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Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* species in pre-weaned sheep in Australia

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Abstract

A total of 477 fecal samples from pre-weaned sheep from 5 different farms in the south west of Western Australia were screened for the presence of *Cryptosporidium* and *Giardia* using PCR. There were substantial differences in prevalence between the farms and overall prevalence was 24.5% and 11.1% respectively for *Cryptosporidium* and *Giardia.* At the 18S locus, 66 *Cryptosporidium* positives were identified, the majority of which were *C. bovis* (n = 52), followed by the cervid genotype (n= 10) and *C. parvum* (n = 2). At a second diagnostic locus, using *C. parvum* and *C. hominis* specific qPCR primers, 63 *C. parvum* positives were identified, some of which were co-infections with *C. bovis*. The *C. parvum/C. hominis* qPCR was more sensitive than the nested 18S PCR at detecting *C. parvum.* This may be due to the low numbers of oocysts present, as quantitation data indicated that all the *C. parvum* detected was present in low numbers (1-10 oocysts). It may also be that using *C. parvum*-specific primers is necessary to determine the true prevalence of *C. parvum*. Amongst *Giardia* positive isolates, *G. duodenalis* genotype E (livestock) was the most prevalent (36/53), with *G. duodenalis* genotype A detected in 5 positive isolates. There were also 11 mixed A and E infections detected. The findings of the present study indicate that pre-weaned lambs are not an important source of zoonotic *Giardia* genotypes in Australia but may be an important source of zoonotic *Cryptosporidium.*

Keywords: *Cryptosporidium*, *Giardia*, pre-weaned sheep 18S rRNA, *gdh*, *C. bovis*, cervid genotype, *C. parvum*, genotype A, genotype E.

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1. Introduction

Cryptosporidium has been reported in sheep worldwide, however most studies have been based on microscopy and reported prevalences ranging from 2.6 to 82% for *Cryptosporidium* (cf. Ryan et al., 2005). There appears to be geographic differences in the prevalence of zoonotic and non-zoonotic genotypes in sheep based on recent molecular characterisation studies. For example, in the United Kingdom, *C. parvum* was the only species found in neonatal lambs and was the predominant species in sheep flocks (Mueller-Doblies et al., 2008). Similarly, in Spain, *C. parvum* was the only species detected from 137 microscopy-positive diarrheic lambs (aged up to 21 days) from 5 farms (Quilez et al., 2008). Conversely, in Belgium, the cervid genotype was the predominant (n = 10) genotype identified in 18 positive lambs (aged 1 day - 10 weeks), from 10 farms (Geurden et al., 2008). In Maryland, USA, the cervid genotype and *C. bovis* were the predominant species identified in both ewes and lambs (Santin et al., 2007), and were also the predominant species infecting post-weaned sheep in Western Australia (Ryan et al. 2005).

Giardia is a protozoan parasite and one of the most common causes of diarrhoea in humans and animals (Caccio and Ryan, 2008). *Giardia duodenalis* (*syn. G. lamblia* and *G. intestinalis*) is the species, which infects most mammals and humans (Caccio and Ryan, 2008). There is considerable genetic variation within *G. duodenalis* and several major morphologically similar but genetically distinct assemblages/genotypes have been identified; genotype A and B in humans and animals; genotypes C and D in dogs, genotype E in cattle, sheep and pigs, genotype F in cats and genotype G in rats (Monis et al., 1999; Van der Giessen et al., 2006; Caccio and Ryan, 2008).

Giardia infection in sheep is relatively common and has been reported worldwide (Xiao et al., 1994, Diaz et al., 1996, Giangaspero et al., 2005, Ryan et al., 2005, Ozmen et al., 2006 and

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van der Giessen et al., 2006). Three genotypes of *G. duodenalis* have been identified in sheep, genotype E, and the two zoonotic genotypes, genotype A and B (Giangaspero et al., 2005, Ryan et al., 2005, Aloisio et al., 2006, van der Giessen et al., 2006; Castro-Hermida et al., 2007; Santin et al., 2007; Geurden et al., 2008).

