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Association of farm soil characteristics with ovine Johne's disease in Australia

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Abstract

Speculation about the association of soil characteristics with the expression of ovine Johne's disease (OJD) prompted this cross-sectional study. We enrolled 92 sheep flocks in Australia during 2004-05 and in each enrolled flock collected pooled faecal samples from an identified cohort (group of same age and sex) of sheep and soil samples from the paddocks grazed by this cohort of sheep. Faecal pools were cultured to create three outcome variables: positive or negative status of faecal pools (pool OJD status, binary); the log number of viable *Mycobacterium avium* subsp. *paratuberculosis* (MAP) organisms per gram of faeces (log pool MAP number, continuous); and the prevalence of faecal shedders (cohort OJD prevalence level, ordinal: low <2%, medium 2-10% and high >10%). Separate statistical models were then developed to investigate the association between soil characteristics and each outcome variable.

Sheep raised on soils with a higher percentage of organic carbon and clay had a higher OJD prevalence whereas, sheep grazing on soils with a higher content of sand and nitrogen had a lower OJD prevalence. Iron content of the soil was positively associated with OJD infection but the association between soil pH and OJD was inconclusive. Parent soil type, the only farm level factor, was not significant in any of the final models.

Study results indicate a higher risk of OJD in sheep raised on soils with greater organic matter and clay content. We hypothesise that this is due to adsorption of MAP to clay and the

consequent retention of the bacteria in the topsoil, thus making them available in higher numbers to grazing sheep.

Keywords: risk factors, paratuberculosis, mycobacterium, cross-sectional study, organic carbon, clay, sand, pH, iron.

1. Introduction

Paratuberculosis or Johne's disease (JD), a chronic enteric disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), causes considerable economic losses to the livestock industries. Losses to the American dairy industry alone were estimated to be US$ 200 to 250 million annually (Ott et al., 1999). Similarly in Australia, paratuberculosis caused an average reduction of income of AU$ 13,715 per farm per year on 12 infected Merino sheep flocks (Bush et al., 2006). Control programmes initiated in many countries to combat the disease have met with limited success, partly due to incomplete understanding of disease epidemiology, particularly the factors that affect disease prevalence and mycobacterial survival. While research into the epidemiology of paratuberculosis is undertaken in order to inform disease control and prevention programmes, a discussion continues among farmers as well as the scientific community about the potential association of soil characteristics with disease prevalence and severity (Lugton, 2004b).

Characteristics of soil, particularly soil pH and iron content are hypothesised to be associated with JD prevalence based on the premise of better MAP survival in soils with high iron content and low pH (Johnson-Ifearulundu and Kaneene, 1997). Like other organisms, MAP requires iron for its continued survival and replication, but unlike others, is a poor chelator of iron (Cocito et al., 1994). However, at low pH, the solubility of iron increases, which enhances the ability of MAP to uptake iron (Johnson-Ifearulundu and Kaneene, 1999) and therefore encourages its survival outside its biological host. On the other hand, MAP is an obligate pathogen and its period of survival in the external environment is finite (Whittington et al., 2004). This creates some uncertainties about the impact of soil pH and iron content on MAP survival.

Studies conducted in dairy cattle in the USA and sheep in South Africa reported increased prevalence of JD in animals reared on acidic soils (Johnson-Ifearulundu and Kaneene, 1999; Michel and Bastianello, 2000). In contrast, in a study conducted in the Netherlands, soil pH was not significantly different between seropositive and seronegative dairy cattle herds (Muskens et al., 2003). Moreover, the application of lime to the soil (to increase pH) appeared not to affect the survival of MAP in an Australian field trial (Whittington et al., 2004). Apart from soil iron content and pH, soil type was also reported to affect the survival of MAP, and hence the distribution of JD by some (Reviriego et al., 2000; Ward and Perez, 2004) but not by others (Turnquist et al., 1991).

Limited scientific information and poor agreement among studies conducted to date encourages speculation by farmers about the potential import of some specific soil characteristics in the control of JD. These concerns have required responses from animal health regulatory authorities, who require more information to understand the effect of such factors on disease prevalence on-farm. Therefore, this study was conducted to identify and quantify significant

associations between soil characteristics and JD prevalence in infected Merino sheep flocks in Australia. The findings will contribute to our understanding of the epidemiology of JD in sheep and therefore to development of better disease control programmes.

2. Materials and methods

2.1. Faecal sample collection and culture

This study was conducted as a part of a larger JD risk factor investigation; farm selection and faecal sample collection procedures are described in detail elsewhere (Dhand et al., 2007). Briefly, to meet the target sample size of 100 flocks, we obtained a list of 233 known JD infected sheep farms from official records and contacted owners or managers of each by phone to request their participation. Details of farmers who agreed to participate were further screened to assess their flock eligibility according to our study selection criteria (self-replacing Merino flocks, infected for 3 or more years; and having $\geq$ 210 non-ovine Johne's disease (OJD)-vaccinated 3-year old, 3- and 4-year old or 4- and 5-year old sheep).

At each enrolled farm, a cohort of sheep (*defined as a group of sheep of a specific age and sex*) was identified from which pooled faecal samples (usually seven pools, each containing one pellet of faeces from 30 or 50 sheep) were collected. On 11 farms sufficient numbers of sheep were available to select a cohort of ewes and a cohort of wethers. On farms where sufficient numbers of sheep to constitute seven pools were not available from one age and sex group, we selected two or more cohorts of sheep from different sex and/or age groups. This resulted in selection of a major sheep cohort (with >3 pools) and a minor sheep cohort (with ≤ 3 pools), but for the analyses reported in this paper the minor sheep cohorts were excluded from the datasets (see Section 2.2.1 below).

