations may be that although soil can influence expression of JD, the effect can be highly variable depending upon a large suite of interacting soil parameters (i.e. it is too simplistic to state pH or any other single parameter affects JD). This would make it difficult to generalise about the effect of soil on JD.

- It is important to note that the biological effect of soil on JD or Mptb was often (not always) observed to be small compared with other more important management factors. Additionally, infection has still been observed in some 'lower risk' soils (at a lower prevalence) even when a significant association was observed.
- Further study would be required to answer this question with any degree of confidence.

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1. Background

At the request of the SheepMeat Council of Australia (SCA), Meat and Livestock Australia (MLA) commissioned a systematic literature review of the role of soil type in clinical expression of Johne's disease. The purpose of this report is to document the work that occurred to complete this project. The report describes the several parts of the project, namely:

1. The systematic literature search that occurred
2. A summary of soil Mptb research
3. A summary of each relevant observational study identified as these provide the highest level of evidence available to support or refute associations
4. A list of several hypothesised mechanisms published in the literature that seek to explain associations that have been perceived or observed. A description of potential biological mechanisms and assessment for plausibility based on published literature occurred
5. A summary of major findings.

2. Objectives of review

The two objectives specified by MLA were:

1. Identify any potential associations between soil type (and in particular soil pH) and the occurrence of Johne's disease in sheep or cattle.
2. If a likely relationship is identified, evaluate and describe the hypothesised pathways and mechanisms underpinning this relationship.

3. Introduction

Mycobacteria are aerobic and nonmotile. Although their cell walls are not typical of most other bacteria, they are usually considered Gram-positive. Mycobacteria have a characteristic hydrophobic and waxy cell wall that is rich in mycolic acids. This structure makes a substantial contribution to the survival of mycobacteria in soil because it provides some resistance to dehydration and many aqueous disinfectants. The biology of mycobacteria in soil is both complex (Falkinham, 2009a; Falkinham, 2009b) and poorly understood. The taxonomy of the *Mycobacterium avium* complex (MAC) is in flux but is generally accepted as containing *M. avium* ssp. *avium*, *M. avium* ssp. *hominisuis*, *M. intracellulare* and *M. avium* ssp. *paratuberculosis* (Mptb). There is much clonal variation and many different genotypes of Mptb (Fernández-Silva et al., 2012; Manning and Collins, 2001).

Johne's disease (JD) refers to the clinical expression of signs of infection with Mptb. Clinical signs such as diarrhoea, emaciation and death are related to chronic granulomatous enteritis. Mptb predominantly enters the soil via faeces and survives for weeks, months and possibly years.

Bovine Johne's Disease (BJD) and Ovine Johne's Disease (OJD) are both forms of Johne's disease. Hence, the papers referenced herein refer to Mptb or JD in both cattle and sheep. Care is taken to specify whether OJD or BJD is under consideration. In general, much of the Australian literature refers to OJD, whereas much of the overseas literature refers to BJD. Whilst there are many strains of Mptb, there are two broad categories, C (cattle, bovine or Type II) and S (sheep, ovine or Type I) (Begg and Whittington, 2008). These can be distinguished, for example with strain typing (Marsh et al., 1999) and strains are typically associated with their named host species (Begg and Whittington, 2008).

Different diagnostic techniques are available for Johne's disease diagnosis, but historically the two most common methods rely on an enzyme-linked immunosorbent assay (ELISA) or culture of faeces on either liquid or solid growth media. The blood test detects antibodies and hence reveals whether the animal has seroconverted to Mptb, but does not indicate disease. Culture methods reveal whether the animal is actively (or sometimes passively) shedding Mptb in its faeces. None of the tests diagnose disease. Test performance is relatively poor (i.e. sensitivity is low).

4. Method of identification and collection of papers

The purpose of this section is to describe the systematic collection of literature that was completed as part of the review.

A list of key words was generated that was intended to be broadly relevant to Johne's disease and its association with soil or pH. Key words/phrases were:

Johne's disease

Johnes disease

Paratuberculosis

Mycobacterium avium subspecies *paratuberculosis*

Mycobacterium paratuberculosis

M. paratuberculosis

Nested with Soil

Nested separately with pH

Two scientific databases were searched: Web of knowledge (Web of Science, Current Contents Connect, BIOSIS Previews, CAB Abstracts, and MEDLINE) and Scopus. A popular search engine, Google scholar was also searched. Relevant citations were collected into reference software (EndNote). Papers in the reference list of retained papers or that were related to retained papers were also examined for relevancy. Retention of these papers occurred whenever the papers dealt with new areas relevant to JD and its association with soil, or whenever a paper expanded usefully upon a theme identified from papers above.

Using the search engines, 308 articles were identified. Many of these were irrelevant indicating the sensitivity of the search was high, but the specificity was low. Table 1 details the search strategies used and the number of papers returned with each strategy. From the 308 articles, 45 articles were relevant to the objectives of the review. A further 17 articles were identified as relevant whilst examining the 45 papers. An additional handful of articles

were provided by MLA on behalf of interested parties to the review. These were opinion pieces in the grey literature. An additional 19 papers documenting the enumeration and survival of *Mptb* in soil were collected using expert knowledge (by Professor Richard Burns). These were not identified in the systematic search as they were basic soil microbiology papers, not dealing with the applied issue of pH and JD. They often dealt with other mycobacterial species.

Table 1: Search strategy, search terms and numbers of papers identified with each strategy in Web of Knowledge and Scopus

Search term (searching topic ¹)	Number of results	Nested search term	Refined results	Relevant results	Comments
Web of Knowledge					
All disease names (Johne's disease Johnes disease Paratuberculosis Mycobacterium avium subspecies paratuberculosis Mycobacterium paratuberculosis M. paratuberculosis)	18 759	Soil	138	40	All 138 nested search results were searched and relevant articles retained. There was a lot of recent research activity, but little research in the early years where most articles were simply departmental reports where soil research and Johne's disease were mentioned separately within the agriculture department's report.
Additional pH nested search					
All disease names (Johne's disease Johnes disease)		pH	102	2	These were generally laboratory or food hygiene based studies looking at culture techniques or food safety respectively.

¹ Topic= Title field, Foreign Title field, Abstract field, Major Concepts field, Concept Code(s) field, Taxonomic Data table, Disease Data table, Chemical Data table, Gene Name Data table, Sequence Data table, Geographic Data table, Geologic Time Data table, Methods and Equipment Data table, Parts & Structure Data table, Miscellaneous Descriptors field

Paratuberculosis Mycobacterium avium subspecies paratuberculosis Mycobacterium paratuberculosis M. paratuberculosis)					
Scopus					
(ALL(johne's disease OR johnes disease OR paratuberculosis OR mycobacterium avium subspecies paratuberculosis OR mycobacterium paratuberculosis OR m. paratuberculosis))	1253	AND (soil)	68	3	There were more relevant results than 3, but replication from the web of knowledge search meant there were only 3 additional results.
Johne's disease Johnes disease Paratuberculosis OR Mycobacterium avium subspecies paratuberculosis OR Mycobacterium paratuberculosis OR M. paratuberculosis OR +			5440	0	The results were ordered by relevance and the first 100 articles examined. Several relevant articles were identified but these had been previously identified in earlier scientific data base searches. The majority of articles were concerned with Crohn's disease. Removal of Crohn's seemed to make little difference as many articles still focused on Crohn's disease. Other topics included genetics of Mptb and diagnosis.

Soil					
Additional pH nested term Johne's disease Johnes disease Paratuberculosis OR Mycobacterium avium subspecies paratuberculosis OR Mycobacterium paratuberculosis OR M. paratuberculosis OR + pH			5800	0	This produced very similar search terms to the previous search and again the articles largely concerned Crohn's disease. Again the first 100 articles were searched.

5. Overview of literature on soil and Johne's disease

Over nearly a century, a large amount of literature exploring the association between soil characteristics and JD or infection with Mptb has been published. These studies are extremely variable in what has been measured, how the study has been conducted and often in findings. This makes comparison between research findings difficult in many cases. Many hypotheses have been presented to seek to explain identified or perceived associations. Broadly, the literature on Johne's disease/ Mptb and soil can be categorised into four types:

1. Descriptive or anecdotal reports
2. Experimental and other studies describing Mptb in soil (e.g. survival and movement of Mptb in soil)
3. Narrative reviews
4. Cross sectional surveys investigating soil as a risk factor for Johne's disease/ Mptb

No randomised controlled trials have occurred, the highest level of evidence that would otherwise be available to assess the role of soil in JD. The literature identified in each category is summarised briefly below, with a greater level of detail provided on the papers describing observational studies (cross sectional), as this is the highest level of evidence published.

5.2 Descriptive or anecdotal reports

These types of reports are often a description of an outbreak(s) or the disease that occurred in a defined time period in a specific area. Sometimes no data are recorded, the report being simply the writer's recollection or collective experience (Smythe, 1931). Alternatively, the description may include some incomplete data, for example if the reports are received second hand (Richards, 1989). A defining feature of these reports is the absence of controls. That is, the investigator may report that a factor causes JD, yet there are no observations of how frequently animals that are not exposed to the cause are afflicted with disease (Richards, 1989). This makes true assessment of a risk factor difficult and conclusions potentially inaccurate. Thus, the level of evidence presented by these types of study is generally poor. They are most useful for identifying areas of interest for further investigation (so called hypothesis generating), for example leading to analytical studies where associations are investigated (Dohoo, 2009).