As sheep may potentially contribute significantly to contamination of watersheds, it is important to understand the public health risk posed by *Cryptosporidium* and *Giardia* infections in sheep. The previous study conducted in Australia examined post-weaned animals only, therefore the aim of the present study was to determine the prevalence of these parasites in pre-weaned sheep from 5 farms in Western Australia and through DNA sequence analysis, to identify the species and genotypes that were present.

2. Materials and Methods

2.1 Animals and faecal sample collection

Faecal samples were collected from lambs from 5 different farms located within a 50km radius of Kojonup in the south west of Western Australia (Table 1). All of the farms had a Mediterranean environment (hot dry summers and cool wet winters) with an average annual rainfall of 450-550mm per annum. The estimated stocking rate on the 5 farms at the time of sampling ranged from 8-12 dry stock equivalents (DSE) per winter grazed hectare. A total of 477 faecal samples were collected were collected directly from the rectum of randomly selected lambs aged up to 8 weeks old between July and August 2007. All of the lambs sampled were Merinos.

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2.2 DNA isolation

Genomic DNA was extracted from 200mg of each faecal sample using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) or from 250mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California). A negative control (no faecal sample) was used in each extraction group.

2.3 PCR amplification.

All samples were screened at the 18S rRNA locus for both parasites and positives were genotyped by sequencing. A two-step nested PCR protocol was used to amplify the 18S rDNA gene of *Cryptosporidium* as previously described (Ryan et al., 2003). Amplification of a fragment of the *Giardia* 18S rRNA gene was performed as described by Hopkins et al., (1997) and Read et al., (2002). All samples were also screened using a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific protein-coding locus previously described (Morgan et al. 1997) and referred to from here on as the diagnostic locus. Briefly, a forward primer, 021F (5' GGTACTGGATAGATAGTGGA 3'), which anneals to both *C. hominis* and *C. parvum* was combined in the same reaction with a *C. hominis*-specific primer, CHR (5' CCTCTTTCCAATTAAAGTTGATG 3') and a *C. parvum*-specific primer CPR (TCCAAATTATTGTAACCTGGAAG 3'). A *C. hominis*-specific probe (5'-FAM-TGATTTTCCAGGCTAC 3') and *C. parvum*-specific probe (5'-JOE-TGATCTTCCAGGTTAC 3') were also included. Each 15 µl PCR mixture contained 1× HotStar Buffer, 5 mM $MgCl_2$, 1 mM dNTP's, 2.0 U Hot Star DNA polymerase (Qiagen), 0.2 µM each of forward and reverse primers and 50 nM each of the *C. hominis* and *C. parvum* probes. The PCR cycling conditions consisted of a pre-melt at 95°C for 10 min and then 45 cycles of 95°C for 30 sec, and a combined annealing and extension step of 61°C for 60 sec. A standard curve for quantifying *C. parvum/C. hominis* DNA was generated using DNA extracted from 10,000 *C. parvum* and/or *C. hominis* oocysts and diluted down to 1,000, 100, 10 and 1

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oocyst equivalents. A spike analysis (addition of 0.5µL of *C. hominis/Giardia duodenalis* assemblage B positive control into each sample) was conducted on randomly selected *Cryptosporidium* and *Giardia* negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition.

The specificity and sensitivity of the qPCR at the diagnostic locus was previously analysed (Yang, unpublished). The assay was tested on a wide range of *Cryptosporidium* species and genotypes from different geographic locations as well as *Giardia, Isospora, Eimeria, Cyclospora*, gregarine, human and sheep DNA and shown to be specific for *C. parvum* and *C. hominis*. The sensitivity was determined to be 1 oocyst and the reproducibility of Ct values was 1.6%-2.3% relative standard deviation (RSD) (Yang, unpublished). In addition, GenBank Blast searches revealed no significant homologies to the diagnostic locus.