Each faecal pool was cultured using a modified BACTEC radiometric culture method (BACTEC 460; Johnston Laboratories, Towson, MD), (Whittington et al., 2000). The growth of MAP was confirmed by polymerase chain reaction (PCR) and restriction endonuclease analysis (REA) to demonstrate the presence of IS*900* (Green et al., 1989; Whittington et al., 1998; Cousins et al., 1999). Faecal samples that were positive to all three tests done in series were considered positive. Pooled faecal culture results were used to create three outcome variables as discussed in Section 2.4.1.

2.2. Soil sample collection and analysis

2.2.1. Identification of paddocks and soil sampling

Three soil samples were collected on each farm, preferentially one each from the paddocks that had been grazed by the cohort sheep when they were lambs, weaners and yearling/adults, respectively. On farms from which two or more cohorts were selected due to insufficient number of animals of one age and sex group, soil samples were collected only from the paddocks grazed by the major cohort, therefore, the minor cohorts were excluded from the datasets used for analyses.

Similarly, on 11 farms from which an ewe and a wether cohort were selected, soil samples were sourced only from the paddocks grazed by the ewe cohort. Therefore, wether cohorts were

excluded from the data except for the datasets representing paddocks shared by both ewes and wethers (lambing paddocks in all 11 farms and weaning paddocks in 7 farms).

After the farmer identified the relevant paddock, the top 10 cm of the soil from approximately 30 sites per paddock was collected and then pooled and mixed thoroughly in a bucket to obtain one representative sample. The sampling sites were selected in a zigzag manner, if feasible, avoiding areas such as fences, roads and dams because soil from these areas is usually not representative of the paddock. Samples were stored at 4^{o}C until transported to the soil laboratory (usually transported within a week). At the time of sample collection, global positioning system (GPS) coordinates of all three paddocks were ascertained using a GPS meter (Garmin^{TM} GPS 12 XL; 12 channels).

2.2.2. Laboratory analyses of soil samples

A commercial laboratory (Incitec Pivot Limited, Werribee, Victoria) analysed the soil samples employing standard methods accredited by the National Association of Testing Authorities. The chemical characteristics of each sample were measured according to standardised procedures using calibrated equipment and further calculated parameters were created using standard industry formulae (Anonymous, 2006).

The proportions of clay, silt and sand in soil samples were measured by particle size analysis (PSA) at The University of Sydney soil physics laboratory. The amount of silt and clay in a dispersed suspension of soil was measured by a calibrated soil hydrometer whereas the amount of sand was measured gravimetrically. We determined soil texture from the proportions of silt, clay and sand for each sample using the international soil triangle (Leeper and Uren, 1993) employing the Texture Auto Lookup software (TAL for Windows version 4.2, http://agri.upm.edu.my/~chris/tal/).

2.3. Data management

Data from faecal and soil sample analyses and the specimen submission forms were entered into a database. After completion of data entry, the continuous variables were sorted to identify the 10 lowest and highest values which were verified against the original results to check for data entry errors.

Two datasets were created for statistical analyses reported in this paper: the cohort-level dataset had one observation for each cohort while the pool-level dataset had one observation for each faecal pool. The minor sheep cohorts comprised of ≤ 3 pools, the 5-year-old sheep cohorts and wether cohorts sourced from some farms, as discussed earlier, were deleted from the datasets. After these exclusions, the datasets used in the analyses reported in this paper represented 87 farms.

2.4. Data analysis

Unless mentioned to be otherwise, all statistical analyses were conducted using SAS statistical software (release 9.1, © 2002-2003, SAS Institute Inc., Cary, NC, USA).

2.4.1. Outcome variables

Based on the pooled faecal culture (PFC) results, three outcome variables were created representing different aspects of OJD infection: positive or negative status of faecal pools (pool OJD status (PSTATUS), binary); the log number of viable MAP organisms per gram faeces (log pool MAP number, continuous); and the prevalence of faecal shedders (cohort OJD prevalence level (CPREV), ordinal: low <2%, medium 2-10% and high >10%). These outcome variables were used in separate statistical analyses to identify the soil factors significantly associated with them.

2.4.1.1. Cohort OJD prevalence level (CPREV)

The prevalence of sheep shedding MAP within the cohort (animal-level cohort OJD prevalence) was estimated from the PFC results employing the variable pool size method of Williams and Moffitt (2001) for calculating animal-level prevalence from pooled results. Categorisation of this prevalence to classify each sheep cohort as either a low (<2% prevalence), medium (2-10% prevalence) or high (>10% prevalence) prevalence cohort, resulted in the creation of the CPREV outcome variable. This variable was used in ordinal logistic regression analyses (cummulative logit models or proportional odds model) to identify factors statistically associated with CPREV and to quantify the magnitude of these associations. In cumulative logit models, the outcome is assumed to have a natural ordering and the parameter estimates (and hence the odds ratios) are considered to be independent of the category of the outcome variable.

2.4.1.2. Pool OJD status (PSTATUS)

PSTATUS, a binary outcome, represented the positive or negative PFC status of each faecal pool. It was used in binomial logistic regression analyses at the univariable level and generalised linear mixed model analyses at the multivariable level to identify the soil factors significantly associated with the positive pools.

2.4.2. Log pool MAP number (MAPNUM)

MAPNUM outcome represented the $\log_{10}$ of viable numbers of MAP per gram of faeces and was based on the method of Reddacliff et al. (2003). This method calculates the number of organisms initially inoculated in the culture media on the basis of the number of days taken by the sample to reach a cumulative growth index of 1000 in BACTEC media. This outcome was used in linear regression analyses at the univariable level and linear mixed model analyses at the multivariable level to identify the soil factors significantly associated with MAPNUM.

2.4.3. Explanatory variables

Two cohort-level variables, sex and age, were considered to be potential confounders because they were expected to confound the association of explanatory variables under investigation with the outcome variables. The prevalence of faecal shedding of MAP was shown to be associated with sex of sheep (Dhand et al., 2007) and is also likely to be associated with soil characteristics due to anecdotal evidence that farmers tend to raise wethers on nutritionally poorer paddocks compared to ewes. Similarly, cohort age might be associated with both the outcome and the explanatory variables.

