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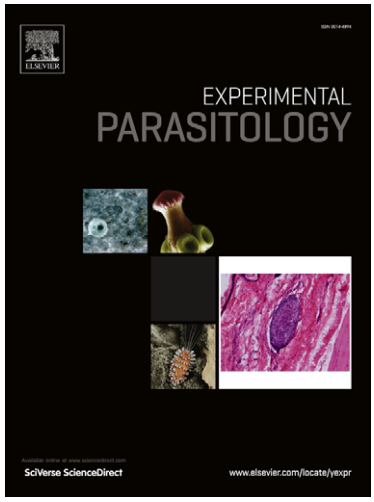

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Evidence of *Cryptosporidium* transmission between cattle and humans in northern New South Wales.

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Abstract

Cryptosporidium is an enteric parasite of public health significance that causes diarrhoeal illness through faecal oral contamination and via water. Zoonotic transmission is difficult to determine as most species of *Cryptosporidium* are morphologically identical and can only be differentiated by molecular means. Transmission dynamics of *Cryptosporidium* in rural populations were investigated through the collection of 196 faecal samples from diarrheic (scouring) calves on 20 farms and 63 faecal samples from humans on 14 of these farms. The overall prevalence of *Cryptosporidium* in cattle and humans by PCR and sequence analysis of the 18S rRNA was 73.5% (144/196) and 23.8% (15/63) respectively. Three species were identified in cattle; *C. parvum, C. bovis* and *C. ryanae,* and from humans, *C. parvum* and *C. bovis*. This is only the second report of *C. bovis* in humans. Subtype analysis at the *gp60* locus identified *C. parvum* subtype IIaA18G3R1 as the most common subtype in calves. Of the seven human *C. parvum* isolates successfully subtyped, 5 were IIaA18G3R1, one was IIdA18G2 and one isolate had a mix of IIaA18G3R1 and IIdA19G2. These findingssuggest that zoonotic transmission may have occurred but more studies involving extensive sampling of both calves and farm workers are needed for a better understanding of the sources of *Cryptosporidium* infections in humans from rural areas of Australia.

Key Words: *Cryptosporidium;* calves; outbreak; humans; genotyping; 18S rRNA; *gp60*; zoonotic transmission.

1.0 Introduction

Cryptosporidium is a protozoan parasite that causes self-limiting diarrhoea in immunocompetent individuals but may be chronic and life-threatening to those that are immunocompromised (Hunter and Nichols, 2002). Humans can acquire *Cryptosporidium* infections through various transmission routes, such as direct contact with infected persons (person-to-person transmission) or animals (zoonotic transmission) and ingestion of contaminated food (foodborne transmission) and water (waterborne transmission) (Xiao, 2010). The relative importance of the different transmission routes is still unclear as most species of *Cryptosporidium* are morphologically identical and cannot be differentiated through routine diagnostics in pathology laboratories (which rely on microscopy and rarely perform genotyping). Molecular characterization tools such as PCR and sequence analysis of the 18S ribosomal RNA (rRNA) gene and the hypervariable 60-kDa glycoprotein (*gp60*) gene, are required to identify species and track transmission (Xiao, 2010; Ng et al., 2008; Ng et al., 2010a, 2010b).

Of the 23 valid species, *C. hominis* and *C. parvum* are responsible for the majority of infections in humans (Xiao, 2010; Ng et al., 2010a). *Cryptosporidium hominis* is predominately found in humans, whereas *C. parvum* can be zoonotic in origin. Cattle are considered one of the main reservoir hosts for *C. parvum*. However, studies worldwide suggest that cattle are infected with at least five *Cryptosporidium* parasites: *C. parvum, C. bovis, C. andersoni, C. ryanae* (previously called deer-like genotype) and *C. suis* (Xiao and Feng, 2008; Xiao, 2010). In case–control studies, contact with cattle was implicated as a risk factor for human cryptosporidiosis in the United States, United Kingdom,

Ireland, and Australia (Robertson et al., 2002; Goh et al., 2004; Hunter et al., 2004; Roy et al., 2004).