A subset of samples (n = 19) were also analysed at the *Giardia* glutamate dehydrogenase (*gdh)* locus as previously described (Read et al., 2004). PCR contamination controls were used including negative controls and separation of preparation and amplification areas. The amplified DNA fragments from the secondary PCR products were separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et al., 2006).

2.4 Sequence and phylogenetic analyses

Purified PCR products were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58ºC. Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp).

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A phylogenetic tree was constructed for the *gdh* locus with additional isolates from GenBank. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1997), based on evolutionary distances calculated with the Tamura-Nei model and grouped using Neighbour-Joining. The confidence of groupings, was assessed by bootstrapping, using 1000 replicates.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 15.0 (Statistical Package for the Social Sciences) for Windows (SPSS inc. Chicago, USA).

3 Results

Cryptosporidium

Cryptosporidium was detected in 24.5% (117/477) of samples from the five farms in Western Australia (Table 2). At the 18S rRNA locus, 66 *Cryptosporidium* positives were identified. At the diagnostic locus, a total of 63 *C. parvum* positives were detected, of which 51 were additional to the positives detected at the 18S locus. Two isolates, which were identified as *C. parvum* at the 18S locus (one from Farm 1 and one from Farm 3), were also identified as *C. parvum* at the diagnostic locus. Ten isolates from Farm 3, which were identified as *C. bovis* at the 18S locus, were identified as *C. parvum* at the diagnostic locus, indicating mixed infections. There were substantial differences in prevalence between the three farms with Farm 2 having the lowest prevalence at 9.3% (3.5-15.1 95% CI) and Farm 3 having the highest prevalence at 56.3% (46.3, 66.2) (Table 2).

Sixty-four partial 18S rDNA sequences were obtained from the 66 18S positives. The results of the sequencing reactions were analysed, edited and compared to existing *Cryptosporidium* 18S rDNA sequences on GenBank™ using BLAST searches. The results of

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the 18S sequencing were combined with the diagnostic locus. Three species/genotypes of *Cryptosporidium* were detected (Table 3). At the 18S rRNA locus, *C. bovi*s was the most common species detected with an overall prevalence of 10.9% (8.1, 13.7 CI) amongst the 5 farms. The cervid genotype was detected in 2.1% (0.8, 3.4 CI) of total samples and *C. parvum* in two samples (0.4%). At the diagnostic locus, 63 *C. parvum* isolates were detected. Twenty-five samples were sequenced at random to confirm this. *Cryptosporidium hominis* was not detected.

The results of genotyping pre-weaned lambs were also compared to the genotyping data previously obtained for post-weaned sheep (Table 4) (Ryan et al. 2005). The prevalence of *Cryptosporidium* in pre and post-weaned sheep was similar at 24.5 and 26.2% respectively. There were however substantial differences in the *Cryptosporidium* species/genotypes identified in pre and post-weaned sheep, with a much wider range of species/genotypes identified in older sheep (Table 4).

Giardia

A total of 11.1% (8.3, 13.9 95% CI) of the samples were shown to be positive for *Giardia* when tested using PCR (Table 1). Farm 3 had the lowest prevalence at 2.1 % (0, 4.9 CI) and farm 5 had the highest prevalence at 18.3 % (10.4, 26.1 95% CI). Fifty-two partial 18S rDNA sequences were obtained from the 53 positives (Table 2). *Giardia duodenalis* livestock genotype (genotype E) was the most common species detected with an overall prevalence of 7.5% (5.2, 9.9 95% CI) amongst the 5 farms. *Giardia duodenalis* genotype A was identified in 1% (0.1, 2.0 95% CI) of total samples and mixed A and E infections were identified in 2.3% (1.0, 3.7 95% CI) of samples. When compared to post-weaned animals, the prevalence of *Giardia* was much lower in pre versus post-weaned animals at 11.1% and 44% respectively.

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Genotype E was the most common species identified in both pre and post-weaned sheep (Table 4).