Information about parent soil type, the only farm level factor, was obtained from the farmer in a questionnaire administered to identify sheep management practices (Dhand et al., 2007) and further verified from geological maps selected using the GPS coordinates of the soil samples. Information from the maps was given precedence when the referenced parent soil type differed from what the farmer had reported.

Except parent soil type, all other explanatory variables were derived from soil analyses. The main set of variables represented average results for the three samples analysed per farm (labelled "3-paddock mean" variables). In addition, we created three subsets of variables based on the results of the samples sourced from the paddocks grazed by cohort sheep when they were lambs, weaners or yearling/adults (labelled "lambing paddock", "weaner paddock" and "adult paddock" variables, respectively). This was considered necessary because descriptive analyses of soil characteristics indicated considerable variability between the results of 3 soil samples collected per farm. Secondly, this avoided confounding due to farmer preference to mark out particular paddocks for lambing or weaning and also aided in the investigation of the association of soil characteristics to which the sheep were exposed at various stages during their lifetime that could determine their disease status as adults.

2.4.4. Descriptive analyses

To assess the distribution of explanatory variables, frequency distributions and bar charts were created for the categorical variables and histograms for the continuous variables. Further, to have a preliminary idea of their association with the outcomes, we prepared contingency tables of the categorical explanatory variables and box-and-whisker plots of the continuous explanatory variables for the categories of CPREV and PSTATUS. Two variables, sodium and chloride, were dropped because of a large number of missing values (Table 1) and all the remaining variables were tested for unconditional associations (20 each for 3-paddock mean, lambing, weaning and adult paddock subsets).

Table 1
Descriptive statistics for the quantitative soil variables measured in 261 soil samples collected from 87 farms (3 samples per farm) in 2004 in Australia.

Variables[a]	Number	Minimum	25P	Mean	Median	75P	Maximum
Aluminium (meq/100 g)	218	0.03	0.10	0.33	0.22	0.44	2.00
Boron (mg/Kg)	255	0.18	0.32	0.58	0.44	0.62	5.40
Calcium (meq/100 g)	261	0.55	2.40	4.67	3.80	6.00	29.00
Cation exchange capacity (meq/100 g)	261	1.95	4.22	7.17	5.81	8.46	35.10
Chloride (mg/kg)[b]	184	10.00	13.50	31.95	20.00	33.00	260.00
%Clay	253	4.05	11.38	16.09	14.83	19.17	48.74
Copper (mg/kg)	261	0.01	0.33	0.99	0.51	0.95	11.00
Iron (mg/Kg)	261	35.00	130.00	195.39	190.00	250.00	470.00
Magnesium (Meq/100 g)	259	0.29	0.62	1.39	0.91	1.60	15.00
Manganese (mg/kg)	261	0.33	19.00	35.38	30.00	47.00	150.00
Nitrate Nitrogen (mg/kg)	253	1.00	5.50	14.21	10.00	19.00	76.00
%Organic carbon	255	0.89	1.70	2.51	2.30	3.00	7.70

pH (CaCl2)	261	3.90	4.40	4.80	4.60	5.10	7.50
Phosphorus (mg/Kg)	261	6.80	18.00	31.74	27.00	39.00	200.00
Phosphorus buffer index	255	4.86	45.00	69.60	57.00	83.00	650.00
Potassium (meq/100g)	261	0.10	0.35	0.59	0.52	0.74	2.30
%Sand	253	30.37	52.83	61.77	63.49	70.42	91.90
%Silt	253	2.67	16.75	22.14	21.00	27.31	41.44
Sodium (meq/100g) [b]	48	0.04	0.24	0.53	0.42	0.72	1.70
Sulphate Sulphur (mg/Kg)	261	1.40	4.30	7.59	6.50	9.20	49.00
Zinc (mg/kg)	261	0.20	0.86	1.68	1.20	1.90	33.00

a. Chemical compounds were assumed to be missing if present at a level lower than the minimum detection limit of the laboratory method. Minimum detection limits reported by the laboratory were: aluminium =0.03 meq/100 g, chloride =10mg/kg, magnesium= 0.2 meq /100 g, Nitrate nitrogen =1mg/kg and sodium =0.2meq/100 g;
b. Excluded from further analysis due to a large number of missing values.

2.4.5. Univariable analyses

Facilitated by in-house developed SAS macros (http://elearn.vetsci.usyd.edu.au/magicmacros), separate univariable analyses were conducted for all four subsets of explanatory variables to investigate their unconditional association with each outcome variable using ordinal logistic regression for CPREV, binomial logistic regression for PSTATUS and linear regression for MAPNUM (Hosmer and Lemeshow, 2000; Stokes et al., 2000; Armitage et al., 2002). Explanatory variables associated with the outcome variables at $P < 0.25$ were subsequently selected for inclusion in the relevant multivariable model.

Linearity of continuous variables was assessed visually by fitting a spline of the variable with the categorical outcome variables CPREV and PSTATUS, facilitated by the PSPLINET macro (available from Vanderbilt University School of Medicine website: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/SasMacros).

2.4.6. Multivariable analyses

2.4.6.3. Ordinal logistic regression analyses for CPREV

Ordinal logistic regression models were constructed for the CPREV outcome variable using the SAS LOGISTIC procedure (Stokes et al., 2000) by a manual forward stepwise approach. Decision on inclusion or exclusion of a variable at each step was based on the individual contribution of each variable using a likelihood-ratio chi-square test (retaining variables with $P < 0.10$) although two confounders (cohort age and cohort sex) were forced into each model as fixed effects. First order interaction terms were then added to all the final models and retained when significant at $P < 0.05$. Random effects models were not considered because only one cohort existed for majority of flocks.