Cryptosporidiosis is a notifiable disease in humans in Australia, and data from the National Notifiable Diseases Surveillance System (NNDSS) show that there have been numerous outbreaks of cryptosporidiosis in recent years across most states, only a few of which have been genotyped (Ng et al., 2010b; Waldron et al., 2011a). A previous preliminary study in New South Wales (NSW), which examined the species/genotypes and subgenotypes of *Cryptosporidium* in 7 human and 15 cattle cases of sporadic cryptosporidiosis in rural western NSW during the period from November 2005 to January 2006, reported that four of the six *C. parvum* subtypes found in humans were also found in the cattle, indicating that zoonotic transmission may be an important contributor to sporadic human cases of cryptosporidiosis in rural NSW (Ng et al., 2008). Here, we report a more extensive study of human and cattle faecal samples from farms in rural NSW to more accurately elucidate the transmission dynamics of *Cryptosporidium* in rural populations.

2. Material and Methods

2.1 Sample selection

In June 2010, invitation letters were sent to dairy farmers in northern NSW to recruit for the study. The study sample included twelve farms from the upper Hunter Valley, and eight from around Tamworth. Farms were selected that had herd sizes with more than 100 milking cows. A total of 20 herds meeting these criteria were selected using a rectangular transect approach expanding in area until the desired sample size was reached. Between dates 12/7/2010 and 29/8/2010 veterinarians collected approximately 10 rectal swabs from individual calves on each farm and conducted a survey requesting information on farming practices, scouring history, management of calves and treatment regimes.

A public health epidemiologist and environmental health officer visited the fourteen farms in the Hunter Valley within seven days of the veterinarians collecting specimens. They requested information on farm worker demographics including those who live on the farm property with direct or indirect contact with the dairy calves, diarrhoea in the past month, association with travel, child care centres, public swimming pools, potable water source, consumption of raw milk, eating near the animals, contact with the animals and washing hands after animal contact.

2.2 DNA extraction

A total of 196 faecal samples were collected from calves on 20 farms and 63

faecal samples were collected from humans from 14 of these farms. All cattle and human specimens were sent to Murdoch University, Western Australia (WA) and refrigerated on receipt (4 °C). Total DNA was extracted using a QIAmp DNA Stool Kit (Qiagen, Hilden, Germany) and DNA was stored at -20 C until testing was completed.

2.2 PCR amplification and sequence analysis

Samples were initially genotyped to species level at the 18S rRNA locus using a two-step nested PCR as described by Ryan et al., (2003). The amplified DNA fragments from the secondary PCR products were purified as described by Ng et al., (2006) and sequenced using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California).

Subgenotyping of *C. parvum* isolates was performed using a two-step nested PCR to amplify a ~832 bp fragment of the *gp60* gene as described (Strong et al., 2000; Peng et al., 2003). The amplified DNA fragments were purified and sequenced as described above. Nucleotide sequences were analysed using Chromas v2.3 (Technelysium) and aligned using Clustal W (Larkin et al., 2007). Subtypes within a particular subtype family differ largely in the number of trinucleotide repeats (TCA / TCG) and subtypes were named according to nomenclature described by Sulaiman et al., (2005). Representative sequences have been deposited in GenBank under accession numbers JQ362488-JQ362497.

2.3 Statistical analysis

Prevalence and 95% confidence intervals were calculated based on the exact binomial method (Ross, 2003). Analysis of risk factors associated with presence of *Cryptosporidium* was limited to humans who submitted faecal specimens and completed the survey (62/63). Diarrhoea, as the primary clinical symptom of cryptosporidiosis, was used in the case definition for cryptosporidiosis and its association with risk factors was analysed. Statistical analyses were performed using SPSS version 17.0 (SPSS inc. Chicago, USA) to investigate associations between the presence of *Cryptosporidium* sp. and the factors surveyed in the questionnaire. Univariable analyses conducted included chi-square test for independence, Fisher's exact test for statistical significance with p-value cutoff points when p=0.2-0.25 and odds ratio (OR) with 95% confidence intervals.

3.0 Results

3.1 Cryptosporidium species and subtypes in calves

The overall prevalence of *Cryptosporidium* in cattle was 73.5% (144/196) (95% CI: 66.7, 79.5). The prevalence on the different farms ranged from 30% to 100% (Table 1). 18S sequences were obtained for 142 of the 144 positives; 85 were *C. parvum*, 29 were *C. bovis* and 14 were *C. ryanae*. Eleven were mixed *C. parvum*/*C. bovis* infection and 3 were mixed *C. parvum/C. ryanae* infections. Sub-typing analysis at the *gp*60 locus was successful for 84 of the 143 positives. The most common subtype identified was IIaA18G3R1 (n =57), followed by IIaA19G3R1 (n=11), IIaA17G2R1 (n=7), IIaA19G2R1 (n=6), IIaA16G3R1 (n=2) and IIaA20G3R1 (n = 1).