Diagnostic methods used to diagnose disease or infection in these descriptive studies variously include observation of clinical signs (Richards, 1989; Smythe, 1931), post mortem examination (Grayson and Letts, 1958; Smythe, 1931), questionnaire surveillance (Richards, 1989), ELISA testing (Bihrmann et al., 2012; Fredriksen et al., 2004; Michel and Bastianello, 2000; Richards, 1989), Agar Gel Immunodiffusion Test (Michel and Bastianello, 2000; Richards, 1989), culture (Fredriksen et al., 2004), histopathology/'microscopy' (Grayson and Letts, 1958; Michel and Bastianello, 2000) and complement fixation test (Richards, 1989). However, the exact diagnostic method used was often poorly defined and only mentioned in passing. Confounders cannot be assessed in these studies as no analysis occurs. The following paragraphs present a brief summary of several of these studies.

Some of the earliest literature describes the situation according to the experience of the author or the results of pseudo-scientific questionnaires. Smythe (1931) describes his observations that BJD in England is rare on sandy soils and more common on granite soils where water contaminated with faeces can pool. He also states that coastal sandy areas, where BJD is rare, has 25% calcium carbonate (presumably alkaline soils) whereas the hilly granite country where BJD is common is lacking in lime (presumably acid soils). De Vries (1933) states questionnaire results that BJD is favoured by moist sandy soil that is intermixed with peat. Grayson and Letts (1958) describe the distribution of BJD in Victoria and explore in greater detail the distribution of BJD and soil type in the Gippsland region. There are purportedly three acidic soils in Gippsland with infection more common on the upland podsolic soil (a shallow grey acidic soil).

Kopecky (1977) reviewed the distribution of BJD in Wisconsin. This descriptive analysis compared the distribution of cases of BJD herds and the major soil type (classification occurred using 1:710 000 Maps). Mptb infected herds were found in every soil type regardless of pH. However, since disease was relatively rarer in alkaline soils, a conclusion that the disease is self-limiting in alkaline soils and that in acid soils one might expect Mptb to become established was made. The major problems with this descriptive analysis included the chance of information bias in classification of farm soil type due to the coarse granularity of maps, and the lack of objective statistical analysis of the population at risk or confounders. Additionally, the method of classification of herds to a status could not be assessed for its validity. It was difficult to tell whether infection status or disease status was relevant throughout.

Richards (1989) reviewed other research, anecdotal reports and the descriptive analysis of outbreaks and local BJD and OJD distributions. They raised the hypothesis that lime application in various areas of the farm (e.g. rearing, housing areas, and pasture) can raise soil pH with the result that Johne's disease is controlled. It is not apparent whether the claimed control was due to reduced prevalence of the bacteria or through reduced expression of disease. As acknowledged by the author, the study is simply a collection of circumstantial evidence intended to stimulate research, but does not have controls or objective analysis.

Michel and Bastianello (2000) observed that OJD was more common in sheep on acid soils in South Africa.

Fredricksen et al (2004) demonstrated an association between risk of infection (in cattle) and crude location data and posited that this may have been due to factors such as soil (but no soil data was available to assess this). Other authors have conducted more recent descriptive studies to examine whether sero-prevalence to Mptb in cattle was spatially clustered (Bihmann et al., 2012). Spatial clustering was evident and it was hypothesised that this was due to soil characteristics. The authors recommend that further analysis be conducted to assess the role of soil characteristics in clustering but have no plans to do this analyses themselves (Kristine Bihmann, personal communication, 20th of August 2012).

The overlap between soil acidity and OJD was recently assessed in Victoria (Anonymous, 2012). A conclusion was reached that OJD was strongly influenced by rainfall and soil pH. This conclusion was made on the basis of spatial visualisation. Spatial visualisation is a descriptive means of analysis that is best used early in the analysis phase as it will generally influence the reader more strongly than statistics but has the potential to mislead the reader due to mapping artefacts (Pfeiffer et al., 2008). A more appropriate means of assessing the impact of a risk factor (such as soil) on disease is with spatial regression modelling as this accounts for dependence (Pfeiffer et al., 2008) and allows for control of confounding (such as time since infection or sheep density). The research would have more credibility if appropriate statistical methods were used to allow objective assessment of inferences and if peer review and publication occurred.

A variety of authors write descriptive pieces that various mineral deficiencies or excesses can increase or reduce an animal's susceptibility to Johne's disease/Mptb or assist treatment of JD. For example, Lomba et al. (1974) states selenium supplementation may be useful to treat BJD disease while Latteur (1962) links cobalt deficiency and JD in ruminants. Fouquet and Delauney (1960) state injection of phosphorus, magnesium, copper and manganese 'improves results' of JD treatment. Morin (1947) agrees that phosphorus is important but believed the mechanism is that cattle on phosphorus deficient soils are more susceptible to 'attack' by Mptb. Generally though, whilst it is recognised that treatment can lead to clinical recovery there is limited evidence that a true bacteriological cure of Mptb can occur (Fecteau and Whitlock, 2011).

5.2 Experimental and other studies describing Mptb in soil

Free living mycobacteria are commonly found in soil and water and their presence can be associated with low pH conditions and several elements including high iron (Norby et al., 2007). However, Mptb are obligate parasites of mammals (Thorel et al., 1990), although recent work suggests that they may also colonise and perhaps replicate in environmentally ubiquitous free living protists (such as amoeba, flagellates and ciliates) (Mura et al., 2006; White et al., 2010). Mptb is shed in several ways from animals, but faecal shedding followed by oral ingestion is a primary means of transmission.

Environmental contamination by Mptb infected faeces can assist faecal oral transmission. Mptb can persist for long periods in faeces as well as soil and water contaminated by faeces (Fecteau et al., 2012; Whittington et al., 2005; Whittington et al., 2003; Whittington et al., 2004). Significantly, Mptb can exhibit dormancy in the soil and other environmental locations outside the host (Whittington et al., 2004). Plants that grow in infected soil can become contaminated and therefore have the potential to infect grazing herbivores (Pribylova et al., 2011; Whittington et al., 2004).

Generally, survival of Mptb in soil declines over time and is finite, consistent with the understanding that it is an obligate parasite (Whittington et al., 2004). However, the survival of Mptb (and other mycobacteria) in soil and on incorporated faecal and plant material is prolonged and much studied in laboratory and field (Moravkova et al., 2012). For example Whittington et al. (2005) reported that Mptb survived in shaded sediments for up to 48 weeks. Reviewing some specific studies, survival of Mptb in soil has been linked with moisture, organic material and clay (Pribylova et al., 2011), diurnal temperature fluctuations, (but not application of lime or dryness of the soil) (Whittington et al., 2004) or soil temperature and moisture content, with hot dry soils reducing survival times (Schroen et al., 2003). Schroen et al. (2003) found the effect of soil pH was minor but may have been confounded. However, the reader should note that Schroen et al. (2003) was an artificial laboratory study and moisture made no difference in other relevant field trials.

The survival of Mptb and other mycobacteria in soil has been attributed to many factors (Falkinham, 2009a; Falkinham, 2009b). These and other possibilities are summarised and evaluated below.

Factors affecting survival of Mptb (and other mycobacteria) in soil

1. *Lipid cell walls*

Mycobacterial cells have strongly hydrophobic cell walls. These impart resistance to toxic organics and heavy metals, dehydration, oxygen stress and microbial antagonism (from extracellular degradative enzymes and antibiotics). The membranes also drive the cells out of the aqueous phase to the protection of soil surfaces and within microaggregates.

2. *Soil surfaces and substrates*

The soil-water interface (in particular those that are associated with negatively-charged clays and humates) concentrates potential microbial substrates, nutrients and energy sources. These may contribute to resting cell maintenance, trigger the

end of dormancy and the switch to viability, and even support growth if it occurs – (see later).

3. *Biofilms*

Mycobacteria readily form polysaccharidic biofilms on plant roots, organic debris and soil particles (Yamazaki et al., 2006) which protect them from predators and adverse physical and chemical pressure. Mycobacteria are likely to be pioneer colonisers of surfaces and the drivers of multispecies biofilm communities – a process that probably involves quorum sensing (a system of stimulus and response associated with bacterial population densities) and other signals (Carter et al., 2003). Prolonged survival on crops and within plant residues and faecal matter is likely to be due, at least in part, to biofilm formation (Arrigoni et al., 2009; Pribylova et al., 2011). Other *Mycobacterium avium* complex organisms are reported to survive in biofilms within irrigation and other water distribution systems (Whiley et al., 2012) and could serve to reinfect pastures. This is less relevant for Mptb as they do not replicate in the environment.

4. *Dormancy*

Mptb can shut down growth and division and form dormant cells. Dormancy is associated with DNA binding protein (*dps* protein), GTP pyrophosphokinase (*relA* gene product) and other proteins (Gumber and Whittington, 2009) and is recorded in many mycobacteria (Gupta et al., 2002). Both *relA* and *dps* have been used to identify the dormancy response in Mptb (Whittington et al., 2004). Research with *M. smegmatis* has shown that there are different forms of dormant cells with different conditions required for growth renewal (Mulyukin et al., 2010). Dormant cells expend maintenance energy and hence cannot exist indefinitely.

5. *Survival in protozoa and amoeba.*

Mptb cells may be ingested by protozoa and amoeba (White et al., 2010) but not digested. In addition, amoebal cells encyst under adverse conditions and this will further protect any enclosed bacteria (Strahl et al., 2001). This intracellular association is suggested by some as similar to the prolonged survival of viable mycobacteria in mammalian host macrophages prior to expression of pathogenicity (Pieters and Gatfield, 2002). There are some reports of Mptb actually growing in protozoa as a secondary host or even forming some kind of symbiotic relationship (Falkinham, 2009a; Falkinham, 2009b). There are many examples of mycobacteria replicating in *Acanthamoeba* and *Dictyostelium*. *Acanthamoeba* in particular, which are well known natural hosts of many mycobacterial pathogens (Lamrabet et al., 2012; Mura et al., 2006; Sandstrom et al., 2011; Wilson et al., 2001). Some reports suggest that intracellular growth of mycobacteria may lead to the lysis of the host cell or in some instances enhance subsequent bacterial virulence (Whan et al., 2006). There is a danger in interpreting the switch from dormant to viable as growth (even though cell numbers remain constant). In addition to these intracellular survival mechanisms, some amoeba and protozoa may act as protective vectors for the bacterium, which are only released when inside the animal's stomach. Nevertheless

Mptb survival duration in dam water, which must have contained amoebae, was still finite (Whittington et al 2005).