Proportional odds assumption of the cumulative logit model was tested by the score test (Clark, 2005) whereas the assumption of linearity for continuous variables was assessed by fitting a spline of the variable with CPREV after adjusting for other variables in the final models, using the

macro PSPLINET as described above. If there were slight departures from linearity, the model was fitted again after creating spline variables as well as after adding a quadratic term (or other appropriate term) to the model. Significance of spline variables or the quadratic term was assessed based on the changes in the log-likelihood and the simpler model based on the linear term was retained if the complex model was not better. However, if there were considerable departures from linearity, then a spline of the non-linear variable was fitted and presented after adjusting for the effect of other variables in the final model.

2.4.6.4. Generalised linear mixed models for PSTATUS

Generalised linear mixed models for PSTATUS were conducted for each variable subset by including flock level random effects to account for clustering of pools within flocks, employing SAS GLIMMIX procedure (Anonymous, 2005; Schabenberger, 2005). Two confounders – cohort age and sex – were forced in all the models as done for CPREV model. In addition, a variable '$\log_{10}$ of the pool size' was forced in all the models because the pools were not of uniform size.

Variable selection was based on chi-square test of fixed effects (cut-off P-value < 0.10). First order interaction terms were added to all the final models and retained when significant at P < 0.05.

Linearity of the continuous variables was tested visually by plotting splines of the variables against log odds of the outcome, similar to the CPREV model.

2.4.6.5. Linear mixed models for MAPNUM

General linear mixed models for MAPNUM were constructed using SAS MIXED procedure (Brown and Prescott, 2000) following a procedure similar to that reported for PSTATUS.

3. Results

Of a total of 233 known OJD-infected farms investigated to identify eligible flocks, 141 were not eligible (because 32 farmers declined to participate and 109 flocks did not meet the selection criteria) and 92 were enrolled by 31 July 2004. Faecal samples from the enrolled flocks were collected by 22 September 2004 and the soil samples by 21 December 2004.

The enrolled farms were located in four states of Australia: New South Wales (77 flocks), Victoria (7), Tasmania (6) and Western Australia (2). Based on figures for published in November 2004 for currently infected flocks in NSW, Victoria and Tasmania and Western Australia (Citer and Sergeant, 2004), the proportion of infected flocks in each state constituted by the enrolled flocks was 21.9% for NSW, 5.4% for Victoria, 14.6% for Tasmania and 66.7% for Western Australia.

Of the 92 enrolled farms, data for 5 farms were excluded due to various reasons discussed earlier. In the remaining 87 farms, the farm area ranged from 230 to 8100 ha (median 1087) of which 40 - 100% (median 100%) was grazed by sheep and 0 - 100% (median 75%) was planted with improved pasture. The farms were located at altitudes ranging from 20 to 1500 m (median 650) above sea level and the topography was flat for 5.8% of farms, gently undulating or undulating for 60.9%, and undulating hilly or hilly for 33.3%.

Table 2
Descriptive statistics of explanatory variables (based on means of 3 soil samples per farm) for categories of the cohort OJD prevalence level (CPREV) outcome variable for 87 sheep cohorts sampled from as many flocks in 2004 in Australia.

Variables (3-paddock mean)	Cohort OJD prevalence Level (CPREV)											
	<2% (n=23)				2-10% (n=48)				>10% (n=16)			
	25P	Mean	Median	75P	25P	Mean	Median	75P	25P	Mean	Median	75P
Aluminium (meq/100 g)	0.13	0.28	0.22	0.43	0.14	0.33	0.26	0.47	0.10	0.30	0.19	0.33
Boron (mg/Kg)	0.32	0.41	0.39	0.45	0.33	0.58	0.46	0.60	0.44	0.81	0.72	0.89
Calcium (meq/100 g)	2.57	3.73	3.57	4.53	2.90	4.65	4.15	5.48	3.92	6.08	5.47	6.90
Cation exchange capacity (meq/100 g)	4.27	5.51	4.97	6.40	4.55	7.23	5.95	8.00	5.56	9.40	9.31	10.96
%Clay	12.52	14.66	14.39	16.94	11.77	15.75	14.33	17.67	15.21	19.08	19.24	23.77
Copper (mg/kg)	0.40	0.85	0.57	0.93	0.34	1.04	0.50	0.82	0.43	1.03	0.79	1.41
Iron (mg/Kg)	133.33	172.12	173.33	196.67	155.00	200.51	203.33	250.00	152.00	213.50	223.33	271.67
Magnesium (meq/100 g)	0.60	0.81	0.74	0.92	0.66	1.47	0.94	1.57	0.86	1.97	1.55	2.80
Manganese (mg/kg)	19.53	38.91	36.00	48.00	23.83	33.05	30.00	42.33	22.00	37.26	34.50	54.67
Nitrate nitrogen (mg/kg)	8.50	14.48	13.47	20.67	7.08	14.64	12.35	18.53	5.70	11.39	9.55	11.48
Organic carbon%	1.57	1.96	1.83	2.10	2.00	2.50	2.37	2.80	2.10	3.39	3.37	4.23
pH (CaCl2)	4.40	4.75	4.67	5.07	4.40	4.80	4.65	5.00	4.67	4.92	4.83	5.20
Phosphorus (mg/Kg)	19.33	30.36	30.00	41.00	21.00	31.65	27.67	39.50	21.67	34.00	34.33	44.83
Phosphorus buffer index	40.00	53.80	46.33	69.33	48.00	68.52	62.67	84.67	59.33	97.24	73.33	102.67
Potassium (meq/100 g)	0.39	0.56	0.54	0.77	0.37	0.57	0.51	0.70	0.51	0.68	0.61	0.85
%Sand	61.01	64.75	64.22	69.99	54.12	62.08	63.40	70.07	46.66	56.84	56.98	63.41
%Silt	17.57	20.59	19.71	22.71	17.97	22.17	21.65	27.40	18.75	24.09	24.88	31.34
Sulphate Sulphur (mg/Kg)	4.60	6.22	5.90	7.10	4.00	7.17	6.82	8.67	7.68	10.80	9.17	12.48
Zinc (mg/kg)	0.78	1.35	1.16	1.70	0.99	1.79	1.42	1.97	1.09	1.84	1.48	2.27

3.1. Soil texture

Classification for soil texture based on PSA (available for 253 soil samples) was clay and silty clay for 1 sample each, clay loam for 9 samples, silty clay loam for 11 samples, loam for 108 samples, silty loam for 68 samples, sandy loam for 6 samples, sand for 4 samples and loamy sand for 45 samples. However, soil texture was not used in statistical analyses because we included its constituent variables (%clay, %sand and %silt) instead.