3.2 Cryptosporidium species and subtypes in humans

A total of 63 faecal samples were collected from humans from 14 of these farms. The overall prevalence of *Cryptosporidium* in humans was 23.8% (15/63) (95% CI:14.0, 36.2) (Table 1). 18S sequences were obtained for 14 of the 15 positives; 12 were *C. parvum* and 2 were *C. bovis*. Of those that were positive for *Cryptosporidium*, 4/15 humans reported having diarrhoea, all 4 of which were infected with *C. parvum*. No clinical symptoms were reported from those infected with *C. bovis*. Sub-typing analysis at the *gp*60 locus was successful for 7 of the 12 *C. parvum* positives. Subtype IIaA18G3R1 was identified in 5 humans, IIdA18G2 in one human and a mixed subtype of IIaA18G3R1 and IIdA19G2 in one human.

3.3 Epidemiological analysis

The survey conducted was completed by 62/63 humans who submitted faecal specimens. The dataset generated from the survey was used for analysing *Cryptosporidium* infections in humans and limited to those who contributed stool samples and were positive for *Cryptosporidium* through screening by PCR (15/62). Humans who were *Cryptosporidium* positive were 8.2 times more likely to have diarrhoea (95% CI: 1.3, 50.5). However, none of those with diarrhoea consulted with a doctor about their symptoms. Univariate analyses of risk factors such as eating close to animals, washing hands after contact with animals, drinking raw milk and contact with calves did not reveal any significant association with the presence of *Cryptosporidium* or having diarrhoea compared with individuals without *Cryptosporidium* and/or without diarrhoea (Table 2). All participants

reported rainwater as the main potable water source and none of the participants reported travelling overseas. Visiting child care centres and public swimming pools could not be determined as risk factors due to low number of respondents in the questionnaire survey.

4.0 Discussion

In the present study, the overall prevalence of *Cryptosporidium* in cattle was 73.5% (95% CI: 66.7, 79.5). This is much higher than reports from previous studies, one of which reported a prevalence of 17.6% (95% CI: 13.0, 22.9) in pre-weaned calves from 5 farms in WA and a prevalence of 39.1% (95% CI: 23.7, 41.1) in a NSW farm (Ng et al., 2011). Another study reported that the total prevalence of *Cryptosporidium* in calves from 84 dairy and dairy beef properties across Australia was 58.5% (95% CI: 54.4, 62.4) (Izzo et al., 2011). In the present study, all the calves were < 2 months old whereas in the previous study in NSW and WA, the age of the calves ranged from 1 week to 4 months old (Ng et al., 2011). A study in the US reported that the highest prevalence of infection occurs in calves <8 weeks of age (45.8%), followed by post-weaned calves (3-12 months of age) (18.5%) and heifers (12-24 months of age) (2.2%) (Santin et al., 2008). In the present study, the prevalence on the different farms ranged from 30% to 100%. Most of the calves on these properties were diarrheic, however, insufficient testing was conducted to conclude that *Cryptosporidium* was the sole cause.

Three species of *Cryptosporidium* were detected in the calves; *C. parvum* accounted for the majority (59.4%), followed by *C. bovis* (20.3%) and *C. ryanae* (9.8%). Mixed *C. parvum*/*C. bovis* and *C. parvum/C. ryanae* infections accounted for 9.8% of

infections. Pig genotype II and *C. andersoni* were not identified in the cattle in the present study. The prevalence of *C. parvum* was higher than a recent previous study in NSW, which reported a prevalence of 42% for *C. parvum* amongst typed species in pre-weaned cattle (Ng et al., 2011). There also appears to be geographical differences in the prevalence of *C. parvum* in calves in Australia with lower *C. parvum* rates in WA compared to Eastern states. For example, a previous study reported that the prevalence of *C. parvum* in pre-weaned cattle in 5 WA farms was 17.6%, whereas another study in Victoria, identified *C. parvum* in 46.3% (124/268) of calves on pasture-based dairy farms in three regions sampled (Nolan et al., 2009).