6. *Growth on persistent organics*

Many environmental mycobacteria metabolise recalcitrant humic and fulvic acids as well as halogenated hydrophobic xenobiotics including alkanes, anthracene and vinyl chloride (Cheung and Kinkle, 2005a, b; Leys et al., 2005a; Leys et al., 2005b; Miller et al., 2007; Singleton et al., 2012). Some of the fast growing mycobacteria are candidates for soil bioremediation (Jurelevicius et al., 2012; Wang et al., 2012). No reports were found that Mptb degrades and grows on persistent organics. However, if carbon and energy inputs are crucial for long term survival, this should be investigated. This may be detected or predictable from genome sequences.

7. *Plasmids and horizontal transfer*

Mycobacteria contain plasmids (Jucker and Falkinham, 1990) that impart resistance to physico-chemical stresses, provide heavy metal resistance and initiate xenobiotic metabolism. Plasmids may be transferred across strains and even species by conjugation (especially in biofilms and at surfaces) thereby extending the survival mechanisms of the population.

8. *Spores*

Bacterial spores, as exemplified by the genera *Bacillus* and *Clostridium*, can survive in soil for years and even decades and still be capable of germination. The formation of Mptb spores resistant to heat, lysozyme and proteinase has been reported recently (Lamont et al., 2012). These spores were positive for 16SrRNA and IS900 and were confirmed as arising from Mptb vegetative cells. This is an important observation that needs further investigation because spores will provide an additional mechanism of long term persistence of viable Mptp in soil which has implications for farm biosecurity.

Retention and movement of Mptb in soil

The retention and movement of Mptb in soil is of significance in the context of availability to grazing animals as well as the lateral and horizontal spread of the bacteria (i.e. to understand disease spread and eradication programs). Several detailed and informative studies of Mptb retention and movement have been conducted using columns packed with soils of different textures and ion exchange capacities as well as pure forms of sand and clay.

Dhand et al. (2009b) reported that the retention of cells was greater in pure silica and silica-clay complexes than in clays and sandy soils and that there was some evidence that retention of cells decreased with increasing pH. It is not known if this was due to changes in the ion exchange capacity of the soil components or the cell wall properties of the MAP. However, this result is somewhat contrary to expectation as the cation exchange capacity of 'clean' clays is little influenced by pH changes but in 'real' soils the organic matter components result in an organo-mineral complex that is strongly affected by pH. This lab-based study was conducted to find objective evidence for observations arising from a risk

factor study (Dhand, 2008; Dhand et al., 2009a). Practically, it was very difficult to work with clay in column retention models, hence use of silica-clay mixture.

Mptb has been shown to be negatively charged and highly hydrophobic (Bolster et al., 2009). They showed that leaching of the predominantly negatively charged MAP through positively charged Fe-coated sands was much less than through negatively charged quartz. This means that Mptb may be bound to upper soil layers where it may be available for ingestion by grazing animals or can be released during run off events (Bolster et al., 2009; Dhand, 2008; Dhand et al., 2009a). This is largely confirmed by other trials that showed that Mptb remains on grass and upper layers of soil and that the speed at which Mptb is leached from soil depends on the soil's composition (Salgado et al., 2011). Leaching is faster at moderately acidic pH and in more sandy soils (Salgado et al., 2011). Higher slopes also lead to faster and greater run off of Mptb (Salgado et al., 2012). An earlier report of increased infectivity related to soil iron content (Johnson-Ifeorunlu and Kaneene, 1999) may be due to increased adsorption and retention of MAP in the top soil.

Raizman et al. (2011) used polymerase chain reaction (PCR) to demonstrate the capacity of MAP to leach through columns containing a sandy loam soil. MAP was detected in the leachate for many weeks. Attempts to culture MAP from the leachate were successful in only one experiment and therefore confirmation of viability was not routinely possible.

Enumeration of Mptb in soil

Enumeration of Mptb in soil is essential for the accurate interpretation of research findings (e.g. investigating the effect of soil on Mptb survival, retention and movement as well as assessment of the infectivity of contaminated soil and recommendations for soil quarantine). Unfortunately techniques available (e.g. culture and viable counts, molecular methods, serological methods and other novel methods, e.g. targeting of Mptb specific unique volatile fatty acid or metabolic profiling of colonies with mass spectrometry) are all deficient in some manner or still await validation. The development of high throughput sensitive and specific diagnostic tools is urgently required to assist research in Mptb survival and movement in soil. DNA microarrays and lab-on-a-chip antigen systems for on-site diagnosis (Wadhwa et al., 2012) are exciting developments but validation of diagnostic tests for Mptb is an essential process.

Despite this, culture and enumeration from soil has been routine since 2004, with analytical sensitivity probably around 100 viable cells (as it is for faecal culture). PCR has little role in survival studies as it does not differentiate live from dead organisms.

Conclusion of this section

Mptb survives and persists for extended periods of time in the environment, including the soil. Mptb is an obligate parasite so its survival time in soil is finite (though long). Soil characteristics do affect the adsorption, survival, leaching and run-off of Mptb. For example, it is likely that hydrophobicity and charge will serve to retain many Mptb cells at soil surfaces (especially in clay and organic soils) for an extended period of time. Mptb is principally transmitted by faecal oral contamination. Oral ingestion of Mptb may occur directly when faecally contaminated soil is ingested during grazing or indirectly due to soil contamination of plants and water. Thus, it is plausible that soil characteristics could affect the level of Mptb environmental contamination. Experimental infection models have revealed that greater and

more frequent doses of Mptb affect clinical expression and even infection outcome (Begg and Whittington, 2008). Thus, it could be hypothesised from a biological standpoint that soil type could influence Mptb loading of soil which could affect infectious dose and hence clinical expression of disease. However, all of the studies in this section are experimental studies investigating Mptb persistence or contamination of the environment and not infection or disease in animals. Observational studies and especially randomised controlled trials would be required to address the issue conclusively.

5.3 Narrative reviews

During narrative reviews literature is purposively collected (or all the papers in a field are collected where the number of papers is small), reviewed and distilled to one or several findings. Since paper selection and interpretation can be subjective, narrative reviews can suffer from subjectivity and are being superseded by systematic literature reviews, with or without meta-analysis. Two narrative reviews have been conducted addressing the association between soil and JD/ Mptb (Johnson-Ifearegulu and Kaneene, 1999; Lugton, 2004b).

Johnson-Ifearegulu and Kaneene (1999) explicitly searched for literature to address the hypothesis that soil pH is related to the prevalence of paratuberculosis. They assessed the existing literature against each of Hill's causal criteria (Hill, 1965). They found that some causal criteria had been satisfied; indicating some support for the hypothesis that prevalence of clinical Johne's disease is affected by soil pH. However, not all causal criteria were satisfied and they suggest that well designed field epidemiological studies and controlled trials be conducted to address the issue and answer the question definitively.

Critical review of the paper suggests it was largely well conducted and written with sensible conclusions but with one or two exceptions. The main error arises from potential inaccuracies in the assessment of some causal criteria. For example, the authors address the criteria of plausibility by hypothesising that Mptb has a poor ability to utilise iron in neutral or high pH soils. Further, they posit that in low pH soils iron uptake is better in the free living stage and that this leads to greater Johne's disease prevalence in low pH soils. However, Mptb is an obligate parasite (Thorel et al., 1990) and it is unlikely that Mptb grows in the soil. Thus the presence of soluble iron (at low pH) for growth is irrelevant, since growth is limited or does not occur (Thorel et al., 1990). However, this does not preclude a true association between soil pH and prevalence of Johne's disease, it simply questions the mechanism raised by the authors.

Lugton (2004b) reviewed the literature for associations between clinical expression of Johne's disease and deficiencies or imbalances of micronutrients. Essentially, he sought to identify and explain any associations between soil pH and Johne's disease using a micronutrient rationale. A conclusion was reached that there is strong evidence that acidification and resulting excesses and imbalances of various minerals (e.g. iron) have a role in the disease process. A final conclusion was that trace element supplementation, liming and the use of shallow rooted pastures may assist in controlling the clinical expression of Johne's disease.

The findings are somewhat tenuous. Apart from citing the paper on Michigan cattle and JD (Johnson-Ifearegulu and Kaneene, 1999), the review is somewhat speculative as it

attempts to relate a number of disparate findings from papers over many years to construct a central thesis. For the central thesis to hold each of the separate hypotheses must be true and causally related. Addressing only one of the concepts, that dietary micronutrients affect clinical expression of Johne's disease (elements other than iron), Lugton (2004b) makes the case in part by demonstrating that dairy cattle fed good quality feed have a lower incidence of clinical paratuberculosis. The first and only modern paper relied upon by Lugton (2004b) to support this argument is a questionnaire based cross sectional survey (Çetinkaya et al., 1997). This paper found an inconsistent association between some feeding practices and JD, and that other management factors may have been responsible for identified associations. More importantly, Lugton (2004a) found no significant association between trace element supplementation or deficiencies in sheep, soil pH and the clinical expression of Johne's disease during a cross sectional survey of infected sheep flocks in Australia. This finding directly questions one of the hypotheses central to the thesis identified in the review.