A subset of samples (n = 19) from all five farms were also analysed at the glutamate dehydrogenase (*gdh)* locus. Sequence and phylogenetic analysis at this locus identified 18 isolates as genotype E and 1 as genotype AII (Figure 1). Genotyping results for the two loci were in agreement with each other with the exception of isolate 49 from Farm 4, which was genotype A as the 18S locus but genotype E at the GDH locus.

4 Discussion

In the present study, the prevalence of *Cryptosporidium* and *Giardia* in pre-weaned sheep was 24.5 and 11.1% respectively. A much higher prevalence of *Giardia* was identified in post-weaned lambs and sheep (>8 weeks old) (44%) in a previous study (Ryan et al. 2005). A higher prevalence of *Giardia* in ewes than in lambs was also reported by Santin et al. (2007), although other studies have reported a higher prevalence in lambs (Olson et al. 1997). The differences may be due to seasonal differences as pre-weaned samples were only taken during July and August 2007, whereas the post-weaned samples were taken over a longer period (September 2002 to January 2003). The post-weaned samples were all collected from lambs and sheep in lairage at an abattoir and it is possible that the stress of mustering, transport and lairage resulted in increased shedding of cysts. It is also important to note that the previous study did not include the testing with the diagnostic locus qPCR and the selection of the sheep was with preference given to lines showing evidence of scouring. This could have biased the results and influenced the genotypes found.

There were significant differences in the prevalences of *Cryptosporidium* and *Giardia* between farms, with Farms 2 and 3 having the lowest and highest prevalences of *Cryptosporidium* at 9.3% and 56.3% respectively. Interestingly, Farm 3, which had the highest

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Cryptosporidium prevalence, had the lowest *Giardia* prevalence (2.1%). Farm 5 had the highest *Giardia* prevalence at 18.3%. There were no obvious differences in breed and stocking densities between the farms (Table 1) and the dams of the lambs sampled had all received an anthelmintic drench during the previous summer (December–February). However, other factors such as breeding strategies and paddock rotations may have differed and contributed to the variable prevalence's and species of parasitic protozoa detected at each of the farms. Also cattle were being grazed on Farm 4 and Farm 5 was located adjacent to an abattoir where cattle, sheep and goats may have been housed prior to slaughter. This may also have impacted on the prevalence and species of protozoa detected.

Three species/genotypes of *Cryptosporidium* were identified in pre-weaned lambs, whereas 8 species/genotypes were identified in postweaned sheep (Ryan et al. 2005) (Table 3). In the present study, the 18S and diagnostic loci used for *Cryptosporidium* produced very different results. The diagnostic locus codes for a C-type lectin containing protein with a transmembrane domain and mucin-like rich regions, which appears to be unique to *Cryptosporidium* (cryptodb.com). The copy number is unknown but the qPCR at the diagnostic locus was more sensitive than the nested 18S PCR at detecting *C. parvum.* This may be due to the low numbers of oocysts present, as quantitation data indicated that all the *C. parvum* detected was present in low numbers (1-10 oocysts). It may also be that using *C. parvum*-specific primers is necessary to determine the true prevalence of *C. parvum*.

At the 18S locus, *C. bovis* was the most common species identified in pre-weaned lambs (< 8 weeks old), whereas the cervid genotype was the most predominant genotype amongst post-weaned lambs and sheep (> 8 weeks old) (Ryan et al. 2005). *Cryptosporidium bovis* does not appear to be zoonotic as it has not been detected in humans. In the USA, the cervid genotype was the most common species identified in both pre-weaned lambs and ewes (Santin et al. 2007) and was also the predominant species detected in lambs aged 1 day - 10