The overall prevalence of *Cryptosporidium* in humans in the present study was 23.8% (15/63) (95% CI: 14.0, 36.2) which is higher than the overall prevalence reported in dariy farm workers in India at 11.8% (Khan et al., 2010). The differences are likely due to the different *Cryptosporidium* detection methods employed. For example, in the present study, PCR-based molecular tools were used whereas microscopy and an Enzyme Linked Immunosorpent Assay (ELISA) were used in the Indian study (Khan et al., 2010). PCR based molecular tools have been shown to be more sensitive than microscopy or ELISA when detecting *Cryptosporidium* in faecal samples, which may have contributed to higher prevalence in the present study (Morgan et al., 1998; Nair et al., 2008). Of the 14 positives that were sequenced at the 18S gene locus, 12 were *C. parvum* and 2 were *C. bovis*, with the latter identified in a 3 year-old child and a 23 year-old adult, from separate farms. *Cryptosporidium bovis* was first described in 2005 and is morphologically indistinguishable from *C. parvum* (Fayer et al., 2005). It has a narrow host range and has previously been described in cattle and sheep. To date there has only been one report of

C. bovis in a dairy farm worker in India, where the infection was asymptomatic (Khan et al., 2010). In the present study, infection of *C. bovis* in both humans was asymptomatic. Both individuals had reported drinking raw milk and had regular contact with calves. One of the individuals reported not regularly washing their hands after contact with calves. Whether the two humans involved were actually infected or passing oocysts as a result of mechanical transmission is unknown but highlights the potential for transmission between cattle and humans in rural areas.

Direct or indirect contact with animals, washing hands after handling animals and drinking raw (unpasteurized) milk have been reported as risk factors for *Cryptosporidium* transmission to humans by numerous researchers (e.g. Harper et al., 2002; Ashbolt et al., 2003; Kiang et al., 2006). In the present study however, we could not infer any statistically significant associations between the risk factors examined with the presence of *Cryptosporidium* or reports of diarrhoea in humans on these farms, due to the small sample size. Further studies with a larger sample are required to provide stronger evidence associating these risk factors with *Cryptosporidium* transmission in rural populations.

Subtype analysis identified that *C. parvum* IIaA18G3R1 was identified in 69% of the 80 cattle isolates subtyped. This was also the most common subtype identified in six of the seven human isolates successfully subtyped. IIaA18G3R1 is a common subtype in both humans and cattle worldwide and has been reported in both calves and humans in NSW (Ng et al., 2008; Waldron et al., 2009) and in WA (O'Brien et al., 2008; Ng et al., 2010a, 2010b; Ng et al., 2011), humans and cattle in Victoria (Jex et al., 2007; Nolan et al., 2009) and humans in South Australia (Jex et al., 2008). A recent study, which

examined sporadic cryptosporidiosis in the Hunter Valley human population between January 2008 to December 2010, identified 7 *C. hominis* and 5 *C. parvum* isolates, with IIaA18G3R1 the most common *C. parvum* subtype (Waldron et al., 2011b). Although the epidemiological analysis was inconclusive, this finding suggests that zoonotic transmission may be occurring. However, a much larger study is required to confirm this.

Interestingly, subtypes IIdA18G2 and IIdA19G2 were identified from two humans who lived on the same farm but these subtypes were not found in any of the cattle isolates genotyped. The IId subtype family is much less commonly identified in cattle, sheep, goats and humans in European studies and dairy calves in Egypt (Amer et al., 2010; Xiao, 2010), and has never been found in humans or cattle in the United States and Canada (Xiao, 2010). IId subtypes (IIdA15G1, IIdA24G1) have been reported previously in humans but not in cattle in NSW (Waldron et al., 2009). Both the IIdA18G2 and IIdA19G2 subtypes have not been reported previously in humans or cattle and represent novel subtypes. The finding of these subtypes in humans in the present study and the finding of *C. hominis* subtypes in sporadic human cases from a recent study in the Hunter Valley (which included the present study area) (Waldron et al., 2011b), suggests that human to human transmission is also common in NSW.

In conclusion, the present study identified *C. parvum* subtypes IIdA18G2 and IIdA19G2 for the first time in humans, showing the genetic diversity of *C. parvum* subtypes infecting humans in NSW. The concurrence of *C. parvum* subtypes and the cattle-specific *C. bovis* in humans and calves provides evidence of zoonotic transmission and the possible association of infected calves and human infection with *Cryptosporidium*. Studies involving more extensive sampling of both calves and farm

workers and collection of more extensive epidemiological data are needed for a better understanding of the sources of *Cryptosporidium* infections in humans in rural areas.

