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Diversity and Abundance of Zoonotic Pathogens and Indicators in Manures of Feedlot Cattle in Australia[▽]

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The occurrence of 10 pathogens and three fecal indicators was assessed by quantitative PCR in manures of Australian feedlot cattle. Most samples tested positive for one or more pathogens. For the dominant pathogens *Campylobacter jejuni*, *Listeria monocytogenes*, *Giardia* spp., *Cryptosporidium* spp., and *eaeA*-positive *Escherichia coli*, 10^2 to 10^7 genome copies g^{-1} (dry weight) manure were recovered.

More than 600,000 tons of feedlot cattle manure are generated each year in Australia, which raises concern for potential water, air, and soil contamination (21, 27). Hence, better monitoring and knowledge of the resulting risks are needed (5, 26). Most zoonotic pathogens associated with cattle are well described in the literature, especially those of major health significance, including the bacterial pathogens *Campylobacter* spp., *Listeria monocytogenes*, pathogenic *Escherichia coli* (particularly serotypes O157 and O111), *Salmonella enterica*, *Yersinia* spp., *Leptospira* spp., *Coxiella burnetii*, *Mycobacterium avium* subsp. *paratuberculosis*, and the parasitic protozoa *Giardia lamblia* and *Cryptosporidium parvum* (2, 21, 27). While studies of pathogen occurrence in manure are numerous, data suited to quantitatively estimating end user risks are still limited. Few surveys quantify multiple pathogens (11, 12, 14, 28), and none have concurrently measured all 10 above in cattle manure. A further constraint on risk assessment is that most data were generated in North America or Europe, where cli-

TABLE 1. Abundance of fecal indicators and pathogens in manures from Australian feedlot cattle

Analyte	Method	No. of organisms g^{-1} [dry wt][a]					
		Fresh feces	Pen manure	Harvested manure	Aged manure	Compost manure	Carcass compost
Total coliforms	MPN	7.4 ± 0.28	6.1 ± 1.1	3.2 ± 1.9	2.7 ± 1.3	2.3 ± 1.2	3.6
E. coli	MPN	7.4 ± 0.33	5.2 ± 1.3	2.5 ± 1.6	1.6 ± 0.54	1.0	1.1 ± 0.32
	qPCR	6.8 ± 0.69	5.1 ± 0.98	3.5 ± 1.5	2.5 ± 0.56	2.8	<DL[d]
Enterococci	MPN	5.8 ± 0.73	5.2 ± 0.88	3.1 ± 1.5	1.7 ± 0.69	2.4 ± 1.6	2.0 ± 1.2
E. faecalis	qPCR	6.2 ± 0.87	6.0 ± 0.84	4.9 ± 0.95	3.9 ± 0.50	3.6 ± 0.38	4.6 ± 1.0
C. perfringens	qPCR	4.5 ± 0.75	3.8 ± 0.87	3.8 ± 0.98	3.7 ± 0.69	<DL	4.3 ± 0.80
Pathogenic *E. coli*[b]	qPCR	5.1 ± 1.3	3.8 ± 1.9	2.6 ± 0.85	2.5 ± 0.59	2.6	2.6 ± 0.78
C. jejuni	qPCR	5.1 ± 0.94	3.3 ± 0.66	<DL	2.9	<DL	<DL
L. monocytogenes	qPCR	3.7 ± 0.53	3.2 ± 0.53	3.0 ± 0.38	3.2 ± 0.61	<DL	3.4 ± 0.64
S. enterica	qPCR	3.4	<DL	<DL	<DL	<DL	<DL
Y. pseudotuberculosis	qPCR	3.4	3.0	2.9	2.9	<DL	<DL
C. burnetii	qPCR	3.4	<DL	2.9	<DL	<DL	<DL
Leptospira spp.	qPCR	<DL	3.0	<DL	<DL	<DL	<DL
M. avium subsp. *paratuberculosis*	qPCR	<DL	<DL	3.0	<DL	<DL	<DL
Cryptosporidium spp.[c]	qPCR	3.1 ± 0.94	2.6 ± 0.71	2.4	2.5 ± 0.67	<DL	<DL
Giardia spp.[c]	qPCR	3.4 ± 1.6	<DL	1.8	2.3 ± 0.98	<DL	2.7 ± 0.97

[a] Log_{10} of arithmetic mean ± standard deviations of triplicate samples. Values shown without standard deviations correspond to less than three samples.
[b] Positive for virulence marker gene *eaeA*.
[c] Numbers corresponding to cysts/oocysts.
[d] DL, detection limit.