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weeks in Belgium (Geurden et al. 2008). It has been suggested that the cervid genotype could possibly emerge as an important human pathogen because current evidence suggests this genotype has a wide host range and zoonotic potential (Santin and Fayer, 2007). Post-weaned sheep in Australia should therefore be considered as an important reservoir for this zoonotic *Cryptosporidium* genotype. *Cryptosporidium hominis* and *C. parvum* are however, by far the most common species found in humans (Xiao and Fayer, 2008). The zoonotic *C. parvum* was not detected in post-weaned lambs and sheep in the previous study (Ryan et al. 2005) but was detected in 63 pre-weaned lambs in the present study, albeit with very low numbers of oocysts. This finding suggests that pre-weaned lambs in Australia may be an important source of *C. parvum*. The diagnostic locus qPCR was not applied to samples from post-weaned sheep and it is possible that *C. parvum* may also be present in these samples in low numbers. Larger numbers of both pre-weaned and post-weaned lambs from different geographic areas within Australia need to be screened at different times of the year at multiple loci and the numbers of oocysts quantitated to confirm this.

Amongst *Giardia* isolates, genotype E was the most common species identified in both pre and post-weaned sheep, which conforms to other studies (Geurden et al. 2007; Santin et al. 2007). Phylogenetic analysis indicated that there is genetic variation within genotype E. Previous allozyme data based on 23 allozyme loci revealed that genotype E comprised three clusters of isolates; a pig cluster, a sheep cluster and a cattle cluster (Monis et al. 2003). In the present study, all the sheep-derived isolates were identical to each other (with the exception of 4 isolates from different farms that had 1 base difference), but there was no clear clustering of isolates into host groups. This is based on phylogenetic analysis of sequence data from only 1 locus (*gdh*) however and further studies at multiple loci are required to understand if there are host-associated genotype E subtypes.

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The zoonotic genotype A was identified in 5 isolates and mixed A and E infections were identified in 11 isolates at the 18S locus. Sequence analysis of the gdh locus of one genotype A isolate identified it as genotype A1. Genotype A1 is generally found in animals, whereas genotype A2 has mainly been identified in humans. However, A2 (and many other A genotypes) have also been detected in animals (cf. Caccio and Ryan, 2008). Assemblage B was not identified in the present study and was previously thought to be largely restricted to humans, however more recently, assemblage B has been reported in various animals including sheep (cf. Caccio and Ryan, 2008). Assemblage B has been associated with severe clinical symptoms in lambs (30-90 days of age), including malabsorption, decreased weight gain, impairment in feed efficiency and malodorous and poorly formed faeces (Aliosio et al. 2006).

In the present study, one isolate (no. 49 from farm 4), was identified as genotype A as the 18S locus but genotype E at the GDH locus. Assignment of *Giardia* isolates to genotypes using different markers is problematic, as frequently different markers can give different results (Caccio and Ryan, 2008, Traub et al., 2004). This phenomenon has been found in both human and animal isolates and has been found using different combinations of gene markers (Caccio and Ryan, 2008). The most likely explanation is that there were mixed genotype infections in the sheep and the different markers used (18S and *gdh*) amplified different genotypes from the same host. The finding of a significant number of mixed A and E infections at the 18S locus (11/53), tends to support this hypothesis. Mixed infections are quite common and have been identified in multiple hosts including dogs where both zoonotic and host adapted genotypes were identified in the same sample using different markers (Caccio and Ryan, 2008; Traub et al., 2004). However, other explanations such as allelic sequence heterozygosity and meiotic recombination (cf Caccio and Ryan, 2008) need to be considered.

In conclusion, the findings of the present study indicate that pre-weaned lambs are not an important source of zoonotic *Giardia* genotypes in Australia but may be an important source

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of zoonotic *Cryptosporidium*. Further research is required to determine if this holds true for pre-weaned lambs across Australia and to understand why there are such dramatic geographic differences in the prevalence of zoonotic genotypes in pre-weaned lambs.

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Table 1: Sheep farms sampled during the present study.

