SPi	Journal Code	Article ID	Dispatch: 09-MAR-16	CE: No
	AVJ	12428	No. of Pages: 9	ME: No

Prevalence, faecal shedding and genetic characterisation of *Yersinia* spp. in sheep across four states of Australia

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Objectives To develop molecular tools for investigation of the prevalence, species and faecal shedding of *Yersinia* spp. in sheep.

Methods A quantitative PCR (qPCR) targeting the β subunit of the *Yersinia* spp. RNA polymerase gene was developed and validated. The prevalence of pathogenic *Y. enterocolitica* was determined by screening for the virulent *yst* gene. These qPCR assays were used to determine *Yersinia* spp. prevalence and faecal shedding concentration from 3412 faecal samples collected from approximately 1189 lambs (100–180 lambs/flock) on eight farms across Australia. This was a longitudinal study, with sheep sampled on three occasions (weaning, post-weaning and pre-slaughter). A subset of up to five positive samples from each sampling on each farm (n = 111) were sequenced.

Results *Yersinia* spp. (including both pathogenic and non-pathogenic species) were identified in all flocks, with 60.7% of lambs shedding *Yersinia* spp. on at least one sampling occasion. Point prevalence ranged from 4% to 91% across farms and sampling occasions. Median *Yersinia* spp. bacterial concentration was 1.1×10^6, 2.8×10^6 and 5.6×10^5 organisms/g faeces at weaning, post-weaning and pre-slaughter, respectively, across all farms. Pathogenic *Y. enterocolitica* was identified in all eight flocks sampled, with 14.8% of lambs shedding pathogenic *Y. enterocolitica* on at least one sampling occasion.

Conclusion *Yersinia* spp. and pathogenic *Y. enterocolitica* in particular were commonly identified in a sample of Australian sheep flocks using molecular techniques. Further studies into associations between faecal shedding of pathogenic *Yersinia* spp. and sheep productivity or clinical disease may utilise qPCR in conjunction with other diagnostic tools.

Keywords genotyping; lambs; qPCR; *Yersinia* spp; *RopB*; *yst*

Abbreviations bp, base pair; BT, biotype; IAC, internal amplification control; NSW, New South Wales; qPCR, quantitative PCR; RDS, relative standard deviation; RSQ, R squared; SA, South Australia; VIC, Victoria; WA, Western Australia

Aust Vet J 2016 doi: 10.1111/avj.12428

Yersinosis caused by *Yersinia* spp. causes gastrointestinal illness in humans and animals and is the most frequently reported zoonotic gastrointestinal disease (typically associated with *Y. enterocolitica*) after campylobacteriosis and salmonellosis in many developed countries.[1–3] Yersiniosis occurs in sheep of all ages, but is more common in younger animals. Clinical signs include scouring (diarrhoea), depression, dehydration, ill thrift and deaths.[4–6] It is caused by *Y. enterocolitica* biotype 5, serotype 0:2,3 and *Y. pseudotuberculosis* serotype III, and although *Y. intermedia* and *Y. frederiksenii* have also been reported in Australian sheep, they are considered non-pathogenic.[4,5,7–9]

Yersinia enterocolitica species exhibit broad biochemical and pathogenic diversity and have been characterised into six biotypes (BTs) according to their pathogenic properties. Of these, five are classed as pathogenic species (BT 2–5 weakly pathogenic and BT 1B highly pathogenic) and one as non-pathogenic (BT 1A), related to the absence of most of the classical virulence markers in BT 1A strains.[3,10] Growing epidemiological, clinical and experimental evidence suggests that some biotype 1A isolates are virulent and can cause gastrointestinal disease in humans, but their pathogenicity is sheep has not been determined.[10]

Yersinia spp. can be detected using microscopy, culture and immunoassays, but these can lack specificity and are time-consuming; in the case of culture, can require 3–5 days.[11] Many of the currently available enrichment and plating media for isolation of pathogenic strains of *Y. enterocolitica* are not selective enough to repress the background flora, which increases the risk of false-negative results.[3] Additionally, *Yersinia* spp. may enter into a viable but non-culturable state.[12]

More recently, PCR assays have been developed that have demonstrated enhanced detection of *Yersinia* spp. in food and water samples[13–15] and faecal samples.[16,17] Quantitative PCR (qPCR) assays for *Yersinia* spp. have the added advantage of being able to enumerate numbers of organisms present by directly monitoring the increasing amount of PCR products during DNA amplification, provide greater specificity and require less time and labour to complete than conventional PCRs.[18–20] *Yersinia enterocolitica* is also known to be an extremely heterogeneous species.[21] The methods that have been used most commonly to assess heterogeneity in *Y. enterocolitica* include biotyping, serotyping, phage typing and more recently, molecular typing. The RNA polymerase (*rpoB*) gene has emerged as a core gene candidate for phylogenetic analyses and identification of bacteria, especially when studying closely related isolates.[22]

Animals have long been suspected of being reservoirs for *Y. enterocolitica* and hence, sources of human infections.[23] There have been few studies that have examined the prevalence and faecal concentrations of *Yersinia* spp. in lambs in Australia and all have relied on culture and/or immunological methods for detection.[1,4,5,8–10] Therefore, the aim of the present study was to

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develop a qPCR for *Yersinia* spp. Targeting the β subunit of the *Yersinia* spp. *rpoB* gene and to use the qPCR assay to determine the prevalence, bacterial shedding concentrations and species of *Yersinia* spp., specifically *Y. enterocolitica*, in flocks of lambs (located over a wide geographical area representing the major sheep growing regions of Australia) over time between birth and slaughter.