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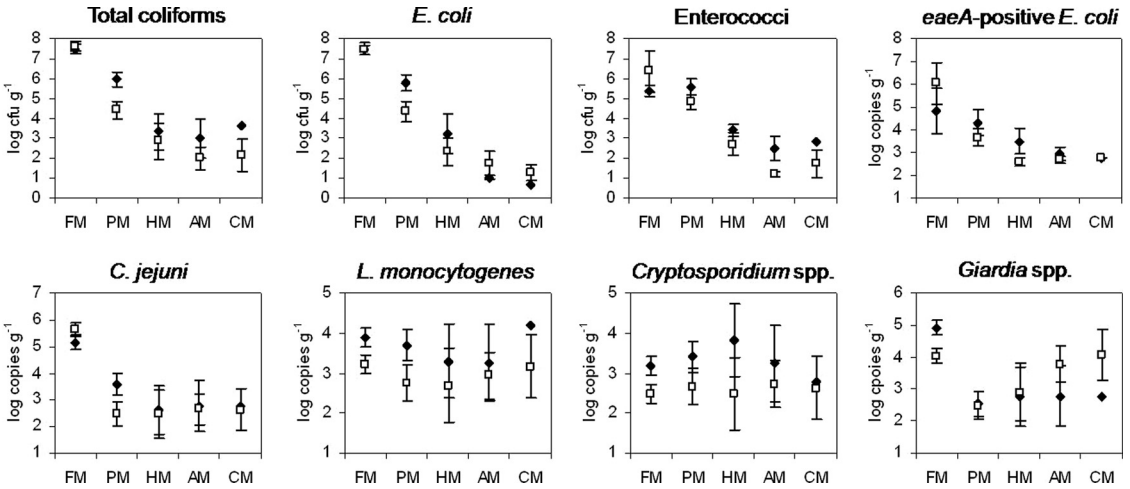

FIG. 1. Occurrence of indicator bacteria and pathogens in wastes of Australian feedlot cattle in winter and summer. Indicators (total coliforms, *E. coli*, and enterococci) were quantified by MPN assay, and pathogens (*eaeA*-positive *E. coli*, *C. jejuni*, *L. monocytogenes*, *Cryptosporidium* spp., and *Giardia* spp.) were quantified by qPCR in fresh manure (FM), pen manure (PM), harvested manure (HM), aged manure (AM), and composted manure (CM). All values represent mean values and standard deviations from all sampling locations in summer (open squares) and winter (filled diamonds).

mate and environment can differ markedly from Australian conditions.

Addressing this knowledge gap now appears feasible, as real-time quantitative PCR (qPCR) can be used as an alternative to culture-based methods for quantifying environmental pathogens (7, 23, 29). Improvements in sample preparation and nucleic acid cleanup methods have largely overcome problems associated with the molecular biology-based analysis of fecal matter (22). Further, qPCR can detect stressed, damaged, and otherwise nonculturable cells persisting in a state of dormancy or indeed dead (15, 17, 29). The aim of this paper is to report on a quantitative survey of zoonotic pathogens and indicators in manures from Australian feedlot beef cattle.

A total of 128 composited samples (five subsamples each) representing fresh feces ($n = 32$), pen manure ($n = 32$), harvested pen manure ($n = 28$), stockpiled manure ($n = 23$), composted manure ($n = 6$), and carcass compost ($n = 7$) were collected from five cattle feedlots in eastern Australia in the winter/summer of 2009 (13). All samples were assayed for the 10 key pathogens listed above and also fecal indicators (total coliforms, *E. coli*, and enterococci).

Quantification of fecal indicators by culture methods. Fecal indicators were quantified by the most probable number (MPN) method as previously reported (13). *E. coli* in fresh feces ranged between 10^7 and 10^8 CFU g^{-1} (dry weight) (majority of total coliforms being *E. coli*), while enterococci ranged between 10^5 and 10^6 CFU g^{-1} (Table 1). Marked reductions in indicator numbers were observed with increasing manure age and processing. No differences ($P > 0.05$) in fresh manure indicator numbers were observed between seasons (Fig. 1) or feedlots. In contrast, pen manure indicator numbers declined more in summer by up to 1.5 logs. Indicator numbers for harvested manure were further reduced by 1 to 3 logs, with the highest inactivation in summer. Analysis of aged manure stockpiles showed further reduction, providing an overall reduction for all three indicators in excess of 5 logs.