5.4 Cross sectional surveys investigating soil as a risk factor for Johne's disease/Mptb

There are several modern epidemiological papers that investigate the association between soil characteristics and JD or Mptb (Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007; Johnson-Ifearegundun and Kaneene, 1999; Johnson-Ifearegundun and Kaneene, 1998; Lugton, 2004a; Muskens et al., 2003; Reviriego et al., 2000; Scott et al., 2007; Scott et al., 2006; Turnquist et al., 1991; Ward and Perez, 2004). These are the strongest sources of evidence available to assess the association between soil and JD. Each of these papers is summarised and reviewed below (Table 2 summary and text).

Herd status (sero-prevalence) and association with loess soil in Louisiana (Turnquist et al., 1991)

Summary

Turnquist et al. (1991) found no association between sero-prevalence of Mptb in beef cattle in Louisiana and loess soils. Loess soils are fertile yet erodible soils that are generally high in silt and bound together with calcium carbonate.

Data and analysis

The study compared loess soils and non-loess soils for sero-prevalence of Mptb with a very simple contingency table (i.e. no adjustment for confounders).

Limitations

Limitations of the study include the case definition. A herd could be classified as positive with a single seropositive in certain circumstances or two sero-positives. Given the relatively low specificity reported for the ELISA used (e.g. 66%) this means the herd specificity was likely less than 10% in either case (although herd sensitivities were high). This may have introduced misclassification bias into the study (false positive herds). Other limitations include the fact that soil sampling did not occur, introducing further possibility of misclassification error in assigning properties to soil type. The default category for non-loess soils is likely to be broad and encompass many soil types and hence the external validity of the results is low, because other areas are likely to have different soils. Additionally,

confounders may be distributed by soil types. That is, it is difficult to generalise from such a broad categorisation of soil to other situations.

Hypothesis

Soil type (loess or non loess) is associated with exposure of beef cattle herds to Mptb. The research did not support the hypothesis.

Environmental risk factors for herd status (sero-prevalence) in Michigan dairy cattle (Johnson-Ifearulundu and Kaneene, 1999; Johnson-Ifearulundu and Kaneene, 1998)

Summary

The first paper by Johnson-Ifearulundu and Kaneene (1998) investigated management factors (including liming) and their association with JD. Cleanliness of calf rearing locations and liming of pasture reduced the odds that a farm would have at least two cows seropositive to Mptb (i.e. be a positive herd).

The second paper by Johnson-Ifearulundu and Kaneene (1999) reported on the herd status and herd sero-prevalence of Mptb and association with soil pH, soil iron² content, history of pasture liming and other management factors. They showed that management factors and prior history of JD were strongly associated with both herd status and herd sero-prevalence. There was also an association between increasing soil iron and the presence of at least two seropositive animals. This association was reversed for pasture liming with a reduced odds of having at least two seropositive dairy cattle if pasture lime application occurred. In the second Poisson model (see below), herd sero-prevalence was positively associated with the acidity (decreasing pH) and increasing soil iron concentration. Herd sero-prevalence was negatively associated with lime application. In essence these papers support the use of pasture liming to reduce JD.

Data and analysis

This discussion concerns the second paper (Johnson-Ifearulundu and Kaneene, 1999). Two models were implemented. One outcome was the herd status (presence or absence of Mptb as indicated by at least two sero-positive animals). Risk factors included liming, soil pH and soil iron as well as soil type (forced into model to account for common management factors) and many management variables. Univariable analysis occurred before a multivariable logistic regression model was constructed with automated procedures (backwards elimination). The second outcome was sero-prevalence of cattle tested and was modelled with a Poisson regression model. Similar risk factors and model construction occurred.

Limitations

There were similar limitations with both papers because the data set was essentially the same. The first is possible selection bias due to a large non-response rate to participation in the study. Additionally, misclassification of herd status may have been possible as the ELISA

² Note for reader on the general nature of iron in soil: Iron exists in soil in two redox states: ferrous (Fe²⁺) and ferric (Fe³⁺). Ferric iron is poorly soluble and its availability to plants and microbes decreases with increasing pH. Iron deficiencies are common in soils of pH 7.5-8.5. Soil aeration and organic matter levels also influence Fe oxidation state and availability. Plants and microbes overcome some of these limitations in available Fe by secreting siderophores which chelate Fe and which makes it more soluble and transportable.

had limited sensitivity, meaning herds with a very low prevalence may have been misclassified as un-infected herds. Some simple errors of understanding appeared to be present in interpretation of statistical models, for example the use of odds ratios from the Poisson regression model, where risk or rate is the usual exponentiated co-efficient. This does not instil confidence. Some potentially important confounders appear not to have been considered in this analysis (e.g. herd size or density of animals).

Hypothesis/es

The authors posit that high pH soils have less available iron and therefore a lower viability of Mptb. They then go on to say that this leads to less Mptb infection. Further, they state that liming is a practical method to increase soil pH and reduce Fe solubility. They then assume that this will reduce Mptb infection. Whilst statistical associations were identified that support the hypothesis, the mechanism inferred to explain the association was not necessarily biologically plausible (Mptb is an obligate parasite).

Association between soil type and herd status (sero-prevalence) in Spanish sheep and goats (Reviriego et al., 2000)

Summary

The association between soil type and herd status was investigated in Spanish sheep and goats with a questionnaire-based cross sectional survey. Flocks on entisols (soils of low pH and low quality) had an increased odds of being infected (OR: 25.9 (95% CI: 1.6-411)) compared with the referent soil category (alfisols which have a high pH, clay enriched sub soil and high native fertility). Flocks on inceptisols (old soils formed by alteration of parent material) had no increased risk of infection compared with the referent category (OR: 3.5 (95% CI: 0.3-45)). The authors' state that the study provides strong evidence that soil quality and soil pH are important in the epidemiology of paratuberculosis.

Data and analyses

The outcome variable was herd Mptb status, as indicated by one or more antibody positive sheep or goats. Risk factors were parent soil type (assessed as an indicator variable). The study appropriately controlled potential confounding factors, by including variables for management and production, herd size and other physical features such as landscape type. Univariable analysis occurred, followed by multivariable analysis using backwards elimination. Interactions were assessed. A logistic regression model was used.

Limitations

Limitations of the study included classification of farms to soil categories. Specifically, no individual soil assessments (such as pH or other measures) were made at each farm. Instead the variable was created using broad scale maps of parent soil category that classified soils into several broad categories. Hence an assumption that all farms located in areas of predominantly entisols were assumed to be poor quality soils of low pH. This may have introduced misclassification bias. Although the sensitivity of the Agar Gel Immunodiffusion Test (AGID) test was low (27%) the large number of samples taken from each flock meant that a high herd sensitivity was likely (+98%), although a relatively low herd specificity was likely (~70%). Very wide confidence intervals for estimates or odds ratios

were present indicating sampling issues. The soil variable was an indicator variable and whilst each category was compared the referent category, a global test for the significance of the indicator variable was not conducted.

Hypothesis

Soil richness and acidity are associated with the epidemiology of Mptb. There was an association between one soil category and herd Mptb status which supports the hypothesis.

Risk factors for herd status (sero-positive) in Dutch dairy cattle (Muskens et al., 2003)

Summary

Using a questionnaire survey and a large sample size (370 herds), Muskens et al. (2003) investigated many risk factors that differed between seropositive and sero-negative herds of dairy cattle. They included a general soil type acid or non-acid soils. Acid soils included peat or peat on clay soils. They found several univariable risk factors that impacted on Mptb herd status and these included management practices associated with reducing transmission and the size of the herd. Being a farm on an acid soil was not a risk factor. Following multivariable analysis soil type was not a risk factor. This does not address clinical signs, merely associations with herd status (as measured by sero-prevalence).

Data and analysis

This was a questionnaire based cross sectional survey. If greater than one cow was sero-positive a herd was considered positive. Thus, the outcome was herd status. Risk factors assessed included management variables linked to transmission of Mptb, general farm features (including soil type), housing, hygiene management and feeding of calves. Univariable analysis was conducted, followed by multivariable logistic regression analysis ($p < 0.30$ inclusion criteria from univariable analysis) with backward elimination.

Limitations

The study was of good quality but not particularly useful as an investigation of the role of soil in JD. The representation of soil was general and only to broad soil type. An assumption of pH was made on this generalisation. Data were not measured individually at the farm level. This introduces a chance of misclassification error for soil pH if, for example, there is localised soil improvement that means the parent soil type can no longer be used to assess soil pH. Soil heterogeneity of this sort is common in agriculturally managed soils.

Hypothesis

Soil type might influence the ability of Mptb to survive in the environment, suggesting risk factors can vary geographically (Kennedy and Benedictus, 2001). This hypothesis was not supported by the analysis.

Mptb sero-prevalence and its association with soil type in Michigan beef and dairy cattle (Ward and Perez, 2004)

Summary

Ward and Perez (2004) conducted an hypothesis-generating study by looking at associations between soil characteristics and a herd's membership of a cluster of higher than median Mptb sero-prevalence. They found that there was an association between being in a cluster of greater than median sero-prevalence and low silt soils. Looking holistically at their results they found that as silt was replaced by sand in loamy soils the risk of being in a cluster of higher sero-prevalence increased. They posited that this association may have been related to the previously reported association between soil pH and Mptb.

Data and analysis

Ward and Perez (2004) identified clusters of greater than median sero-prevalence of Mptb using cluster analysis techniques (SATScan). They conducted bivariate logistic regression analysis with the outcome being membership within a cluster or not (i.e. controls were herds outside a cluster). Explanatory variables were various soil characteristics (mostly documented for industrial, development and agricultural planning). Multivariable logistic regression models were built using stepwise procedures (unspecified). Confounding effects of dairy or beef status were assessed using Mantel Haenszel adjusted odds ratios.