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Table 1. Prevalence of *Cryptosporidium* species and subtypes in cattle and farm workers on the 20 farms analysed in the upper Hunter Valley and Tamworth NSW.

Farm no	Cattle					Humans		
	Calf age	No. positive	*Cryptosporidium* prevalence (95% CI)	Species	*gp60* subtype	No positive	Species	*gp60* subtype
1	<2 months	9/10	90% (55.5, 99.7))	*C. parvum* (n = 7) *C. bovis* (n = 2)	IIa A17G2R1 (n = 4) IIa A18G3R1 (n = 1) IIa A19G2R1 (n = 1) IIa A19G3R1 (n = 1)	3/5	*C. parvum* (n = 3)	IIa A18G3R1 (n = 2)
2	<2 months	10/10	100% (69.2, 100)	*C. parvum* (n = 10)	IIa A18G3R1 (n = 7) IIa A19G2R1 (n = 1)	-		
3	<2 months	9/10	90% (55.5, 99.7)	*C. bovis* (n = 7) *C. ryanae* (n = 2)	-	-		
4	<2 months	10/10	100% (69.2, 100)	*C. parvum* (n = 2) *C. ryanae* (n = 6) Mixed *C. parvum*/*C. ryanae* (n = 1) ND (n = 1)	IIa A16G3R1 (n = 2)	0/1	-	-
5	1-2 months	7/10	70% (34.8, 93.3)	*C. parvum* (n = 3) *C. bovis* (n = 3) Mixed *C. parvum*/*C. bovis* (n = 1)	IIa A17G2R1 (n = 3) IIa A18G3R1 (n = 1)	0/2	-	-
6	<2 months	7/10	70% (34.8, 93.3)	*C. bovis* (n = 2) Mixed *C. parvum*/*C. bovis* (n = 5)	IIa A19G2R1 (n = 4)	1/10	ND	-
7	<2 months	7/9	77.8% (40.0, 97.2)	*C. parvum* (n = 7)	IIa A18G3R1 (n = 7)	0/3		
8	<2 months	7/10	70% (34.8, 93.3)	*C. parvum* (n = 7)	IIa A18G3R1 (n = 6)	0/3		
9	1-2 months	3/10	30% (6.7, 65.2)	*C. parvum* (n = 2) Mixed *C. parvum*/ *C. ryanae* (n = 1)	-	5/10	*C. parvum* (n = 4) *C. bovis* (n = 1)	IIa A18G3R1 (n = 2) IIa A18G3R1+ IId A19G2 (n = 1) IId A18G2 (n = 1)
10	<2 months	10/10	100% (69.2, 100)	*C. parvum* (n = 10)	IIa A18G3R1 (n = 10)	0/3		