3.2. Soil descriptive information

Descriptive information for 261 soil samples collected from 87 farms is presented in Table 1 and the soil explanatory variables for the mean of 3-paddocks derived from these results in Tables 2 and 3. Due to a large number of missing values, sodium and chloride were not used for analytical analyses.

3.3. Ordinal logistic regression analyses for cohort OJD prevalence (CPREV model)

Of the 20 soil variables investigated for unconditional association in each of the four subsets, 13, 15, 13 and 13 were significant in the univariable models ($P<0.25$) of 3-paddock mean, lambing, weaning and yearling/adult variable subsets, respectively. Final model results for 3-paddock mean, weaning paddock and adult paddock variable subsets presented in Table 4 and Figs. 1a and b indicate a positive linear association of CPREV with organic %carbon while a negative linear association with %sand.

Models for the lambing paddock variable subset had a continuous variable (sulphate sulphur) that showed a non-linear association with CPREV (Fig. 1c and d). The association appears relatively linear except at low sulphur content in Fig. 1c, but a temporary deletion of one outlier (with the sulphur content of 49 mg/kg) highlighted the non-linear nature of the association (Fig 1d). The other continuous variable in this model, nitrate nitrogen, had an approximately linear association (Fig. 1e).

Table 3: Contingency tables of categorical explanatory variables with the cohort OJD prevalence level (CPREV) for 87 sheep cohorts sampled from as many flocks in 2004 in Australia

Variables and categories	Cohort OJD prevalence level (CPREV)			Total
	<2% (n=23)	2-10% (n=48)	>10% (n=16)	
Parent soil type[a]				
Basalt	1	6	1	8
Granite	12	11	4	27
Shale and sand stone	3	19	6	28
Mixed without limestone	2	5	2	9
Mixed with limestone	5	7	3	15
Cohort age[b]				
3-year-old	13	30	9	52
4-year-old	10	18	7	35
Cohort sex[b]				
Ewes	20	43	11	74
Wethers	3	5	5	13

a. Farmer reported; b. Confounders forced in all models.

Table 4
Final ordinal logistic regression models for three variable subsets[a] for cohort OJD animal level prevalence (CPREV) categorised as low (<2%), medium (2-10%) and high (>10%) based on faecal pools collected from sheep flocks in Australia in 2004.

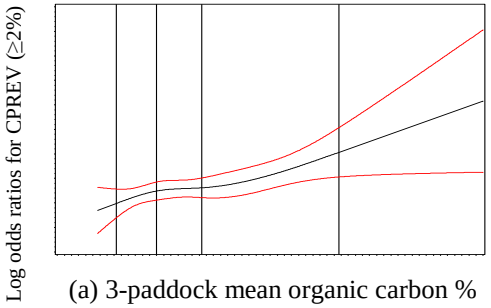
Parameters	3-paddock mean variables (n= 85)					Weaning paddock variables (n = 68)					Adult paddock variables (n = 69)				
	b	*SE(b)*	OR and 95% CL		*P*	*b*	*SE(b)*	OR and 95% CL		*P*	*b*	*SE(b)*	OR and 95% CL		*P*
Constant															
>10% versus 2-10% and <2%	-2.02	1.61	-	-	-	-0.98	1.59	-	-		-2.12	1.73			-
>10% and 2-10% versus <2%	1.25	1.60	-	-	-	2.01	1.62	-	-		1.13	1.71			-
Cohort age	-	-	-	-	0.6	-	-	-	-	0.5					0.9
3 years	-	-	1.0	-	-	-	-	1.0	-						-
4 years	0.23	0.46	1.3	0.5, 3.2	-	-0.36	0.50	0.7	0.3, 1.9		0.09	0.51	1.1	0.4, 3.1	
Cohort sex	-	-	-	-	0.6	-	-	-	-	0.03					0.6
Ewes	-	-	1.0	-	-	-	-	1.0	-						-
Wethers	0.40	0.68	1.5	0.4, 6.1	-	1.42	0.65	4.1	1.1, 17.1		0.39	0.76	1.5	0.3, 7.0	-
%Organic carbon	1.16	0.30	3.2	1.9, 5.9	<0.001	0.88	0.32	2.4	1.4, 4.7	0.002	1.08	0.34	3.0	1.5, 6.0	0.001
%Sand	-0.05	0.02	0.9	0.9, 0.99	0.02	-0.05	0.02	0.95	0.9, 0.99	0.01	-0.04	0.02	0.96	0.9,0.99	0.04

Notes:

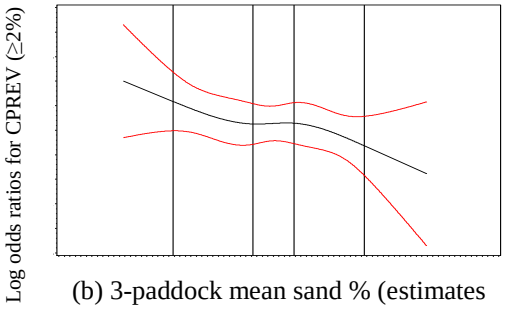
a. Results for the lambing paddock variable subset are presented separately in Fig. 1 because the effects of some variables were not linear.

b. Proportional odds assumption was valid for all models except that based on the weaning paddock variable subset.