Materials and methods

Animals and faecal sample collection

Faecal samples were collected from cross-bred lamb flocks from eight different farms (one flock per farm) across four states of Australia (Table 1). Farms were located in Western Australia (WA), New South Wales (NSW), Victoria (VIC) and South Australia (SA). Farms were selected to represent the wide range of environmental conditions under which sheep are typically farmed in Australia, including summer dominant, winter dominant and Mediterranean (hot dry summer, cool wet winter) rainfall patterns (Table 1). Flocks were selected for inclusion in the study on the basis that ewe numbers in a single mob were sufficiently large to supply at least 110 lambs for the study from birth until slaughter and that sheep were managed under normal husbandry conditions for slaughter lambs.

Lambs were born and reared in paddocks and were not housed indoors at any stage of the study. Lambs were selected at random at lamb marking for inclusion in the study. Lambs were individually identified with ear tags at lamb marking and faecal samples were collected on three occasions (i.e. the same animals were sampled on each occasion): (1) weaning (≈12 weeks of age); (2) post-weaning (≈19 weeks); and (3) pre-slaughter (≈29 weeks) (Table 2). A total of 3412 faecal samples from 1189 lambs were collected directly from the rectum (Table 2).

All sample collection methods used were approved by relevant animal ethics committees in each state, with the overall methodology approved by the Murdoch University Animal Ethics Committee (approval no. R2352/10).

DNA isolation

Total DNA was extracted from 200 mg of each faecal sample from NSW, SA, and VIC using a Power Soil DNA Kit (Mo Bio, Carlsbad, CA, USA) with some modifications as described by Yang et al.[26] Briefly, the faeces for DNA extraction were subjected to four cycles of freeze/thaw (liquid nitrogen followed by boiling water) to ensure efficient lysis of bacterial cells before being processed using the manufacturer's protocol. A negative extraction control (no faecal sample) was used in each 24-sample extraction group. DNA from faecal samples from WA ~~were~~ extracted using the protocol previously described.[24,25]

PCR amplification, quantitation and sequencing

Primers and probes for *Yersinia* spp. were designed using Primer 3 and Real-Time design software available from Biosearch Technologies (Petaluma, CA, USA). A 78-base pair (bp) fragment was amplified from the β subunit of *rpoB* of the *Yersinia* spp. using the forward primer rpoBF1 5′-GGT GCT TCT CTG ATT CCA TTC TTG-3′, the reverse primer rpoBR1 5′-CGC CTG ACG TTG CAT GTT C-3′ and the probe RpoB-Sb 5′-dFAM-AAC ACG ATG ACG CCA ACC GTG C-BHQ1-3′.

An internal amplification control (IAC) ~~consisted~~ of a fragment of a coding region from Jembrana disease virus ~~(JDV)~~ cloned into a pGEM-T vector (Promega, NSW, Aust) was used as previously described.[27] The IAC primers were JDVF (5′-GGT AGT GCT GAA AGA CAT T-3′) and JDVR (5′-ATG TAG CTT GAC CGG AAG T-3′) and the probe was 5′-(Cy5) 5′-TGC CCG CTG CCT CAG TAG TGC-BHQ2-3′. Each 15-μL PCR mixture contained 1 × PCR Buffer, 2 mmol/L $MgCl_2$, 1 mmol/L dNTPs, 1.0 U Kapa DNA polymerase

Table 1. Sheep farms sampled during the present study of *Yersinia* spp. across Australia

Farm	Farm location	Mean annual rainfall (mm)	Farm size (ha)	No. of sheep	Breed of sheep	Commencement of lambing	Goats and/or cattle on property	Winter stocking rate (DSE/ha)
SA1	Wirrega, SA	430	1040	1800	Suffolk	Mid-April	No	10
SA2	Struan, SA	550	1500	5500	BL/Merino × Suffolk	June	Yes	15
VIC1	Rosedale, VIC	620	30	300 ewes*	BL/Merino × Dorset	Mid-July	No	10
VIC2	Ballarat, VIC	750	1960	7000	Merino × Suffolk	Early August	Yes	13
NSW	Armidale, NSW	495	2958	1000	BL/Merino	May–August	No	20
WA1[a]	West Arthur, WA	500	1250	1750	Merino × Suffolk	Early August	No	10
WA2[a]	Pingelly, WA	450	1500	1350	Merino × Suffolk	Mid-July	No	12
WA3[a]	Frankland, WA	550	560	3300	Merino × Suffolk	Mid-July	No	21

[a]DNA from samples from Western Australia were extracted by J. Sweeny.[24,25]
*Breeding ewe numbers only because of large fluctuation in overall numbers related to trading.
BL, Border Leicester; DSE, dry sheep equivalent; ha, hectare; NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia

Table 2. Point prevalence and longitudinal prevalence (animals sampled on at least two occasions and positive on at least one sampling occasion) for *Yersinia* spp. and pathogenic *Y. enterocolitica,* and concentration (range and median) of *Yersinia* spp. (g^{-1}) in faecal samples collected from eight Australian farms over three sampling occasions