TABLE 2. Target sequences, cycling conditions, and oligonucleotide primers

Target organism	Target gene; GenBank no.	Primer annealing temp (°C); primer extension time (s)	Primer names (reference)
E. coli	Glucuronidase; S69414	60; 15	Eco-F, Eco-R (23)
E. faecalis	23S rRNA gene; AE016830	58; 15	ECF, ECR (10)
C. perfringens	Alpha-toxin; AY277724	60; 15	cpaF, cpaR (8)
EHEC and EPEC[a]	Intimin; AF081182	55; 15	EAE-a, EAE-b (7)
C. jejuni	VS1; X71603	55; 20	forward, reverse (24)
L. monocytogenes	Listeriolysin; M24199	60; 15	forward, reverse (19)
S. enterica	Invasin; U43272	68; 15	invA139, invA141 (7)
Y. pseudotuberculosis	Invasin; M17448	62; 15	inv-F, inv-R (25)
C. burnetii	*com1*; AF318146	60; 15	FAF216, RAF290 (1)
L. interrogans	Lipoprotein L32; AF181553	60; 30	270F, 692R (16)
M. avium subsp. *paratuberculosis*	IS*900*; X16293	60; 15	F2, R2 (3)
Cryptosporidium spp.	COWP[b]; AF248743	60; 15	P702F, P702R (9)
Giardia spp.	β-Giardin; M36728	60; 15	P241F, P241R (9)

[a] EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli.*
[b] COWP, cryptosporidium oocyst wall protein.

TABLE 3. Quantification of microbial pathogens after spiking into cattle manures

Target organism	Inoculum (log_{10} copies)	Recovery			
		Fresh manure		Aged manure	
		Range (log_{10} copies)	%[a]	Range (log_{10} copies)	%[a]
E. coli O157:H7	7.0	6.0–6.1	11	5.9–6.2	12
S. enterica	7.0	6.6–6.7	45	6.6–6.7	46
G. lamblia cysts	4.4	4.0–4.1	44		
C. parvum oocysts	4.7			4.2–4.6	48
C. burnetii	6.1	5.0–5.1	9.2	4.9–5.1	8.0
C. jejuni	6.0	4.8–5.1	8.5	4.9–5.1	10
L. monocytogenes	7.4	7.1–7.3	49	6.7–7.2	38
Y. pseudotuberculosis	7.0	6.1–6.5	22	6.1–6.4	21

[a] Averages of two duplicate samples.

Quantification of pathogens and fecal indicators by qPCR. Concurrently, qPCR was used to enumerate the genome copies for the 10 pathogens and the indicators *E. coli*, *Enterococcus faecalis*, and *Clostridium perfringens*. As quantification standards, genomic DNA was isolated from pure cultures of *E. coli* K-12, *E. coli* O157:H7 strain EDL933, *E. faecalis* ATCC 19433, *C. perfringens* ATCC 13124, *S. enterica* serotype Typhimurium ATCC 13311, *L. monocytogenes* NCTC 11994, *Campylobacter jejuni* NCTC 11351, *Yersinia pseudotuberculosis* strain ATCC 29833, *C. burnetii* (QVax; CSL), *M. avium* subsp. *paratuberculosis* strain 316V, and *Leptospira interrogans* serovar Pomona and from *G. lamblia* cysts and *C. parvum* oocysts (Giardi-a-Glo; Waterborne). Genome copy numbers were calculated from DNA concentration, molecular weight, and target gene frequency. After sample preparation and isolation of total DNA, qPCR was performed using published primer sequences (Table 2) and analyzed as published previously (13). Similar copy numbers of *E. coli* and *E. faecalis* were estimated in fresh feces by qPCR compared to MPN; however, up to 100-fold-higher numbers were detected in harvested and aged manures (Table 1). This differential implies that most indicators were nonculturable but sufficiently intact to be detectable by qPCR.

To estimate the efficiency of qPCR to recover pathogens, reference materials from *E. coli* O157:H7, *S. enterica*, *Y. pseudotuberculosis*, *L. monocytogenes*, *C. jejuni*, *C. burnetii* (Q-Vax; CSL), *G. lamblia* cysts, and *C. parvum* oocysts (Giardi-a-Glo; Waterborne) were inoculated into fresh and aged manure that had tested negative for each pathogen. Overall, satisfactory recoveries of inoculated cells were observed, and qPCR appeared to be a reliable tool for quantifying pathogens in manure. Recoveries ranged between 8.0% and 49% with no significant changes between different manures or targets (Table 3). However, high-titer *L. interrogans* and *M. avium* subsp. *paratuberculosis* stocks were not available for recovery estimation, and yet efficient isolation of nucleic acids from *Leptospira* and *Mycobacteria* has been reported elsewhere (3, 16).