Limitations

Limitations of the study include the nature of the variables used as risk factors. They appear to have used an off-the-shelf soil data base (the US national cooperative soil survey) and as such it is not constructed specifically to test relevant hypotheses that were evident in the literature at the time (e.g. soil pH was not included in the risk factors). Additionally, the local nature of soil was not assessed for each herd and it is possible that information bias of soil information could have occurred. Some potential confounders not assessed in the study are productivity, stocking density, contacts and management factors that may vary by region. For example, the soils identified as being higher risk for Mptb are those that are rich in organic matter and more productive for agricultural use. It is possible that this may lead to higher stocking rates which may be a risk factor.

Hypothesis/es

Survival of Mptb may be enhanced by silt or sand content in loamy soils.

Association of risk factors and clinical disease in infected Australian sheep flocks (Lugton, 2004a)

Summary

Lugton (2004a) conducted a cross sectional questionnaire survey of sheep graziers in the southern tablelands of NSW, all of whom had known infected sheep flocks. The aim of the study was to explore associations between clinical JD and various risk factors. The study had an important advantage in that it collected data from an extremely important data source (producers). The study established five separate multivariable models and found that many variables were associated with measures related to clinical expression of disease. The study concluded that clinical expression appears to be primarily associated with light infertile soils and their associated pastures and pasture improvement practices which may lead to micronutrient deficiencies or imbalances.

Data and analysis

The paper uses five outcome variables and numerous explanatory variables, sometimes categorised and sometimes uncategorised. Variables were screened using descriptive and univariable analyses and offered to a variety of multivariable models in an automated forward stepwise manner. The outcome variables were recorded at flock level. The type of multivariable model used was dependant on the outcome variable.

Limitations

Although the study sampled a very valuable data source, the study had several limitations which restricted interpretation. These included:

- Reliance on subjective data (outcome and explanatory variables were subjective with few objective measurements collected).
- Statistical issues (for example deletion of data points to improve model fit, large proportions of missing data points for each model and crude imputation methods)
- Inferences: One of the more important inferences in the paper is that clinical expression of disease is associated with light infertile soils and associated pastures, and that this indicates micro-nutrient deficiencies or imbalances which lead to OJD. However, this appears to be contradicted by statistical analyses. That is, trace element supplementation and farmer identified trace element deficiency were included in the analysis as risk factors and were not found to be significantly associated with disease.
- Parameter space: The study did not include farms located on alkaline soils (≤ 5 (64.1%), 5.1-7 (35.9%)). Thus, potentially important biological relationships between pH and clinical JD were not investigated. For example, if a relationship between decreasing pH and clinical JD existed, then the inclusion of many alkaline soil data points may have revealed this relationship.

Hypotheses

Clinical expression of JD is environmentally driven (associated with light infertile soils that have undergone pasture improvement). Further that this association is not due to macro-nutrients but instead is due to micro-nutrients and other unapparent nutritional factors associated with the composition of the pasture. There was an inconstant association (across models) between OJD and soil type and pasture. There was no association between micro-nutrient deficiencies or supplementation and OJD. The evidence in support of the hypothesis was limited.

Alberta beef and dairy cattle and association herd status (sero-prevalence) and agro-ecological data (Scott et al., 2007; Scott et al., 2006)

Summary

These paired studies investigated a variety of infectious cattle diseases (including Mptb) with several risk factors such as agro-ecological features in either dairy or beef cattle. Soil features investigated included soil order, texture, pH and soil moisture as well as a more general agro-ecological zone.

Individual beef cattle status (sero-prevalence) was weakly associated (negatively) with grassland and montane regions of southern Alberta (southern latitudes, aridity and acidic soils). There was no significant association between individual constituents of the agro-ecologic zone: such as between soil pH and odds/likelihood of disease in bivariable analysis (OR=0.4, p=0.15) or in other models.

Dairy cattle herd status was associated with agro-ecological region (parkland had a higher risk than grassland and montane regions, but was equivalent risk with boreal forest). In separate models increased odds of infection was associated with a wetter climate and also declining soil pH.

Data and analysis

In the beef paper (Scott et al., 2007) saturated mixed logistic regression models were established using risk factors significant in bivariable analyses. Backwards elimination was used to derive a final model. Multiple models were used, but the most relevant used individual animal status with a herd random effect. Risk factors were herd size and individual features (e.g. breed) as well as agro-ecological region and individual components of the agro-ecologic zoning system.

In the dairy paper (Scott et al., 2006), similar analyses were followed. Here, several separate models were implemented to avoid collinearity. Agro-ecological zone were significant covariates.

Limitations

Both of these studies were well conducted but with some limitations. The main limitation is the gross nature of the agro-ecological variable and its individual constituent parts (e.g. pH). It is uncertain whether the classification of the location for each of the 100 sampled herds (77 in dairy cattle) into a broader agro-ecological region or pH accurately captures the soil characteristics of the location where calves were raised (i.e. soil improvements, local variability etc. could have influenced it). Considerable information bias is thus possible. Additionally, sample sizes are small relative to the results observed; meaning confidence intervals within indicator variables are very wide. Potential confounders (for example herd management factors associated with agroclimatic zone) may not have been adjusted for.

Hypothesis/es

One objective was to provide an examination of the major agro-ecological factors associated with sero-prevalence of antibodies to Mptb. By implication there is a hypothesis that sero-prevalence is associated with agro-ecology. Soil pH, aridity and other features are part of this categorisation of agro-ecology. There was an association between agro-ecological zone and Mptb.

Association of Mptb shedding and management and soil risk factors in Australia (Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007)

Summary

Dhand et al. (2009a) conducted a cross sectional survey and examined the association of several outcome measures of OJD with soil characteristics (parent soil type and parameters

from soil samples such as composition, pH, carbon etc.). They demonstrated an association between higher prevalence of OJD and increasing organic carbon or clay and an association between lower prevalence of OJD and sand or nitrogen. There was an association between infection and increasing iron content (although most cations showed this relationship indicating possible confounding). No relationship between soil pH and OJD was identified. They hypothesise that the associations were due to adsorption of Mptb to clay and the consequent retention of the bacteria in the topsoil, thus making Mptb available in higher numbers to grazing sheep.

Importantly, these relationships were further investigated by including sheep management factors (e.g. density of sheep) to determine if any of the identified associations were actually the result of confounding (Dhand, 2008). This adjusted analysis demonstrated that most of their previously identified relationships between soil and OJD were the result of confounding. The only soil factor associated with infection/level of infection was organic carbon. Organic carbon is an indicator of soil carbon and is linked to soil fertility. Dhand (2008) summarised the effect of soil in his PhD thesis by stating that carbon:

'could increase the risk of disease either by increasing Mptb survival (either directly by providing essential nutrients or indirectly by increasing pasture-growth and shade) or by increasing its availability to the grazing sheep (by keeping Mptb in the upper layers of the soil through adsorption). However, further studies are required to test these associations.'

In contrast to soil factors, management factors remained important even after adjusting for soil factors.

Data analysis

Three outcome measures were created from pooled faeces:

1. positive or negative status of faecal pools (pool OJD status, binary);
2. the log number of viable Mptb organisms per gram of faeces (log pool Mptb number, continuous); and
3. the prevalence of faecal shedders (cohort OJD prevalence level, ordinal: low <2%, medium 2–10% and high >10%).

Soil risk factors were derived from soil parameters from three soil samples collected from the location of lamb, weaner and yearling/adult paddocks for the 92 sheep flocks enrolled in the study. The parameters measured were numerous but included proportions of sand, silt and clay as well as many other quantitative soil variables (e.g. pH, elements etc.). Management risk factors were derived from questionnaires.

Univariable analyses were conducted then separate statistical models developed with automated approaches to investigate the association between soil characteristics and each outcome variable (therefore, ordinal logistic regression, mixed logistic regression and linear mixed models were established).

Limitations

The quality of these studies appears high. Sampling appears appropriate for the stated aim, although risk factors for transmission onto a property cannot be inferred and, instead, inferences are limited to the effect of soil characteristics on clinical expression of JD (Dhand

et al., 2009a). The statistical analysis appears adequate and the authors note that caution in inference is required where a significant variable is found only in one or two models. Inferences were limited to situations where multiple models showed the same result. This implicitly acknowledges the nature of their analyses whereby multiple testing occurred and an elevated type 1 (α) rate was possible. The research is superior to most other studies on this issue as field data collection minimised information bias. That is, samples of soil and faeces were collected in a systematic manner and analysed comprehensively. Confounders were adjusted for.

A limitation is that the paper aimed to investigate the association of OJD and risk factors. However, the outcome measures used are not perfectly representative of OJD as shedding could occur in clinically normal sheep. Despite this, it is an objective measure that is arguably more closely related to disease than other commonly recorded measures, such as sero-prevalence.

Another limitation is that the study largely occurred on acidic soils (median pH= 4.6 (Q1,Q3: 4.4, 5.1)). Thus, there is a possibility that important biological relationships between pH and clinical JD may have been missed. For example, it could be speculated that inclusion of data from alkaline soils may have provided important additional information on associations between OJD risk and soil pH. It is noted that a wide variety of OJD prevalence (0-59%) was observed across the limited parameter space in the (pH: 3.9-7.5) (Dhand et al., 2009a).

Hypothesis/es

Higher organic carbon in soil provides: (i) more nutrients to Mptb to directly enhance Mptb soil survival (but see discussion on growth in soil above) which leads to greater infective doses or greater exposure of animals to Mptb; (ii) greater pasture growth and more shade, thereby indirectly enhancing Mptb survival in soil. This leads to greater infective doses or greater exposure of animals to Mptb; and, (iii) greater adsorption of Mptb hence retaining Mptb in the upper layers of the soil resulting in greater infective doses or greater exposure of animals to Mptb. These hypotheses were inferred from the identified association between soil carbon and OJD and hence all are potentially supported.

Table 2: Summary of main observational studies conducted.