Farm	Sampling period	Lambs sampled (n)	*Yersinia*-positive (n)	% prevalence *Yersinia* spp. (95% CI)	pathogenic *Y. enterocolitica* (95% CI)	Conc. *Yersinia* spp. (organisms/g faeces)	
						Median	Range
SA1	Weaning	165	41	24.8 (18.3–31.4)	5.5 (2.0–8.9)	3.2×10^6	4.0×10^3–8.1×10^{10}
	Post-weaning	148	50	33.8 (26.2–41.4)	4.1 (0.9–7.2)	6.1×10^5	3.5×10^3–4.1×10^7
	Pre-slaughter	159	42	26.4 (19.6–33.3)	0.6 (0.0–1.9)	1.4×10^5	250–1.8×10^7
	Longitudinal	**160**	**112**	**70.0 (63.2–77.0)**	**10.0 (5.4–14.6)**		
SA2	Weaning	169	46	27.2 (20.5–33.9)	5.9 (2.4–9.5)	6.3×10^6	1.3×10^3–8.1×10^9
	Post-weaning	156	69	44.2 (36.4–52.0)	4.5 (1.2–7.7)	6.4×10^7	7.8×10^3–5.5×10^{11}
	Pre-slaughter	147	50	34.0 (26.4–41.7)	0.0 (0.0–0.0)	2.4×10^5	500–3.9×10^7
	Longitudinal	**135**	**107**	**60.7 (58.5–62.9)**	**8.1 (3.5–12.8)**		
Vic1	Weaning	180	26	20.0 (14.2–25.8)	7.8 (3.9–11.7)	1.8×10^6	750–2.9×10^8
	Post-weaning	172	60	34.9 (27.8–42.0)	0.0 (0.0–0.0)	3.1×10^4	250–3.0×10^6
	Pre-slaughter	160	108	67.5 (60.2–74.8)	0.0 (0.0–0.0)	1.4×10^5	500–2.4×10^7
	Longitudinal	**178**	**130**	**73.0 (65.9–79.4)**	**8.4 (4.3–12.5)**		
Vic2	Weaning	176	16	9.1 (4.8–13.3)	6.8 (3.1–10.5)	5.1×10^6	3.8×10^3–4.2×10^9
	Post-weaning	173	62	35.8 (28.7–43.0)	0.0 (0.0–0.0)	7.6×10^7	2.3×10^3–7.2×10^9
	Pre-slaughter	128	77	60.2 (51.7–68.6)	0.0 (0.0–0.0)	1.4×10^7	1.1×10^4–3.3×10^9
	Longitudinal	**176**	**140**	**80.0 (65.9–79.4)**	**6.3 (2.9–9.8)**		
NSW	Weaning	160	89	55.6 (47.9–63.3)	48.8 (41.0–56.5)	1.3×10^6	750–1.8×10^{10}
	Post-weaning	160	145	90.6 (86.1–95.1)	31.3 (24.1–38.4)	4.0×10^6	750–4.6×10^{10}
	Pre-slaughter	167	103	61.7 (54.3–69.1)	0.0 (0.0–0.0)	9.9×10^5	222–2.1×10^{10}
	Longitudinal	**160**	**158**	**98.8 (95.6–99.8)**	**66.3 (58.9–73.6)**		
WA1	Weaning	124	9	7.3 (2.7–11.8)	0.8 (0.0–2.4)	1.2×10^6	1.3×10^3–9.6×10^7
	Post-weaning	122	15	12.3 (6.5–18.1)	0.0 (0.0–0.0)	6.3×10^3	250–1.3×10^8
	Pre-slaughter	121	10	8.3 (3.4–13.2)	0.0 (0.0–0.0)	4.2×10^6	5.9×10^4–6.9×10^7
	Longitudinal	**124**	**30**	**24.2 (17–32.7)**	**0.8 (0.0–2.4)**		
WA2	Weaning	109	23	21.5 (13.7–29.3)	0.0 (0.0–0.0)	3.8×10^5	300–2.7×10^7
	Post-weaning	107	6	5.5 (1.2–9.8)	0.0 (0.0–0.0)	9.3×10^5	3.5×10^3–3.2×10^7
	Pre-slaughter	107	24	22.4 (14.5–30.3)	7.5 (2.5–12.5)	7.8×10^5	8.0×10^3–6.5×10^7
	Longitudinal	**110**	**45**	**40.9 (31.6–50.7)**	**6.4 (1.8–10.9)**		
WA3	Weaning	101	6	5.9 (1.3–10.6)	2.0 (0.0–4.7)	1.3×10^4	6.3×10^3–3.1×10^9
	Post-weaning	101	12	11.9 (5.6–18.2)	1.0 (0.0–2.9)	3.2×10^5	1.3×10^3–1.2×10^8
	Pre-slaughter	100	4	4.0 (0.2–7.8)	0.0 (0.0–0.0)	7.9×10^6	500–1.6×10^7
	Longitudinal	**101**	**18**	**17.8 (10.9–26.7)**	**3.0 (0–6.3)**		
All farms	Total (n)	3142	1093	32.0 (30.5–33.6)	5.8 (5.0–6.6)	1.2×10^6	250–5.5×10^{11}
	Longitudinal	**1144**	**740**	**60.7 (58.5–62.9)**	**14.8 (12.7–16.8)**		