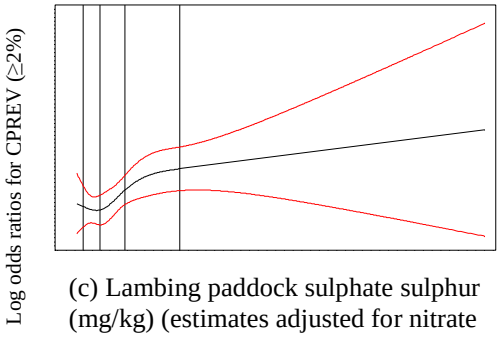
Figure 1.
Estimated spline transformation and 95% confidence limits of the association of some continuous soil variables with JD in sheep based on the study conducted in Australia in 2004-05.



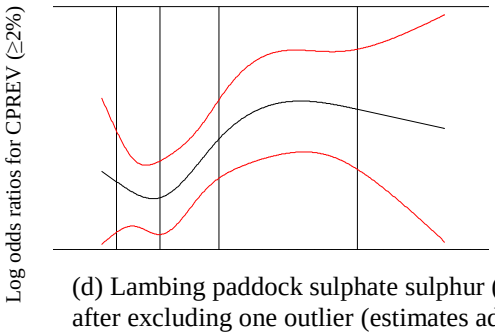
(a) 3-paddock mean organic carbon % (estimates adjusted for sand%, cohort age and cohort sex)



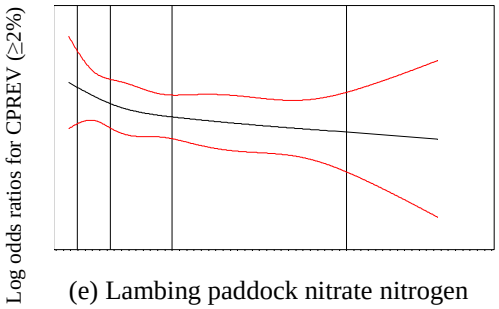
(b) 3-paddock mean sand % (estimates adjusted for organic carbon %, cohort age and cohort sex)



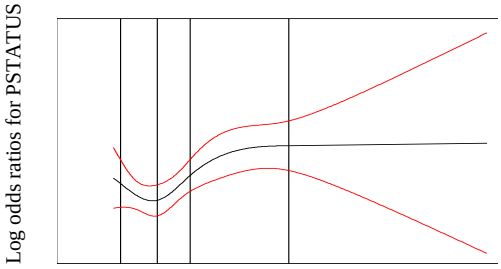
(c) Lambing paddock sulphate sulphur (mg/kg) (estimates adjusted for nitrate nitrogen (mg/kg), cohort age and cohort sex)



(d) Lambing paddock sulphate sulphur (mg/kg) after excluding one outlier (estimates adjusted for nitrate nitrogen, cohort age and cohort sex)



(e) Lambing paddock nitrate nitrogen (mg/kg) (estimates adjusted for sulphate sulphur (mg/kg), cohort age and cohort sex)



(f) Adult paddock pH ($CaCl_2$) (estimates adjusted for organic carbon %, cohort age and cohort sex)

3.4. Generalised linear mixed models for pool OJD status (PSTATUS model)

Of the 20 soil variables investigated in each subset, 16, 16, 15 and 13 were significant in the univariable binary logistic models (P<0.25) of 3-paddock mean, lambing, weaning and yearling/adult variable subsets, respectively.

The multivariable models for two variable subsets (3-paddock mean and weaning paddock) presented in Table 5 suggest a higher probability of pools being MAP positive in sheep raised on soils with higher organic %carbon and a lower probability in those raised on soils with higher

%sand. The association of continuous variables reported in Table 5 was linear (figures not shown).

Table 5

Final generalised linear mixed model for 2 subsets[a] of explanatory variables for pool OJD status (PSTATUS) outcome, created based on the culture results of faecal pools (positive or negative) collected from sheep flocks in Australia in 2004.

Parameters and categories	3-paddock mean variables (n= 553)					Weaning paddock variables (n = 440)				
	b	*SE(b)*	OR and 95% CL		*P*	*b*	*SE(b)*	OR and 95% CL		*P*
Intercept	0.13	2.80	-	-	-	-1.02	3.50	-	-	-
Cohort sex	-	-	-	-	0.9	-	-	-	-	0.2
Ewes	-	-	-	-	-	-	-	-	-	-
Wethers	0.06	0.49	0.8	0.4, 1.9	-	1.62	0.60	-	-	-
Cohort age	-	-	-	-	0.7	-	-	-	-	0.04
3 years	-	-	-	-	-	-	-	-	-	-
4 years	0.12	0.33	1.1	0.6, 2.1	-	-0.01	0.42	-	-	-
Log of pool size	-0.38	0.8	1.1	0.4, 2.8	0.6	0.61	1.00	1.8	0.3, 13.2	0.5
Organic carbon%	0.77	0.20	2.2	1.5, 3.2	<0.001	0.66	0.24	1.9	1.2, 3.1	0.007
Sand %	-	-	-	-	-	-0.03	0.02	0.97	0.9, 0.99	0.03
Cohort sex* Cohort age (4-year-old wethers)	-	-	-	-	-	-2.15	0.90	-	-	0.02
Flock variable variance estimates (SE)	1.18 (0.33)					1.37 (0.45)				

a. Results of the lambing and the adult paddock variable subset are discussed separately because the effect of some continuous variables was not linear.

For the lambing paddock variable subset, two soil variables sulphur and nitrate nitrogen were significant after adjusting for the confounders forced in the model. Their association with the outcome was similar to that in the CPREV model (Fig. 1c-e), and therefore not shown here.

For the adult paddock variable subset, %organic carbon and soil pH were the significant soil variables. The association of %organic carbon was similar to as described before, but that of soil pH was not linear, therefore, a spline of the variable is presented after adjusting for the effect of other variables in the model (Figure 1f).