In the main survey, DNA from all 10 pathogen groups was detected at least once (Tables 1 and 4). Most abundant were *eaeA*-positive *E. coli*, *C. jejuni*, and *L. monocytogenes*. Least abundant were *S. enterica*, *C. burnetii*, *M. avium* subsp. *paratuberculosis*, pathogenic *Leptospira*, and *Y. pseudotuberculosis*. *E. coli* strains carrying the *eaeA* virulence marker gene (presumptively enterohemorrhagic or enteropathogenic) were detected in 81% of fecal samples at numbers up to 10^7 g^{-1}. High counts of *Giardia* and *Cryptosporidium* ($>10^5$ g^{-1}) were sporadically identified in all manures, indicating high persistence of their DNA targets. *C. jejuni* was initially abundant but was rapidly inactivated. *L. monocytogenes* appeared widespread and persistent but low in numbers. *C. burnetii* and *M. avium* subsp. *paratuberculosis*, major concerns for the beef industry, were rare. Yet, frequency of detection was partly a function of assay sensitivity. For all pathogens, depending on dilution, dry matter content, and gene frequency, the detection limit varied between 10^2 and 10^3 copies g^{-1}. Higher numbers of *C. jejuni*, *L. monocytogenes*, *Cryptosporidium*, and *eaeA*-positive *E. coli* organisms appeared more common in winter, but no such trend was detected with *Giardia* (Fig. 1).

Concluding remarks. The applied qPCR assays were largely based on published oligonucleotide primer sequences originally developed for clinical samples and pure cultures, most of them in conjunction with additional reporter probes (Table 2). It was uncertain if these primers were suitable for cattle manures, where high levels of interfering substances and uncharacterized nucleic acids occur (18, 22). Overall, we overcame the constraints of traditional indicators and culture methods by directly assaying key pathogens of concern by qPCR. The abundance data appear suited to quantitative microbial risk assessment (QMRA). A limitation was the unknown number

TABLE 4. Frequency of DNA detection by qPCR of zoonotic pathogens in manures of Australian feedlot cattle

Target organism	% detection (no. of analyses)[b]					
	Fresh feces	Pen manure	Harvested manure	Aged manure	Composted manure	Carcass compost
Pathogenic *E. coli*[a]	81 (32)	69 (32)	32 (25)	20 (20)	17 (6)	14 (7)
C. jejuni	94 (32)	38 (32)	**0 (25)**	5 (20)	**0 (6)**	**0 (7)**
L. monocytogenes	31 (32)	34 (32)	16 (25)	35 (20)	**0 (6)**	43 (7)
S. enterica	6 (32)	**0 (32)**	**0 (25)**	**0 (20)**	**0 (6)**	**0 (7)**
Y. pseudotuberculosis	3 (32)	6 (32)	4 (25)	5 (20)	**0 (6)**	**0 (7)**
C. burnetii	3 (32)	**0 (32)**	4 (25)	**0 (20)**	**0 (6)**	**0 (7)**
Leptospira spp.	**0 (32)**	3 (32)	**0 (25)**	**0 (20)**	**0 (6)**	**0 (7)**
M. avium subsp. *paratuberculosis*	**0 (32)**	**0 (32)**	8 (25)	**0 (20)**	**0 (6)**	**0 (7)**
Cryptosporidium spp.	13 (32)	16 (32)	8 (25)	15 (20)	**0 (6)**	**0 (7)**
Giardia spp.	34 (32)	**0 (32)**	8 (25)	30 (20)	**0 (6)**	43 (7)

[a] Positive for virulence marker gene *eaeA*.
[b] Data for assays under the detection limit are shown in boldface.

of intact dead cells detected by qPCR that may lead to an overestimation of risk (15, 20). However, the abundance and diversity of pathogens and indicators were in general agreement with existing data (4, 6, 11, 12, 28). Most importantly for QMRA, the abundance estimates were necessarily conservative. We conclude that qPCR offers much promise as a routine tool for monitoring zoonotic pathogens in livestock manures.

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