CI, confidence interval; NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia

(MolBio), 0.2 µmol/L each of forward and reverse *rpoB* primers, 0.2 µmol/L each of forward and reverse IAC primers, 50 nmol/L of the *rpoB* probe, 50 nmol/L each of forward and reverse IAC primers and probe, 10 copies of the IAC template and 1 µL (≈50 ng) of sample DNA. The PCR was performed on a Rotor-gene Q real-time cycler (Qiagen, VIC, Aust). The cycling conditions consisted of a pre-melt at 95 °C for 3 min and then 45 cycles of 95 °C for 30 s, and a combined annealing and extension step of 60 °C for 45 s. PCR contamination controls were used, including negative controls (no template and blank extraction controls) and separation of preparation and amplification areas.

A standard curve for quantifying *Yersinia* spp. DNA was generated by cloning the PCR product amplified from *Y. enterocolitica* isolate AS-11–2403 (which was originally isolated from a pig in WA), into a pGEMT-vector (Promega) and transforming into *Escherichia coli*-competent cells. Plasmid DNA was isolated by alkali–SDS lysis, followed by column purification using QIAprep Spin Columns (Qiagen) in accordance with the manufacturer's protocol. Plasmid mini-preparations were sequenced using T7 and SP6 sequencing primers (Stratagene, La Jolla, CA, USA) and clones with the correct sequence then used as positive controls for generating a standard curve.

Pathogenic *Y. enterocolitica* were screened by qPCR using primers and probe sequences (specific to virulent *Y. enterocolitica yst* gene), as previously described.[28]

Specificity and sensitivity testing of the qPCR

The analytical specificity of the qPCR assays was assessed by testing DNA from *Y. enterocolitica, Y. pseudotuberculosis, Y. intermedia* and *Y. frederiksenii* (Department of Agriculture, WA), for inclusivity by testing *Campylobacter jejuni, Salmonella typhimurium, Chlamydia pecorum, Chlamydia abortus, Streptococcus bovis* (ATCC 33317), *Enterococcus durans* (ATCC 11576), *E. coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Serratia marcescens* (ATCC 14756 pigmented), *Citrobacter freundii* (NCTC 9750), *Enterobacter cloacae* (ATCC 13047), *Coxiella burnetii* and non-bacterial species; *Giardia duodenalis* assemblage A (n = 1) and assemblage E (livestock) isolates (n = 1) from sheep, *Cryptoporidium hominis, Cryptoporidium parvum, Isospora* spp., *Tenebrio* spp., *Cyclospora* spp., *Toxoplasma gondii, Trichostrongylus* spp., *Teladorsagia circumcincta, Haemonchus contortus* and *Eimeria* spp., as well as human, sheep and cattle DNA for exclusivity. (Note the validity of non-ATCC isolates used for specificity analysis was previously verified in our laboratory by sequencing.)

In order to determine the sensitivity of the assay, 10-fold serial dilutions (n = 5) of plasmids containing the cloned PCR products amplified from *Yersinia* spp. as described were conducted from 1,000,000 copies down to 100 copies of the plasmid template. These were then spiked into faecal samples and the DNA extracted and amplified as described. Mean detection limits, R squared (RSQ) values and % relative standard deviation (RDS) were then calculated. Template copy numbers were converted to numbers of organisms present on the basis that the targeted gene (*rpoB)* is a single copy gene[29] and the bacterial genomes are haploid. Therefore, the detected plasmid numbers were approximately equivalent to the numbers of *Yersinia* spp.

Investigation of inhibition and efficiency

Inhibition in faecal samples was measured using the IACs because the IACs were added to all faecal DNA samples to detect any PCR inhibitors present in the extracted DNA. If any inhibition is present in a sample, the IAC will not produce a signal. Amplification efficiency (*E*), (which is a measure of inhibition) was estimated by using the slope of the standard curve and the formula $E = -1 + 10^{(-1/\text{slope})}$. A reaction with 100% efficiency will generate a slope of −3.32. A PCR efficiency less than or greater than 100% can indicate the presence of inhibitors in the reaction, but reaction efficiencies between 90% and 110% are typically acceptable.[30] To estimate amplification efficiency on faecal samples, serial dilutions of individual DNA samples (neat, 1 : 10, 1 : 100) were performed and three qPCR reactions were conducted on each dilution. The *Ct* values were then plotted versus the log base 10 of the dilution and a linear regression was performed using Rotor-Gene 6.0. software (Qiagen). AQ4