	Farm location	**Mean annual rainfall**	**Farm size**	**Number of ewes**	**Breed of sheep**	**Commencement of lambing**	**Goats and/or cattle on property?**	**Winter stocking rate**
Farm 1	north west of Kojonup	550mm	1000Ha	7000	Merino	Last week of June	no	8-10 DSE/Ha
Farm 2	south of Kojonup	550mm	3000Ha	6500	Merino	1 July	no	12 DSE/Ha
Farm 3	north west of Kojonup	450mm	1000Ha	2500	Merino	1 July	no	8.5 DSE/Ha
Farm 4	north west of Kojonup	540mm	230Ha	1400	Merino	mid July	cattle	8-10 DSE/Ha
Farm 5	north west of Kojonup	450mm	3000Ha	7500	Merino	mid July	located adjacent to abattoir	10 DSE/Ha

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Table 2: Prevalence of *Cryptosporidium* and *Giardia* in pre-weaned lambs in five farms in Western Australia by PCR analysis of the 18S rRNA and unidentified diagnostic locus for *Cryptosporidium* and 18S locus for *Giardia*. Upper and lower 95% confidence intervals are given in parenthesis.

	No. positive for *Cryptosporidium*	**No. positive for *Giardia***	***Cryptosporidium* prevalence**	***Giardia* prevalence**
Farm 1	15/97	12/97	15.5 (8.3, 22.7)	12.4 (5.8, 18.9)
Farm 2	9/97	12/97	9.3 (3.5, 15.1)	12.4 (5.8, 18.9)
Farm 3	54/96	2/96	56.3 (46.3, 66.2)	2.1 (0, 4.9)
Farm 4	18/94	10/94	19.1 (11.2,27.1)	10.6 (4.4,16.9)
Farm 5	22/93	17/93	23.7 (15.0, 32.3)	18.3 (10.4, 26.1)
Total	**117/477**	**53/477**	**24.5 (20.7, 28.4)**	**11.1 (8.3, 13.9)**

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Table 3: *Cryptosporidium* and *Giardia* genotypes in pre-weaned lambs in five farms in Western Australia.

	Cryptosporidium					***Giardia***		
	C. bovis (18S)	cervid genotype (18S)	*C. parvum* (18S)	*C. parvum* (diagnostic locus)	Mixed *C. bovis* and *C. parvum* 18S + diagnostic locus	A	E	mixed A and E
Farm 1	8	0	1	5	0	1	10	0
Farm 2	4	0	0	5	0	0	4	8
Farm 3	21	0	1	33	10	0	2	0
Farm 4	6	4	0	8	0	2	6	2
Farm 5	13	7	0	2	0	2	14	1
Total	**52/66**	**10/66**	**2/66**	**53**	**10**	**5/53**	**36/53**	**11/53**

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Table 4. Comparison of *Cryptosporidium* and *Giardia* species in pre and post-weaned sheep in Western Australia. Post-weaned data was taken from Ryan et al. (2005), in which post-weaned sheep were selected with preference for scouring lines and were not tested with the diagnostic qPCR for *C. parvum* and *C. hominis*

Parasite	Preweaned	Postweaned
Cryptosporidium		
C. hominis	0	1
C. parvum	53	0
C. andersoni	0	1
cervid genotype	10	33
C. bovis	42	14
C. fayeri	0	4
Pig genotype II	0	4
C. suis	0	2
Novel genotype	0	1
Mixed C. bovis + C. parvum	10	
Total	115/447	131*/500
Giardia		
G. duodenalis Livestock genotype	36	33
G. duodenalis Livestock genotype variant	0	2
G. duodenalis assemblage A	5	11
Total	53/477	220**/500

* = only 60 out of 131 positive isolates were genotyped

** = only 46/220 positive isolates were genotyped.

Figure 1. Phylogenetic relationships of *Giardia* isolates inferred by Neighbor Joining analysis of Kimura's distances calculated from pair-wise comparisons of *gdh* sequences. Percentage bootstrap support (>70%) from 1000 replicate samples is indicated at the left of the supported node.