Study	Hypothesis investigated (and support for hypothesis) or main finding
(Turnquist et al., 1991)	Soil type (loess or non loess) is associated with exposure of beef cattle herds to Mptb. The research did not support the hypothesis.
(Johnson-Ifeorulundu and Kaneene, 1999; Johnson-Ifeorulundu and Kaneene, 1998)	The authors posit that high pH soils have less available iron and therefore a lower viability of Mptb. They then go on to say that this leads to less Mptb infection. Further, they state that liming is a practical method to increase soil pH and reduce Fe solubility. They then assume that this will reduce Mptb infection. Whilst statistical associations were identified that support the hypothesis, the mechanism inferred to explain the association was not necessarily biologically plausible (Mptb is an obligate parasite).
(Reviriego et al., 2000)	Soil richness and acidity are associated with the epidemiology of Mptb. There was an association between one soil category and herd Mptb status which supports the hypothesis.
(Muskens et al., 2003)	Soil type might influence the ability of Mptb to survive in the environment, suggesting risk factors can vary geographically. This hypothesis was not supported by the analysis.
(Ward and Perez, 2004)	Conducted a hypothesis-generating study by looking at associations between soil characteristics and a herd's membership of a cluster of higher than median Mptb sero-prevalence. Looking holistically at their results they found that as silt was replaced by sand in loamy soils the risk of being in a cluster of higher sero-prevalence increased.
(Lugton, 2004a)	Clinical expression of JD is environmentally driven (associated with light infertile soils that have undergone pasture improvement). Further that this association is not due to macro-nutrients but instead is due to micro-nutrients and other unapparent nutritional factors associated with the composition of the pasture. There was an inconstant association (across models) between OJD and soil type and pasture. There was no association between micro-nutrient deficiencies or supplementation and OJD. The evidence in support of the hypothesis was limited.
(Scott et al., 2007; Scott et al.,	One objective was to provide an examination of the major agro-ecological factors associated with sero-

2006)	prevalence of antibodies to Mptb. By implication there is a hypothesis that sero-prevalence is associated with agro-ecology. Soil pH, aridity and other features are part of this categorisation of agro-ecology. There was an association between agro-ecological zone and Mptb.
(Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007)	Higher organic carbon in soil provides: (i) more nutrients to Mptb to directly enhance Mptb soil survival (but see discussion on growth in soil above) which leads to greater infective doses or greater exposure of animals to Mptb; (ii) greater pasture growth and more shade, thereby indirectly enhancing Mptb survival in soil. This leads to greater infective doses or greater exposure of animals to Mptb; and, (iii) greater adsorption of Mptb hence retaining Mptb in the upper layers of the soil resulting in greater infective doses or greater exposure of animals to Mptb. These hypotheses were inferred from the identified association between soil carbon and OJD and hence all are potentially supported.

6. List of hypotheses that require assessment for biological plausibility

1. **Associations variously between low soil pH, available iron, liming and JD prevalence lead to an hypothesis that, at alkaline pH, the limited availability of iron inhibits the survival (or growth) of Mptb, thus reducing the prevalence of JD in grazing animals (Johnson-Ifearegulu and Kaneene, 1999).**

Hypothesised mechanism: Generally ferrous iron (Fe^{2+}) availability to plants and microbes in soil is reduced at alkaline pH values. Mptb certainly requires iron for growth and its acquisition will be pH influenced (Hinsinger et al., 2009). Hence, the hypothesised mechanism was that Mptb survival and viability is greater in soil of acid pH because Fe^{2+} availability is increased.

Plausibility and published evidence: Mptb is an obligate parasite of ruminants (Thorel et al., 1990) and therefore (by definition) does not replicate in soil. Hence, there is little likelihood that pH will directly affect replication of Mptb in soil. One can speculate that if Mptb does not replicate in soil then Fe^{2+} can only be important for bacterial survival or retention if Fe^{2+} (and other micronutrients) have an impact on: (i) the soil ion exchange capacity and its adsorptive characteristics; (ii) the survival of Mptb in its dormant state; and (iii) the shift in the cells from dormant to viable state (and vice versa); or (iv) the capacity of cells to sporulate. There is little evidence to assess this.

2. **Light textured soils with improved pastures induce micronutrient deficiencies or imbalances in ruminants resulting in clinical expression of disease (Lugton, 2004a). This association is not due to macro-nutrients but instead is due to micronutrients and other unapparent nutritional factors associated with the composition of the pasture and soil pH (Lugton, 2004a).**

Hypothesised mechanism: The hypothesised mechanism of action proposed by Lugton (2004b) is not clear because as Lugton (2004b) identifies, there are several issues where knowledge is uncertain, including:

- The high complexity of mineral nutrition in animals means that researchers have been unable to demonstrate that minerals actually influence paratuberculosis
- The action and interaction of micronutrients and effects on the immune system remain a poorly explored area of scientific endeavour.

Plausibility and published evidence:

Support cited for the hypothesis appears somewhat speculative and insubstantial in the review paper. For example, the key paper cited to support the idea that dietary micronutrients (besides iron) affect expression of JD is a paper in British dairy cattle (Çetinkaya et al., 1997). This paper showed an association between good quality food and expression of BJD but noted the association may have been explained by other management factors (Çetinkaya et al., 1997). Other papers cited for this point were very old and suffered from poor study design or antiquated diagnostic techniques. More importantly, research in Australian sheep found no significant association between trace element

supplementation or deficiencies in sheep or soil pH and the clinical expression of Johne's disease in several separate models during a cross sectional survey of infected sheep flocks in Australia (Lugton, 2004a).

Iron is specifically mentioned as an element of interest and there was one observational study that offered support for the hypothesis that iron in particular affects infection with Mptb (Johnson-Ifearegulu and Kaneene, 1999). Disparate facts have also been used by proponents of this issue to support this hypothesis, (Turner, 2011). These disparate facts include that iron is important in the virulence of other mycobacterial diseases (Ratledge, 2004) and that mycobactins are required to successfully culture MAP (Janagama et al., 2009). Dealing with each in turn:

1. Iron is critical for virulence in many systemic bacterial pathogens (Ratledge, 2007) including *Mycobacteria tuberculosis* and *Mycobacteria leprae* (Ratledge, 2004).

This is an intra-host mechanism where the infected animal attempts to starve the invading micro-organism of iron (for example with iron sequestration in macrophages) and the organism competes by attempting to harvest iron. Ratledge (2004) raised the prospect that chemotherapeutic agents could therefore be developed to treat human mycobacterial disease. More recent research confirms that MAP infection of cattle has a role in intra-host iron metabolism (Senturk et al., 2009). However, these facts are separate to the hypothesis that dietary iron can affect clinical expression of JD and is not evidence in support of this hypothesis. For example research has demonstrated that dietary iron does not affect the clinical expression of JD in cattle (Lepper et al., 1989). In summary, these papers demonstrate that mycobacteria can affect host iron metabolism (and that chemotherapeutic agents targeting iron metabolism by Mptb may be feasible), but that dietary iron has not been demonstrated to affect JD during experiments.

2. Mycobactins are required to successfully culture MAP (Janagama et al., 2009).

The dietary micronutrient (e.g. iron) hypothesis should not be confused with Mptb's requirement for mycobactin during *in vitro* culture. Mptb has a biochemical quirk whereby it doesn't produce mycobactin to assist iron uptake (Janagama et al., 2009). This is important during *in vitro* culture (laboratory). However, alternative iron uptake pathways exist for use in the host ((Janagama et al., 2009)) and Mptb is a successful pathogen in many species around the world, suggesting that limitations occurring during *in vitro* growth (culture) are not the same as *in vivo* growth (i.e. growth in a host). However, this is an extremely complex area and would require a separate review by a microbiologist experienced in biochemistry to fully detail this area.

Despite the scarce data the hypothesis presented in this section (even including iron) is not necessarily implausible. There is just little evidence to assess the hypothesis either way.

3. **JD is more prevalent on soils high in organic carbon. The mechanisms for this were hypothesised to be (Dhand, 2008; Dhand et al., 2009a):**

- organic carbon provides additional essential nutrients for Mptb survival outside its host

- organic carbon causes greater plant growth leading to shading and survival of Mptb (Whittington et al., 2004)
- organic matter increases water holding capacity of soil resulting in greater survival of Mptb.

Plausibility and published evidence: The research was well conducted and the association is likely to have been real (i.e. un-confounded). All three hypotheses proposed as explanations for the association are possible, but again there is limited evidence to support or refute them. Whittington et al. (2004) did not find any evidence that soil moisture prolonged survival of Mptb in soil.

4. Soil characteristics affect retention and survival of Mptb in soil and hence affect infectious doses available for grazing ruminants.

Mptb is predominantly negatively charged, highly hydrophobic and moves more slowly through positively charged mineral surfaces (Bolster et al., 2009). High adsorption rates have been observed in some soils and attributed to electrostatic and van der Waals forces and Mptb cell surface hydrophobicity (Dhand et al., 2009c; Salgado et al., 2011). These forces increase at acidic pH and at high electrolyte concentrations (Salgado et al., 2011). Soil particle size is also important as filtration can occur (Mawdsley et al., 1995). Rainfall and slope can also affect leaching and runoff of Mptb (Salgado et al., 2012; Salgado et al., 2011). All these factors have the potential to modify retention and survival of Mptb in the upper levels of soil and plant material. This means that Mptb may be bound to upper soil layers where it may be available to be consumed by grazing animals or can be released during run off events (Bolster et al., 2009; Dhand et al., 2009a). This is largely confirmed by laboratory trials that showed that Mptb remains on grass and upper layers of soil and that the speed at which Mptb is leached from soil depends on the soils' composition (Salgado et al., 2011).

Thus, an alternative and general hypothesis is that complex interactions of soil and environmental features can lead to variable retention and survival of Mptb in upper soil levels. This can affect the infectious dose of Mptb consumed by grazing ruminants (depending on soil and environmental characteristics). A mechanism such as this would tend to be revealed during observational studies as an inconstant association between soil characteristics and disease (in terms of size and direction). Inconstant associations have been observed across many cross sectional surveys, thus supporting this conjecture.