Molecular typing

A subset of up to five qPCR positives were randomly chosen from each sampling on each farm (n = 111) and were amplified at the *rpoB* locus using a nested PCR with the following nested primers (designed for this study using Primer 3): YSNexF: 5′-GGT GAA AGA GTT CTT TGG TTC C-3′ and YSNExR: 5′-AAG ATG GAG TCT TCG AAG TTG-3′, which produce a PCR product size of 948 bp; and the internal primers YSNinF: 5′-CAA CCC GTT GTC TGA GAT TAC G-3′ and YSNinR 5′-ATT GGC TCA CCC AGA TTC AC-3′, which produced a PCR product size of 779 bp. The 25-µL PCR reaction contained 2.5 µL of 10 × Kapa PCR buffer, 1.5 µL 25 mmol/L $MgCl_2$, 1 µL 1 mmol/L dNTPs, 10 pmol/L of each primer, 1 U of KapaTaq, 1 µL of DNA and 16.9 µL of H_2O. Both primary and secondary PCRs were conducted with the same cycling conditions: 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min. Aerosol-resistant pipette tips, negative controls, routine decontamination of pipettes and surfaces and other standard contamination controls were used to prevent cross-contamination. Secondary PCR products were purified using an in-house filter tip method and used for sequencing without any further purification, as previously described by Yang et al.[27]

Sequence and phylogenetic analysis

Purified PCR products were sequenced using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems, VIC, Aust) according to the manufacturer's instructions, with the exception of using an annealing temperature of 58 °C. Nucleotide sequences were analysed using Chromas lite version 2.0 and aligned with reference sequences from GenBank using Clustal W. AQ5

Statistical analysis

Prevalence is expressed as the percentage of samples positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0.[31] Prevalence was determined using point prevalence (prevalence at a single

sampling occasion) and longitudinal prevalence (proportion of animals positive on at least one sampling occasion). Animals with faecal samples collected on at least two sampling occasions were included in analysis of longitudinal prevalence. Chi-square and ANOVA analyses were performed using SPSS 21.0 for Windows (SPSS Inc. Chicago, IL, USA) to determine if there was any association between the prevalence and concentration of bacterial species at different sampling times and across states.

Results

Specificity, sensitivity and efficiency testing of the Yersinia spp. qPCR assay

Evaluation of the specificity of the *Yersinia* spp. qPCR assay revealed no cross-reactions with other genera and only amplified *Yersinia* spp. (data not shown). The mean minimum detection for *Yersinia* was 10 organisms/μL. The mean RSQ value for *Yersinia* spp. was 0.99. The % RDS for *Yersinia* was 7.6%. In our study, the incidence of PCR inhibition, as determined by the IAC amplification, was approximately 2%. If inhibition was evident, then the sample was diluted and re-amplified. The mean efficiency for *Yersinia* spp. was 102.3%.

Prevalence of Yersinia spp. in eight sampled flocks

Yersinia spp. were identified in 32.0% of faecal samples from all eight farms over the three sampling periods (weaning, post-weaning and pre-slaughter) (Table 2, Figure 1). The point prevalence on all farms at weaning was 21.6%, 38.5% at post-weaning and 38.4% at pre-slaughter (Table 2). Overall, 61% of lambs were identified as shedding *Yersinia* spp. on at least one sampling occasion (Table 2).

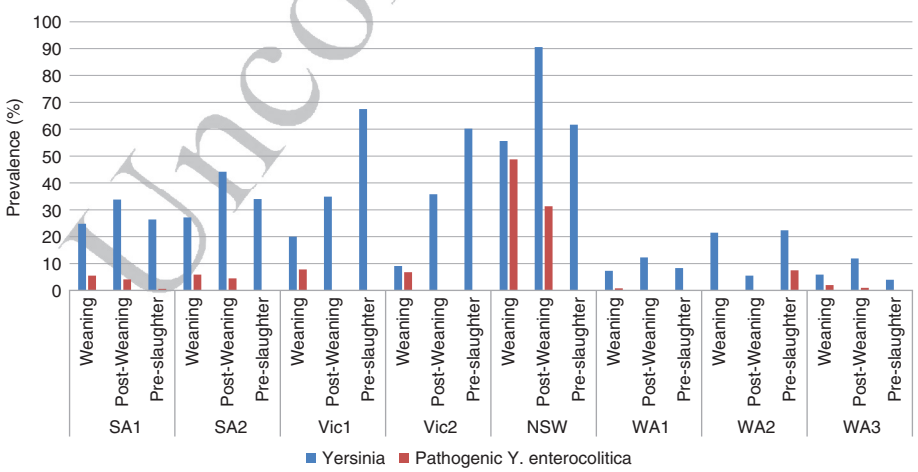
There were significant differences in prevalence of *Yersinia* between flocks for different states ($P < 0.01$). For example, the prevalence of *Yersinia* spp. observed was lower in the WA flocks compared with eastern states' flocks (Figure 1). The highest *Yersinia* spp. point prevalences observed were in the NSW flock at post-weaning (90.6%) and in two Victorian flocks at pre-slaughter (67.5% and 60.2%; Table 2). Of the three WA flocks, the highest prevalence for *Yersinia* was at pre-slaughter (22.4%; Table 2). Similarly, longitudinal prevalences for the three WA farms (18–41% lambs positive on at least one occasion) were lower than for eastern states' farms (61–99% lambs positive on at least one occasion; Table 2).

There was no relationship between *Yersinia* spp. prevalence and sampling occasion ($P > 0.05$), as the peak prevalence for *Yersinia* spp. occurred at different sampling occasions across the flocks tested (Table 2). A total of 65, 53, 2 and 0 individual lambs were positive for *Yersinia* spp. at all three samplings (weaning, post-weaning and pre-slaughter) across the four states (SA, VIC, NSW and WA), respectively.