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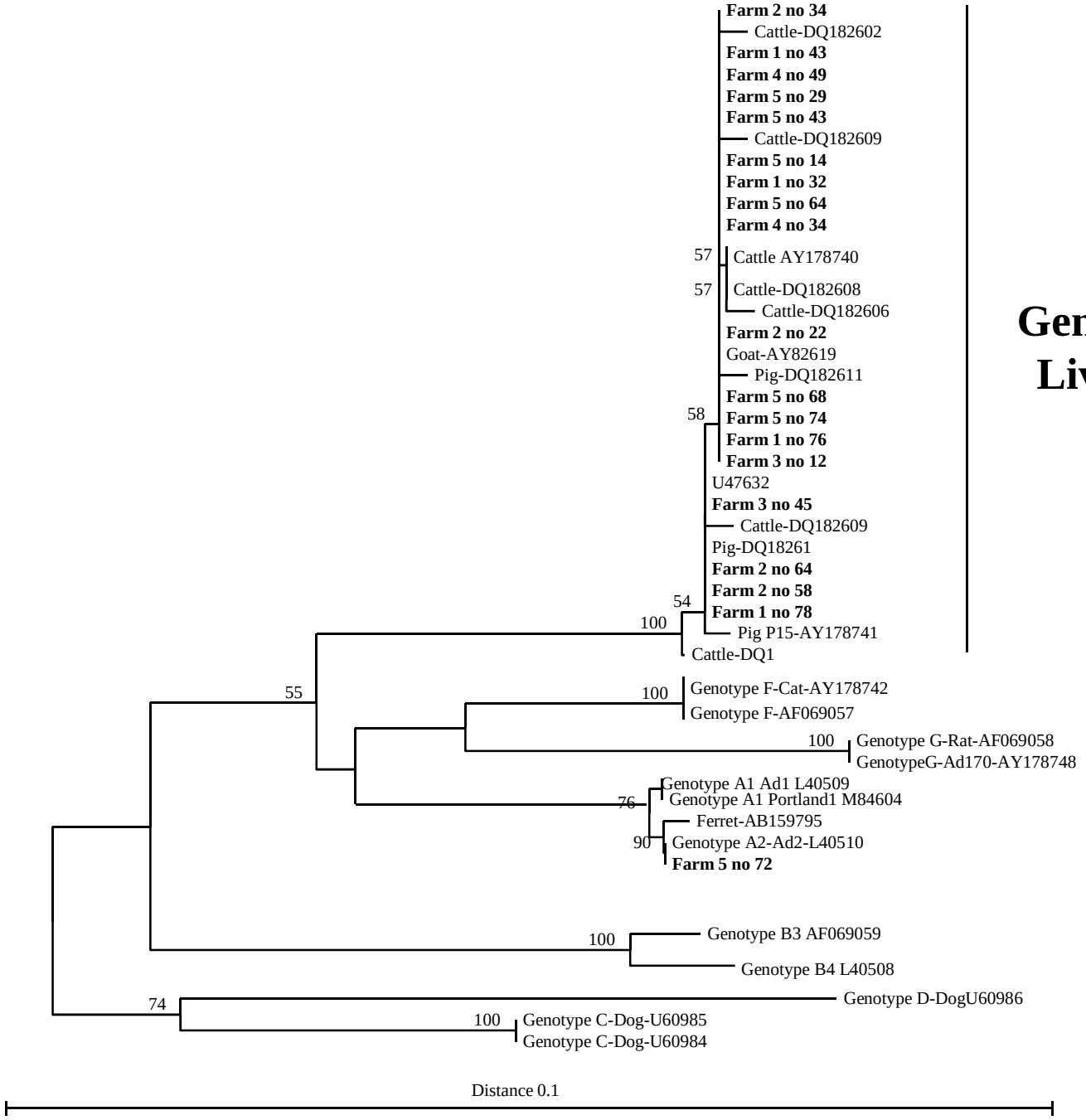

Genotype E Livestock