Table 6

Final linear mixed model for log pool MAP number (MAPNUM) based on faecal pools collected from sheep flocks in Australia in 2004

Parameters	*3-paddock mean variables (n=553)*				*Lambing paddock variables (n=504)*				*Weaning paddock variables (n=440)*				*Adult paddock variables (n=445)*			
	b	*SE(b)*	95% CL of *b*	*P*	*b*	*SE(b)*	95% CL of *b*	*P*	*b*	*SE(b)*	95% CL of *b*	*P*	*b*	*SE(b)*	95% CL of *b*	*P*
Constant	1.67	2.08	-	-	4.07	2.27	-0.5, 8.6	-	-0.78	2.56	-5.9, 4.4		3.33	2.26	-1.2, 7.8	
Cohort sex	-	-	-	0.9	-	-	-	0.2	-	-	-	0.05				0.5
Ewes	0.00	-	-	-	0.00	-	-	-	0.00	-	-	-	0.00	-	-	-
Wethers	-0.03	0.34	-0.70, 0.64	-	0.79	0.30	0.2, 1.4	-	1.09	0.30	0.5, 1.7		-0.26	0.37	-0.99, 0.5	
Cohort age	-	-	-	0.3	-	-	-	0.4	-	-	-	0.3				0.6
3 years	0.00	-	-	-	0.00	-	-	-	0.00	-	-		0.00	-	-	-
4 years	0.22	0.23	-0.24, 0.7	-	0.25	0.28	-0.3, 0.8	-	0.25	0.29	-0.3, 0.8		0.15	0.27	-0.4, 0.7	
Log of pool size	-0.51	0.58	-1.7, 0.64	0.4	-0.9	0.6	-2.2, 0.3	0.2	0.10	0.71	-1.3, 1.5	0.9	-0.83	0.65	-2.1, 0.5	0.2
Iron	-	-	-	-	0.003	0.002	0.0001, 0.006	0.05	0.003	0.001	-0.0002, 0.006	0.07	-	-	-	-
Nitrogen	-	-	-	-	-0.02	0.01	-0.04, 0.002	0.07	-	-	-	-	-	-	-	-
%Organic carbon	0.57	0.13	0.3, 0.8	<0.001	0.29	0.14	0.003, 0.6	0.05	0.33	0.15	0.04, 0.6	0.03	0.59	0.15	0.3, 0.9	0.0001
%Clay	0.04	0.02	-0.005, 0.07	0.08	-	-	-	-	0.05	0.02	0.01, 0.09	0.009	-	-	-	-
Cohort sex×Cohort age (4-year-old wethers)	-	-	-	-	-0.96	0.47	-1.9, -0.04	0.04	-1.14	0.53	-2.2, -0.1	0.03				
Flock variable variance estimates	0.69 (0.17)				0.74 (0.19)				0.76 (0.22)				0.74 (0.20)			
Residual variance	2.41 (0.16)				2.41 (0.16)				2.24 (0.16)				2.52 (0.18)			

3.5. General linear mixed model analyses for pool MAP number (MAPNUM model)

Of the 20 soil variables investigated in each subset, 17, 16, 16 and 16 were unconditionally associated with the MAPNUM models of 3-paddock mean, lambing, weaning, and yearling/adult variable subsets, respectively. Final model results shown in Table 6 suggest that %organic carbon and nitrate nitrogen had a similar direction of association as in the models discussed previously. In addition, %clay and iron content of soil had a positive association with the log pool MAP number. None of the continuous variables had significant departures from linearity though some deviations from normality were observed in the histograms of studentised residuals, but the studentised conditional residuals were approximately normal.

All the flock level random effects were significant and accounted for 22% to 25% of the total variability (Table 6).

4. Discussion

The results suggest a positive linear association between %organic carbon and JD prevalence in sheep. Organic carbon, an indicator of soil organic matter content (Baldock and Skjemstad, 1999) might favour the survival of MAP, either directly by providing essential nutrients for its continued existence outside the biological host or indirectly by increasing pasture growth and thus shading which is reported to be favourable for MAP survival (Whittington et al., 2004). Organic matter also increases the water holding capacity of soil (Krull et al., 2004) but soil moisture has not yet been demonstrated to influence survival of MAP (Whittington et al., 2004). Regardless of the uncertainty about moisture, MAP would be available in greater numbers to the animals grazing on soils with a higher organic matter content.

Apart from its effect on increased mycobacterial survival, organic matter content could also be a confounder for higher stocking rates because the owners of the farms with more fertile soil might tend to increase sheep numbers per unit area of pasture. In our previous investigation, stocking rate in the lambing paddock was found to be detrimental for JD (Dhand et al., 2007); we intend to investigate this association further after adjusting for soil variables.

Similar to organic carbon, higher clay-content soils were associated with higher JD prevalence. Clay could adsorb MAP thus increasing its availability to sheep by retaining it in the upper soil layers rather than allowing it to be leached to the deeper layers. Clay particles are very small (<2µm) allowing a large number of clay particles to be packed in a specified volume of soil, as a result they offer a large surface area for bacterial attachment (Marshall, 1975). Adsorption of various bacteria and viruses of public health significance to clay particles has been reported previously (Moore et al., 1981; Taylor et al., 1981; Banks et al., 2003; 2005). This is mediated by electrostatic and van der Waals' forces or by cell surface hydrophobicity (Taylor et al., 1981). At present there is no direct evidence of mycobacaterial adsorption to clay particles, although one study reported recovery of only 3.5% of non-tuberculous mycobacteria inoculated into soil samples and attributed this to adsorption (Brooks et al., 1984). Whittington et al (2003) inferred a similar phenomenon for MAP, but further studies are required to substantiate this.