Pathogenic *Y. enterocolitica*, as determined by screening for the *yst* gene, was identified in 5.8% faecal samples. Pathogenic *Y. enterocolitica* was identified in all eight flocks sampled, with the highest point prevalence in NSW at weaning (48.8%) and post-weaning (31.3%; Table 2, Figure 1). Overall, 15% of lambs tested positive for pathogenic *Y. enterocolitica* on at least one sampling occasion (Table 2). Longitudinal prevalence of pathogenic *Y. enterocolitica* ranged from 0% to 10% across all farms, except in NSW, where pathogenic *Y. enterocolitica* was identified in 66% of lambs on at least one occasion.

Yersinia spp. faecal shedding concentrations

Faecal bacterial concentration (organisms/g faeces) was determined using the qPCR (Tables 2, 3). The largest median concentration of *Yersinia* spp. organisms/g detected was at VIC2 (7.6×10^7 organisms/g) and SA2 (6.4×10^7 organisms/g) ~~during~~ at post-weaning, and SA2 had the highest concentration of organisms shed by an individual during this period (5.5×10^{11} organisms/g). This coincided with the peak *Yersinia* spp. point prevalence of 44.2% for SA2 at post-weaning. For the three sampling occasions in the NSW flock, the highest concentrations of *Yersinia* spp. organisms shed by individual lambs were 1.8×10^{10}, 4.6×10^{10} and 2.1×10^{10} organisms/g, which coincided with point prevalences of 55.6%, 90.6% and 61.7% for *Yersinia* spp., respectively.



Colour Figure - Print and Online

Figure 1. Overall prevalence (%) of *Yersinia* spp. and prevalence (%) of pathogenic *Y. enterocolitica* in sheep faecal samples from eight flocks across four states (NSW, SA, VIC and WA) over three sampling times (weaning, post-weaning and pre-slaughter) as determined by quantitative PCR analysis of the *rpoB* and *yst* gene loci respectively.

Table 3. Prevalence and number of *Yersinia spp.* organisms across four states (pooled values for farms)

State	Sampling period	% prevalence (95%CI)	No. *Yersinia* spp. (organisms/g faeces)	
			Median	Range
SA	Weaning	26.0 (21.3–30.8)	2.0×10^6	$250–8.1 \times 10^{10}$
	Post-weaning	39.1 (33.7–44.6)	1.9×10^6	$3.5 \times 10^3–5.5 \times 10^{11}$
	Pre-slaughter	30.1 (24.9–35.2)	2.2×10^5	$500–3.9 \times 10^7$
VIC	Weaning	11.8 (8.4–15.1)	2.1×10^6	$750–4.2 \times 10^9$
	Post-weaning	35.4 (30.3–40.4)	3.1×10^6	$250–7.2 \times 10^9$
	Pre-slaughter	64.2 (58.7–69.8)	6.9×10^5	$500–3.3 \times 10^9$
NSW	Weaning	55.6 (47.9–63.3)	1.3×10^6	$750–1.8 \times 10^{10}$
	Post-weaning	90.6 (86.1–95.1)	4.0×10^6	$750–4.6 \times 10^{10}$
	Pre-slaughter	61.7 (54.3–69.1)	9.9×10^5	$222–2.1 \times 10^{10}$
WA	Weaning	11.4 (8.0–14.9)	3.4×10^5	$300–1.8 \times 10^9$
	Post-weaning	9.9 (6.7–13.2)	3.6×10^4	$250–1.3 \times 10^8$
	Pre-slaughter	15.2 (11.4–19.1)	1.9×10^6	$500–6.9 \times 10^7$
All states	Weaning	21.7 (19.3–24.0)	1.1×10^6	$300–8.1 \times 10^{10}$
	Post-weaning	36.7 (33.9–39.5)	2.8×10^6	$250–5.5 \times 10^{11}$
	Pre-slaughter	39.1(36.2–42.0)	5.6×10^5	$221–2.1 \times 10^{10}$

CI, confidence interval; NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia

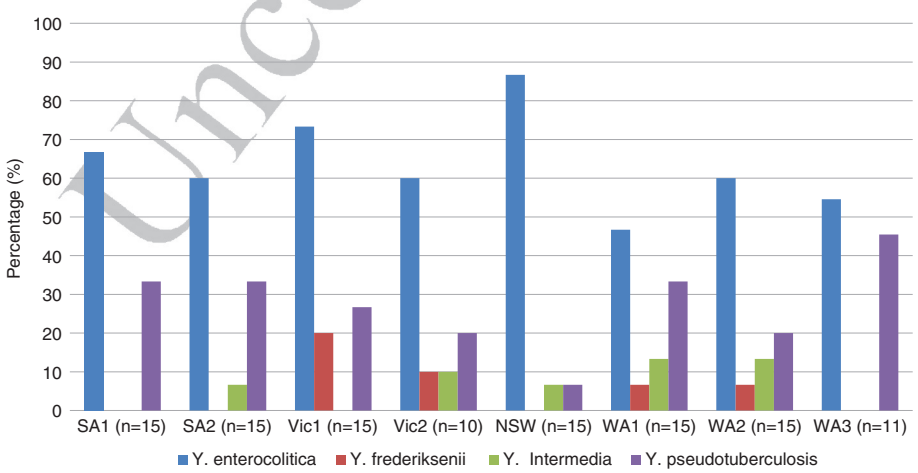
The range of *Yersinia* spp. shedding concentration observed at weaning overall across all states was 300 to 8.1×10^{10} organisms/g and the median was 1.1×10^6 organisms/g. At post-weaning, the range was 250 to 5.5×10^{11} and the median was 2.8×10^6. At pre-slaughter, the range was 221 to 2.1×10^{10} and the median was 5.6×10^5 (Table 3).