7. Discussion of main findings of literature review

There is little doubt that soil plays an important role in the epidemiology of Johne's disease (ovine or bovine), given that ingestion of contaminated soil is a major transmission pathway. There is good evidence that Mptb can persist in soil for many weeks or months after a shedding animal defecates. Mptb contaminated soil can lead to contaminated leachates, run-off, and plants. These contaminated products (including soil directly) can then be consumed by grazing animals, potentially leading to faecal/ oral transmission of Mptb which can give rise to JD. Additionally, there are a number of research papers looking at the influence of different parameters of soil on the fate of Mptb in soil. These provide evidence that soil type may be important in the epidemiology of Mptb (and hence JD since Mptb infection is the necessary cause of JD).

However, the primary objective of this review was to assess whether there was an association between soil type (in particular soil pH) and the occurrence of JD in sheep or cattle. There was much literature purporting to investigate this issue. However, many of these papers provide little objective evidence as they were based on opinion, descriptive analyses or unpublished 'grey' literature (these are reviewed in the main body of the report). If these papers are removed there only remain two types of research papers that provide objective evidence. These can be broadly categorised into experimental microbial soil research papers and epidemiological observational studies (9).

The experimental soil papers reveal that there are a large number of soil characteristics that can lead to greater retention of Mptb in upper soil horizons. From here, Mptb can be ingested by grazing ruminants. However, the large number of soil properties and the interactions between them indicates that any soil relationship with JD is likely to be complex, difficult to predict and not necessarily generalisable. This would be expected to be reflected in observational studies as an inconsistent association between soil and JD/Mptb infection. These papers are presented in the sections entitled 'Experimental and other studies describing Mptb in soil' and 'List of hypotheses that require assessment for biological plausibility' and are not discussed further here.

The observational studies are all cross sectional surveys of varying quality and provide the best level of evidence available to address the objectives of this study. They use a diverse array of methodologies, outcomes and soil risk factors making comparison between them difficult. Unfortunately there are no papers describing randomised controlled trials to assess potential effects of soil on JD. These would offer the most robust means of testing relevant hypotheses.

Having assessed the available literature and addressing the objective, there are two critical questions addressed by these observational studies:

1. Is there an association between soil type and infection of herds with Mptb?
2. Is there an association between soil type and clinical expression of Mptb infection (JD)?³

7.1 Is there an association between soil type and infection of herds with Mptb?

Study design: These observational studies assessed herd or flock Mptb status by sampling individual herd members and testing them using an antibody ELISA (Johnson-Ifearegulu and Kaneene, 1999; Muskens et al., 2003; Reviriego et al., 2000; Scott et al., 2007; Scott et al., 2006; Turnquist et al., 1991; Ward and Perez, 2004). Whilst this approach results in an objective outcome, its disadvantage is that the outcome of the test is sero-prevalence and not clinical disease (although there is likely to be a correlation between the two). However, this introduces the chance of misclassification bias, although some studies dealt with this by adjusting the number of reactors required to classify a herd or flock as 'infected'. The approach was to investigate the association between herd serological status and various soil characteristics. Johnson-Ifearegulu and Kaneene (1999) actually sampled soil on farm and investigated some specific soil parameters, whereas all other papers investigated soil association using convenient and not necessarily entirely applicable soil data sets. The other studies also used aggregated soil data, such as district level soil type and hence may have misclassified soil type on any particular farm. Soil risk factors assessed varied and included a broad classification of parent soil type (Turnquist et al., 1991), parent soil type and pH (Muskens et al., 2003; Reviriego et al., 2000), agro-ecological zone (incorporating soil type and parameters as well as landscape and climatic information) (Scott et al., 2007; Scott et al., 2006) and more detailed soil parameters such as soil pH and soil iron (Johnson-Ifearegulu and Kaneene, 1999). The studies concentrate almost exclusively on BJD with only one study on OJD (Reviriego et al., 2000).

The inclusion of potential confounders in the studies was highly variable. Frequently, important potential confounders were not included in the analysis. For example, key risk factors, such as density of animals or herd size (Johnson-Ifearegulu and Kaneene, 1999) or in most studies proxies for contact rates between farms and risk factors to account for spatial and temporal dependence of farms were not included in analysis. This may result in important bias in results of observational studies conducted thus far. If considered, the treatment of confounders generally included screening with univariable analysis before offering confounders to models during automated model building procedures.

Consistency of findings: Importantly, there were no consistent findings across all of the studies. Three studies found an association between soil features and herd status (Johnson-Ifearegulu and Kaneene, 1999; Reviriego et al., 2000; Ward and Perez, 2004). Two studies found no association (Muskens et al., 2003; Turnquist et al., 1991). One study found an association between agro-ecological type and herd status, but no consistent association with

³ To some extent, the distinction between sero-prevalence (previous infection) and disease is somewhat arbitrary but this reflects different study methodologies. Additionally, the SCA interest was in the role of soil in the expression of clinical disease. Hence this distinction was somewhat arbitrarily imposed.

individual soil parameters (although one model from 5 had a weak association ($p=0.03$) between pH and individual sero-prevalence) (Scott et al., 2007; Scott et al., 2006).

Summary of findings: Several cautious generalisations can be made:

- No consistent association between soil characteristics and herd status (sero-prevalence) is evident in the observational studies identified in this systematic literature review.
- The highest quality study (Johnson-Ifeorunlu and Kaneene, 1999) did find an association between soil type and herd infection status. However, the effect observed was smaller than other risk factors assessed in that study such as important hygiene practices. The external validity of the study may be low, or put another way, the results may not easily be generalised to Australia where a very different farming system is present. The study appears not to consider many important potential confounders.
- Where associations were identified between soil type and infection, they were sometimes of moderate size and hence of questionable biological importance⁴, but this was not always true (Reviriego et al., 2000).
- Where an association between soil type and infection was present, infection was often present in herds/animals without the risk factor, just at lower prevalence (Reviriego et al., 2000; Ward and Perez, 2004).
- Some studies observed an effect that was inconsistent with findings of other papers – even though direct comparisons are difficult. For example Ward and Perez (2004) found that as silt was replaced by sand in loamy soils the risk of being in a cluster of higher sero-prevalence increased. Dhand et al. (2009) found an association between lower prevalence of Mptb shedding and sand.

Conclusion: the inconsistent nature of findings, the sparse number of studies using appropriate data, and the limited scientific quality of some studies, mean that the evidence is insufficient to definitively assess whether there is an association between soil type and infection with Mptb. Despite this, one relatively good quality study demonstrated an association between soil iron and soil pH (Johnson-Ifeorunlu and Kaneene, 1999), although the association was only of a moderate size and the study had several limitations.

7.2 Is there an association between soil type and disease (OJD or BJD)?

Study design: Two observational studies were identified that looked at the association between soil, management factors and clinical disease (Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007; Lugton, 2004a). Lugton (2004a) looked at the association between several owner reported parameters that were proxies for clinical disease and many risk

⁴ For example Johnson-Ifeorunlu and Kaneene (1999) reports an odds ratio of 1.01 for iron in the logistic regression model. Thus, if a herd was located in wetland soil with a mean iron content of 91.2 in comparison with a herd on well drained loam soil with a mean of 119.3 iron content (the most divergent soil types in terms of iron content) then iron content would increase by 27.1 from one to the other. Hence the change in log odds of herd infection is $27.1 \times 0.01 = 0.271$, which is an increase in odds $e^{0.271} = 1.31$ (95% CI: 1.08, 2.25). This is a modest increase in 'risk' considering you have compared the most likely iron content in the lowest iron soil to the most likely iron content in the highest iron content soil.

factors, including some basic soil factors. The study had several limitations, for example the subjectivity of data collected, statistical and inferential limitations. Dhand (2008) investigated the association between shedding of Mptb in sheep and various risk factors representing management and soil. The soil data collected in the study was the most comprehensive and detailed of all 9 observational studies identified in this review. Dhand's work appears the highest quality and most detailed research on this topic and is directly relevant to the objective of this systematic review. Both studies attempted to control confounding by including potential confounders.

Consistency and summary of findings: The two studies are difficult to compare due to differences in methodology. Lugton (2004a) found an association between pasture type/quality, 'light soils' and clinical disease. He hypothesised that these associations may indicate micronutrient deficiencies or imbalances and that these may affect clinical expression of OJD. However, at odds with his hypothesis, was the lack of association between trace element deficiencies or treatment of deficiencies and clinical disease. Dhand (2008) found an association between soil organic matter and OJD (but no other associations when control of management confounders occurred).

Both studies found an association between soil characteristics and OJD. However, the results seem to be inconsistent in that Dhand's (2008) findings suggest that higher quality soils (i.e. with more organic matter) lead to greater OJD, whereas Lugton's (2004a) study indicated that lighter infertile soils (that likely have lower organic matter) have greater OJD. However, this is purely speculative as not enough data on the definition of 'light infertile soils/ lightly textured sandy soils' was provided.

Conclusion: The studies of Dhand (2008) demonstrated an association between JD and certain soil characteristics (soil organic matter). This study was a good quality study and occurred in a relevant population of sheep. This study did not find an association between soil pH or iron and OJD but like Lugton (2004a) occurred across a limited range of soil pH (mostly acid soils).

8. Overall findings of literature review

1. There is a complex relationship between soil and Mptb. Reliable generalisations of this relationship are likely to be difficult across the vast range of soils where JD is found.