In contrast, sand particles are larger in size and offer less surface area for bacterial adsorption. This might account for the lower JD prevalence in flocks raised on soils with a higher % sand. These results are contrary to a previous study (Ward and Perez, 2004) that reported higher JD

prevalence in dairy cattle on farms with sandy soils. This might reflect the variability in management between enterprises and/or countries or confounding of soil variables by other factors. It could also reflect methodological differences between studies, because soil factors in the present study are based on analyses of samples collected from paddocks grazed by sheep, while in the previous study, soil type was characterized on the basis of herd location rather than analysis.

There was high negative correlation between soil %clay and %sand in both datasets, but we decided to test both variables in multivariable models because of no prior evidence of supremacy of one above the other. However, only one of the two variables was significant in the multivariable models, the other being probably deleted due to high correlation with the retained variable.

Similar to a previous study (Johnson-Ifearulundu and Kaneene, 1999), our results indicated an increase in JD prevalence with an increase in iron content of the soil. This association might be due to increased survival of MAP in soils with high iron content as suggested previously. However, it is interesting to note that, apart from iron, most of the cations investigated in this study (including the cation exchange capacity) had a positive association with JD at the univariable level (results not shown). The association of cations with JD observed in this study might in fact be due to their association with clay as clay usually holds a large number of cations because of being negatively charged (Marshall, 1975). Nevertheless, it is worth noting that iron content was significant in the multivariable models after adjusting for organic carbon whereas most other cations were not.

Our finding of no association with parent soil type stands in contrast to that of Reviriego et al. (2000) in Spain, who found soil type was one of the two predictor variables included in the final multivariable model for flock seroprevalence, but aligns with that of Johnson-Ifearulundu and Kaneene (1999) in Michigan who also found no association with soil type. Soil type affects many soil characteristics and therefore can be a surrogate variable for unmeasured factors.

The association of sulphur with JD was complex because it was detrimental for sheep at both lower and higher levels (Fig. 1d). The unfavourable effects of sulphur deficiency in the soil might be as a consequence of its ensuing deficiency in sheep, whereas the effects of higher soil sulphur levels might be due to its association with organic matter because most sources of sulphur in soil are actually constituents of organic matter. Sulphur was positively correlated with organic carbon in this study (Pearson correlation 0.5); in fact, organic carbon was only non-significant in two models where sulphur was present.

The protective association of nitrogen might reflect better protein synthesis and hence immune protection of the sheep but could also be a confounder for other agronomic practices and therefore warrants further investigation.

The previous reports of increased paratuberculosis prevalence with decreased soil pH (Johnson-Ifearulundu and Kaneene, 1999) could not be substantiated in this study because our results suggest a detrimental effect at both lower and higher levels of soil pH. However, this result should be interpreted with caution as pH was significant only in one model. The predominantly acidic soil pH on all farms except one in this study is worthy of note (range 3.9-7.5; median 4.6; Table 1) and might have limited detection of an association, yet the study farms demonstrated a

wide range of cohort OJD prevalence (0 to 59%) in the limited soil pH range. Please note that only JD-positive farms were studied in this project, and the associations cannot be extrapolated to imply being protective/detrimental for *introduction* of infection to uninfected flocks, because that was not the objective of the study.

We conducted this cross-sectional study in order to identify soil risk factors for JD in infected flocks. Within the limitations imposed by this study type, substantial efforts were made to maximise the ability of our study to investigate proposed explanatory variables and identify those strongly associated with JD infection. The soil explanatory variables, based on laboratory analyses and geological maps (for parent soil type), are objective measurements of topsoil composition at the time of collection that is more accurate than the reliance on producer reports or reference to regional soil survey data approaches used in some other studies (Kopecky, 1977; Reviriego et al., 2000; Lugton, 2004a). As a result the likelihood of information bias in explanatory variables is minimal. The objective nature of the explanatory variables was also one of the reasons that we did not favour their categorisation even when they had a non-linear association (such as sulphur) since we believe that categorisation would have caused a loss of information, potentially masking real associations.

However, some soil components might have changed between the time of grazing and the time of sample collection in 2004 due to weather conditions and fertilizer application and might have created information bias. For example, weather data collected in the study suggested substantial changes in rainfall across years in most regions, and majority of the farmers had made some changes in the type of fertilizer used or their rate/frequency over the past 10 years (results not shown). It is acknowledged that such changes would have biased our study results.

Similar to the explanatory variables, all three outcome variables in this study were based on objective data, the culture of faecal samples. The sensitivity of PFC is considerably greater than serology (Sergeant et al., 2002) and so use of PFC provides a more accurate estimate of prevalence than is achieved using serology. Furthermore, culture of faecal samples provides more specific results than serology/producer opinion used in previous studies to create outcome variables (Reviriego et al., 2000; Lugton, 2004a). However, we acknowledge that estimation of prevalence based on pooled faecal culture results involves assumptions about test sensitivity and independence between animals within a pool which might not be completely true, thus producing biased results. In addition, the logistics of sample collection and numbers of 3-5-year-old-sheep present in the study flocks resulted in variation between flocks in the number of pools collected and to a lesser extent variation in the size of collected pools. We overcame this by use of the Williams and Moffitt (2001) method to estimate individual prevalence from pool results. This method accounts for variable pool size and provides valid results for low and high proportions of positive pools including a lower confidence limit above zero for low prevalence estimates.

Variability in the results of analyses of three soil samples collected from three different paddocks on each farm necessitated development of separate models for lambing, weaning and adult paddocks to remove the confounding effect of paddock type and their associated management practices. Although the significance of variables differed slightly among the multivariable models, the subset of variables identified to have an association with JD infection was generally consistent. However, due to the large number of variables tested, caution should be observed while interpreting the associations that were significant only in one or two models.

5. Conclusions

The results of statistical models developed to investigate the association of soil characteristics with JD suggest higher disease prevalence in sheep raised on soils with a higher content of organic carbon, clay and iron and a lower content of sand and nitrogen. This might be due to the greater survival of MAP in such soils because of better availability of nutrients, or adsorption of MAP to clay particles, making them available to potential animal hosts. The association of pH with JD could not be substantiated in this study.

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