Yersinia spp. typing

A subset of up to five positive samples randomly chosen from each sampling period for each flock (n = 111) were sequenced. A total of four species were identified: *Y. enterocolitica* (n = 69), *Y. pseudotuberculosis* (n = 32), *Y. intermedia* (n = 7) and *Y. frederiksenii* (n = 3). Of the subset of positive isolates analysed, *Y. enterocolitica* was the most common species identified across all flocks, ranging from 46.7% at WA1 to 86.7% in NSW (Figure 2). *Yersinia pseudotuberculosis* was the second most common species identified in positive samples across all flocks and ranged from 6.7% in NSW to 45.5% at WA3. *Yersinia intermedia* was only detected at SA2, VIC2, NSW, WA1 and WA2, ranging from 6.7% to 13.3% of positive samples. *Yersinia frederiksenii* was detected at VIC 2 and at WA1 and 2, and ranged from 6.7% (WA2) to 10% (VIC2) of positive samples (Figure 2). A representative subset of sequences was submitted to GenBank under the accession numbers KJ507391–KJ507403.

Discussion

The present study describes the development of a qPCR and subsequent evaluation of longitudinal prevalence, faecal bacterial concentration and species of *Yersinia* spp. in lambs from eight flocks located across four states of Australia at three sampling periods.



Color Figure - Print and Online

Figure 2. *Yersinia* spp. species detected in a subset of isolates (n = 11) from eight flocks across four Australian states at three sampling periods.

Although previous studies have assessed single point prevalence analysis by sampling a random selection of sheep within a flock at a specific time, few studies have estimated longitudinal prevalence.

The qPCR for *Yersinia* spp. described in this study can be used for *Yersinia* epidemiology investigations, with positives then sequenced to identify species. The qPCR assay was very specific for *Yersinia* spp. as it did not cross-react with the non-*Yersinia* species analysed in this study. The sensitivity of the assay was determined by cloning the PCR amplicons from a *Y. enterocolitica* (isolate AS-11-2203) into a plasmid vector and then spiking known amounts of plasmid into faecal samples, extracting the DNA and screening by qPCR. The mean minimum detection for *Yersinia* spp. was 10 organisms/μL of faecal DNA extract. This detection limit is similar to or better than published studies on qPCR detection of *Yersinia* spp.[16,19,32] Inhibitors in stool specimens, including bile acids, bilirubins, haem and complex carbohydrates, sometimes hinder PCR.[33] In the present study, PCR inhibition (as determined by IAC amplification) occurred in only 2% of the examined samples during the qPCR assay, enabling reliable quantification of the concentration of organisms in faeces for 98% of the analysed samples. One important limitation of the qPCR described was that it did not distinguish between pathogenic and non-pathogenic *Y. pseudotuberculosis.*

Yersinia spp. (including both pathogenic and non-pathogenic strains), and more specifically pathogenic *Y. enterocolitica*, were identified in all eight flocks included in this study. The prevalence of *Yersinia* in sheep in Australia has not been well studied, but the prevalences observed in this study were generally higher than previously described. A study in NSW recovered 53 isolates of *Yersinia* spp. by culture from 45 sheep ~~in~~ 37 flocks ~~from sheep~~ in southern NSW from 1981 to 1989.[9] Another study in Victoria reported that *Y. enterocolitica* was isolated by culture from one or more sheep in 78 of 449 (17%) flocks and that *Y. enterocolitica* infection was most common in sheep less than 1 year old.[8] A more recent study of 19 flocks of slaughter-age lambs in New South Wales and Queensland failed to identify *Y. enterocolitica* by culture.[1] It has been suggested that culture methods may underestimate the prevalence of *Yersinia* spp.[34] and this may, in part, explain the differences in prevalence observed between studies utilising culture or molecular methodologies. Conversely, the prevalence of infection detected by the sensitive qPCR assay may also be overstated because of the detection of non-viable bacteria.

Pathogenic *Y. enterocolitica*, as determined by screening for the *yst* gene, was identified in all eight flocks, with the highest prevalence in NSW. The chromosomal *yst* gene encodes a low-molecular-weight, heat-stable enterotoxin that belongs to a family of structurally and functionally related enterotoxins produced by several species of diarrheagenic bacteria.[35,36] Although the *yst* gene is confined to pathogenic bioserotypes of *Y. enterocolitica* and hence is a useful marker of potential virulence, a homologous gene is found in some isolates of *Y. intermedia* and *Y. kristensenii*,[28,37] which are considered non-pathogenic. However, the primers used in the present study were designed to be specific to the *yst* gene in *Y. enterocolitica*.[28]

Yersinia pseudotuberculosis was identified in the subset of positive samples that were sequenced. Overall, the prevalence or point prevalence of pathogenic *Y. pseudotuberculosis* serotype III in the eight flocks could not be determined in this study. Future studies could include qPCR to specifically identify pathogenic *Y. pseudotuberculosis* serotype III, as well as pathogenic *Y. enterocolitica,* to better describe the prevalence of known pathogenic *Yersinia* spp. and identify associations with scouring and production loss in sheep.