Many soil and environmental parameters (e.g. pH, electrolyte concentration, soil structure and composition, slope and rainfall) have been shown to affect adsorption and the general retention of Mptb in soil, which is likely to affect ingestion of Mptb by grazing ruminants. Unfortunately, the complexity of interactions between soil chemical, physical and biological parameters is high and still not well understood. When this complexity is combined with the vast range in soil (and climate and management etc.) parameters across the area of the globe endemic for JD, it becomes very difficult to generalise the effect of soil on JD/Mptb. It is possible the inconsistent and widely divergent associations recorded in observational studies are accurate in themselves, but a reflection of this complexity, as well as indicative of divergent study designs and methodologies (see below).

2. Evidence to assess the association between soil and JD is limited.

The objective studies (observational) that investigate the association between soil and JD are:

- limited in number;
- have a cross sectional study design that only provides a limited ability to investigate the issue;
- limited or at best variable in quality. For example, sometimes pre-existing aggregated regional soil data appears to have been used because it is convenient, which limits the analysis and inferences possible. Other quality issues sometimes included subjectivity and poor control of confounding;
- widely divergent in design, limiting their ability to be compared or combined in a comprehensive meta-analysis; and
- of limited external validity. The ability to extrapolate findings from overseas farming systems to the Australian context is sometimes limited.

3. Contradictory cross sectional surveys

The study findings investigating the association between soil and JD are contradictory with essentially an even split between an association and no association. This suggests that the effect investigated was not constant across the study populations investigated or that methodological issues in research led to biased results in some studies.

4. The two high quality cross sectional surveys suggest an association but are not consistent in findings

An association between soil and JD was evident in the two highest quality studies.

The study in Michigan dairy cattle found significant associations that suggest management risk factors are very important and that soil risk factors (pH and iron) are important (but less biologically important than management factors). Some potentially important confounders

appear not to have been considered in this analysis (e.g. herd size or density of animals). The ability to extrapolate findings of this study to certain parts of the Australian production system may be limited (i.e. the external validity is limited).

The most comprehensive study of JD infected Australian sheep flocks utilised relevant, accurate and comprehensive data (Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007). Its external validity for Australian sheep production is high. It collected data on many potential confounders. This study found no association between soil pH or micronutrients and JD. It did find an association between soil organic matter and JD with several mechanisms of action hypothesised. On balance, this study of Australian sheep is more valid and relevant to Australian producers than any other study identified in the review. It suggests an association between organic carbon and JD but no association between JD and other soil parameters (such as texture, soil nutrients including Fe and soil pH).

5. Hypotheses

Published or perceived associations between soil characteristics and JD or Mptb were identified. The hypotheses posed to explain such associations were also investigated.

The association between Michigan dairy cattle and soil pH, iron and liming was hypothesised to reflect a greater survival or viability of Mptb in soils of low pH (due to iron availability) (Johnson-Ifearegulu and Kaneene, 1999). This hypothesis was poorly supported as it implies that Mptb replicates in the soil when it is known that Mptb is an obligate parasite. This hypothesis could only be possible if pH (iron) affected Mptb dormancy or Mptb whilst in a dormant state. There is little published evidence to assess this.

The association between soil carbon and OJD (Dhand, 2008; Dhand et al., 2009a) was suggested to occur due to three hypotheses. Each of these was possible, although other hypotheses may also explain the association (see below).

The hypothesis that light textured soils with improved pastures induce micronutrient deficiencies or imbalances in ruminants resulting in clinical expression of JD (Lugton, 2004a) was not supported by a substantial observational study or by other more recently published research. Nonetheless, the hypothesis was not implausible, there was just little evidence to assess it one way or the other. Care should be taken not to confuse iron metabolism during *in vitro* culture and *in vivo* (host growth) where alternative pathways for iron uptake by Mptb exist. Also, care should be taken to not confuse the effect of mycobacteria on host iron metabolism and the hypothesis that iron deficiency is associated with clinical expression of JD.

An alternative and general hypothesis is that complex interactions of soil and environmental features can lead to variable retention and survival of Mptb in upper soil levels. Further, different infectious doses of Mptb may be available to grazing ruminants depending on soil and environmental characteristics. A mechanism such as this would tend to be revealed during observational studies as an inconstant association between soil characteristics and disease (in terms of size and direction). Inconstant associations have been observed across many cross sectional surveys, supporting this conjecture.

6. Further study required to assess the association

In order to provide a valid assessment of the association between soil and JD, further research is required. However, care will be needed to ensure studies have good external validity (i.e. results are generalisable as far as possible). Such studies would need to account for spatial and temporal autocorrelation. This is especially important as the time since infection was introduced is important for prevalence and detectability of disease, and spatial proximity to established endemic areas is likely to be a risk factor. Failure to account for this would introduce bias into measured associations.

Randomised controlled trials in relevant areas would provide the best assessment but would be expensive, time consuming and maybe fraught with social and financial problems depending on study design and location.

Other more practical study designs, such as observational studies of large (e.g. state or national data sets of JD and soil), whilst adjusting for spatial and temporal relationships, would be a less expensive and likely an effective means of further assessing the proposed associations. The advantage of such an approach would include that the data set could be sub sampled to look for associations that are more local in nature. Some jurisdictions may have existing records that may contain most of the data, but it is unlikely that all the data would routinely be available, or possible to collect. Data quality may be low.

Data required for such a study would include:

- location and size of affected and unaffected farms (e.g. GPS coordinates and flock/herd size)
- detailed soil data at these locations
- underlying population information (density, size, movements etc.)
- time that these herds/flocks were infected (i.e. not just a detection date)

Other basic soil biology and bacterial research would assist answering the question. For example any proposed relationship between Fe availability and growth in soil could be tested by targeting the microbes' siderophore based import systems (Moolji et al., 2012) and measuring their expression in soil.

However, such studies must also be assessed in terms of practical applications. If an association between soil pH and infection or disease was identified, would it practically influence management of the disease? Only when such questions are addressed should further research money be devoted to the issue.

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Appendix 1: Assessment of meta-analysis potential of observational studies

A meta-analysis of the disparate observational studies identified was attempted in order to provide a single summary measure of the effect of key risk factors (e.g. soil pH) and to allow assessment of the heterogeneity in published studies. Unfortunately this was abandoned for several reasons. In essence outcomes, data reported and methodologies were not comparable and hence not conducive to meta-analysis. In assessing soil pH, 2/3rds of studies would have been excluded.

For example, outcomes recorded varied from farmer reported clinical disease (Lugton, 2004a), in most studies presence or absence of infection in a herd (classified using different numbers of sero-reactors in a herd), herd sero-prevalence (Johnson-Ifearegundun and Kaneene, 1999) or other measures (Dhand, 2008; Ward and Perez, 2004). Many of the studies used automated model building procedures to model the relationship between outcomes and risk factors. This meant that non-significant risk factors were not included in the final model (this frequently included soil pH), which meant no measure of association and sampling variance for the risk factor was available for inclusion in a meta-analysis (Dhand, 2008; Johnson-Ifearegundun and Kaneene, 1999; Lugton, 2004a). Frequently important data that would be required to construct a measure of association was missing (for example the numbers of un-infected beef herds on acid and non-acid soils in Louisiana (Turnquist et al., 1991).

The assessment of each observational study for meta-analysis is listed below.

- Herd status (sero-prevalence) and association with loess soil in Louisiana (Turnquist et al., 1991)

Data could not be reconstructed.

- Environmental risk factors for herd status (sero-prevalence) in Michigan dairy cattle (Johnson-Ifearegundun and Kaneene, 1999; Johnson-Ifearegundun and Kaneene, 1998)

Iron and lime application in the logistic regression model (herd status) and soil pH, soil iron and lime application were significant in the Poisson model (sero-prevalence). Hence any meta-analysis that uses a similar outcome and wishes to explore those variables and uses standard modelling approaches would be compatible with this data.

- Association between soil type and herd status (sero-prevalence) in Spanish sheep and goats (Reviriego et al., 2000)

If a broad meta-analysis of low quality, acid soils is being assessed as a risk factor then the data and outcome odds ratios may be suitable.

- Risk factors for herd status (sero-positive) in Dutch dairy cattle (Musken et al., 2003)

	Acid soil	Non-acid soil
D+	39	36
D-	127	107

Using extrapolation from data in the paper an odds ratio of 0.91 (95% CI: 0.54, 1.54) was evident.

- Mptb sero-prevalence and its association with soil type in Michigan beef and dairy cattle (Ward and Perez, 2004)

The data are not very compatible with other studies due to the interpretation of odds ratios (that is it is not presence or absence, but instead odds of membership of a higher than median sero-prevalence cluster).

- Association of risk factors and clinical disease in infected Australian sheep flocks (Lugton, 2004a)

There are few data present that can be used in a meta-analysis of soil iron and pH. Soil pH had 32% missing values with the rest imputed with crude techniques; so much caution would be required to use this variable. Additionally, the automated modelling approaches mean an estimate of the adjusted (or univariate) odds of disease cannot be determined. The description of soil type may potentially be used, but the description is vague, subjective and open to bias. Soil texture: heavy, medium or light was included in one model (3) and could be used if the outcome the youngest age at which deaths occurred was relevant to the meta-analysis.

- Alberta beef and dairy cattle and association herd status (sero-prevalence) and agro-ecological data (Scott et al., 2007; Scott et al., 2006)

Dairy= pH; OR 1.92 (1.14-3.22) for acid relative to alkaline soils. Adjusted for herd size adjusted and GEE approach used to account for lack of independence. Beef= pH; (2.5 (not reported but cross 1)) for acidic relative to alkaline soils.

- Association of Mptb shedding and management and soil risk factors in Australia (Dhand, 2008; Dhand et al., 2009a; Dhand et al., 2007)

This presents a problem as an estimate of the association between iron, pH etc. and JD is unstated in the paper because automated model building approaches were used and these variables were not significant and hence were not included in the final models. All that can be stated with certainty is that the confidence interval of the estimated adjusted odds ratio must have crossed 1.