The pattern of bacterial shedding in faeces across the three sampling occasions varied among the flocks. The highest median concentration of *Yersinia* spp. organisms observed was at VIC2 and SA2 at post-weaning sampling for both flocks, but for other flocks the highest faecal concentration was observed at weaning (SA1, VIC1) or pre-slaughter (WA3). *Yersinia* spp. shedding concentration in the NSW flock was high across all three sampling periods. The observations from the present study were likely to be influenced by the peaks and troughs of individual species. Seasonal patterns of excretion of individual species cannot be determined with the qPCR test described. Furthermore, all sheep in this study were under 1 year of age and this may have affected the pattern of shedding observed. Further investigation would be required to describe longitudinal changes in shedding patterns for specific species relating to factors including sheep age, season etc. Factors affecting bacterial shedding of *Yersinia* spp. in sheep are not well described. Slee et al. reported that *Yersinia* appeared to be less severe in favourable years with good feed, whereas summer colitis was more severe in wet summers regardless of nutrition.[7] The reasons for the high *Yersinia* spp. output observed are unknown, but may be related to stress or to mixed infections affecting host immunity,[26,38–40] as these sheep were known to be co-infected with other potentially pathogenic organisms, including *Giardia*, *Cryptosporidium*, *Eimeria*, other bacteria and strongylid nematodes. It is possible that there are differences in the pattern of shedding between different *Yersinia* spp., including differences between pathogenic versus non-pathogenic species or strains, which could not be identified in this study.

Of the subset of 111 positive isolates derived from across all eight flocks for sequencing, *Y. enterocolitica* was most commonly identified (62%), followed by *Y. pseudotuberculosis* (29%), *Y. intermedia* (6%) and *Y. frederiksenii* (3%). This was consistent with a study by Philbey et al.,[9] in which *Y. pseudotuberculosis* (49%) and *Y. enterocolitica* (38%) were most commonly identified from 53 isolates cultured from 37 sheep flocks in NSW, with *Y. intermedia* (9%) and *Y. frederiksenii* (4%) identified at lower prevalence. In that study, 20 *Y. enterocolitica* isolates were categorised biochemically as biotype 5 strains and, of six isolates serotyped, all belonged to serogroups 2 and 3.[9]

Previous studies have assessed single point prevalence analysis using a sample of sheep within a flock at a specific time point, but this does not provide an indication of the overall (longitudinal) prevalence in flocks over an extended period of time. This has relevance for infections that may affect animal productivity or have public health significance. In our study, *Yersinia* spp. was identified in 32% of lambs on at least one occasion. However, the point prevalence varied widely among flocks and at different sampling occasions. For example, in the VIC2 flock prevalence of *Yersinia* spp. peaked at 60.2% at pre-slaughter but was only 9.1% at weaning. The point prevalence of pathogenic *Y. enterocolitica* also varied and on seven of the eight

farms ~~where~~ pathogenic *Y. enterocolitica* was not identified at every sampling occasion~~, suggesting~~ that sampling on a single occasion would be likely to underestimate prevalence across flocks or farms. Differences in prevalence could be related to a wide range of factors, including environmental conditions, stocking density, potential for contamination of feed/water and acquisition of host immunity. Further, only a subset of positive samples was identified to species level by sequencing and it is possible that differences in epidemiology exist among the different *Yersinia* spp.

Conclusion

The present study identified *Yersinia* spp., and specifically pathogenic *Y. enterocolitica*, in all eight flocks sampled. Bacterial shedding of *Yersinia* spp. was high. The prevalence of pathogenic *Y. enterocolitica* was generally low, with the exception of the NSW flock. Further work is required to better describe the epidemiology of *Yersinia* spp., including pathogenic strains of both *Y. enterocolitica* and *Y. pseudotuberculosis*, using species-specific qPCR to estimate genetic diversity among sheep-derived *Y. enterocolitica* in Australia and to determine the extent of animal production loss and public health significance associated with *Yersinia* spp. infections in sheep. Further refinement of the qPCR described would provide a useful tool to complement other microbiological tools and allow further study into associations between pathogen load, clinical disease and the effects on productivity and welfare of lambs, as well as the public health significance of these organisms.

Acknowledgments

This study was funded by Meat and Livestock Australia, Australian Wool Innovation Limited and the Australian Government. We thank the participating farmers for their support and providing access to sheep for sample collection. We thank Justin Hoad for providing faecal samples from NSW. Samples from the WA farms were collected and DNA extracted by Joshua Sweeny. Thanks also go to Josephine Ng-Hublin for DNA extraction of faecal samples collected from the eastern states.

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(Accepted for publication 16 August 2015)

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AQ2. Original Table 2 shows WA1 having 1350 sheep and WA2 having 1750. Which is correct? There is no need to duplicate data between tables.

AQ3. please indicatein a footnote the significance of data set in bold type.

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AQ5. please give details for software

AQ6. meaning unclear: point prevalence varied across all farms or only on the 7 of 8 farms? please clarify this sentence

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