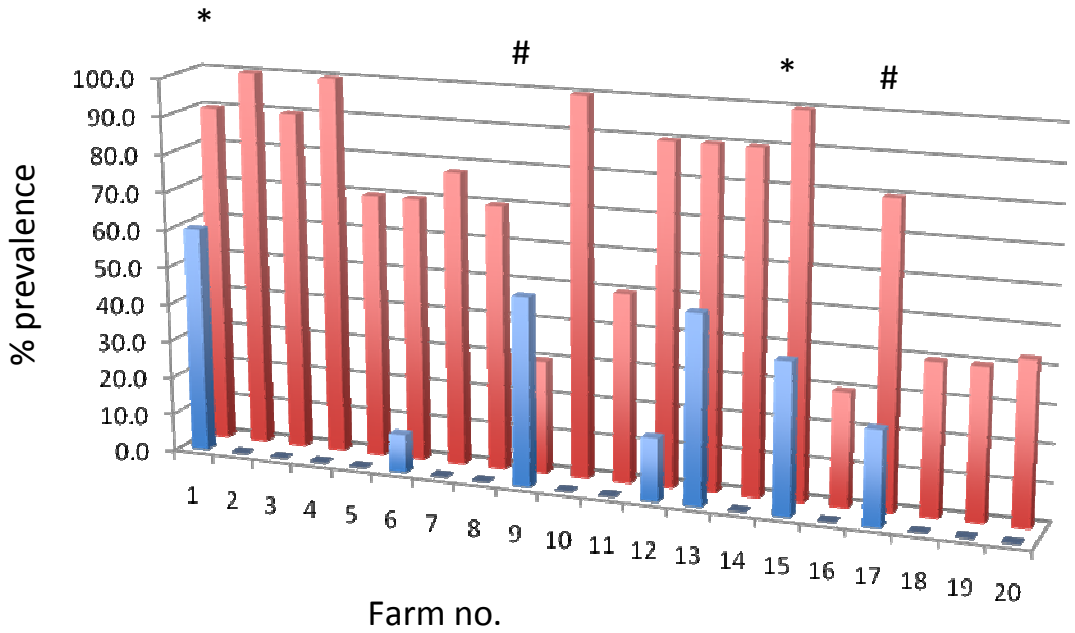
11	**<2 months**	**5/10**	**50% (18.7, 81.3)**	***C. parvum* (n = 2)** ***C. bovis* (n = 3)**	**IIa A18G3R1 (n = 1)**	**-**		
12	**<2 months**	**9/10**	**90% (55.5, 99.7))**	***C. bovis* (n = 4)** ***C. ryanae* (n = 4)** **Mixed *C. parvum*/*C. ryanae* (n = 1)**	**IIa A18G3R1 (n = 1)**	**1/6**	***C. parvum* (n = 1)**	**-**
13	**<2 months**	**9/10**	**90% (55.5, 99.7)**	***C. parvum* (n =9)**	**IIa A18G3R1 (n = 6)**	**1/2**	***C. parvum* (n = 1)**	**-**
14	**1-2 months**	**9/10**	**90% (55.5, 99.7)**	***C. parvum* (n = 9)**	**IIa A19G3R1 (n = 8)**	**0/2**		
15	**<2 months**	**10/10**	**100% (69.2, 100)**	***C. parvum* (n = 4)** ***C. bovis* (n = 1)** **Mixed *C. parvum*/*C. bovis* (n = 5)**	**IIa A18G3R1 (n = 9)**	**2/5**	***C. parvum* (n = 2)**	**IIa A18G3R1 (n = 1)**
16	**<2 months**	**3/10**	**30% (6.7, 65.2)**	***C. parvum* (n =2)** ***C. bovis* (n=1)**	**IIa A18G3R1 (n = 1)**	**0/3**		
17	**<2 months**	**8/10**	**80% (44.4, 97.5)**	***C. parvum* (n = 2)** ***C. bovis* (n = 5)** ***C. ryanae* (n = 1)**	**IIa A18G3R1 (n = 1)** **IIa A19G3R1 (n = 1)**	**2/8**	***C. bovis* (n = 1)** ***C. parvum* (n = 1)**	**-**
18	**<2 months**	**5/10**	**40% (12.2, 73.8)**	***C. parvum* (n =3)** ***C. ryanae* (n=1)** **ND (n = 1)**	**IIa A18G3R1 (n = 2)**	**-**		
19	**<2 months**	**4/10**	**40% (12.2, 73.8)**	***C. parvum* (n = 3)** ***C. bovis* (n = 1)**	**IIa A18G3R1 (n = 1)** **IIa A19G3R1 (n = 1)** **IIa A20G3R1 (n = 1)**	**-**		
20	**<2 months**	**3/7**	**42.9% (6.7, 65.2)**	***C. parvum* (n = 3)**	**IIa A18G3R1 (n = 3)**	**-**		
Total		**144/196**	**73.5% (66.7, 79.5)**			**15/63**		

Table 2: Associations between risk factors examined and the presence of *Cryptosporidium* in humans or report of humans with diarrhoea.

	***Cryptosporidium* Infection**		**Diarrhoea**	
Risk Factors	**Odds Ratio (95%CI)**	**p-value**	**Odds Ratio (95%CI)**	**p-value**
Eating among the animals	0.87 (0.25, 3.02)	0.54	1.69 (0.35, 8.13)	0.39
Washing hands after contact with animals	1.52 (0.42, 5.55)	0.38	0.57 (0.13, 2.61)	0.36
Drinking raw milk	0.45 (0.07, 3.16)	0.38	0.2 (0.03, 1.22)	0.12
Contact with calves	0.31 (0.18, 5.30)	0.43	0.26 (0.02, 2.83)	0.31

Prevalence of *Cryptosporidium* in calves (red) and humans (blue) from the same farms in rural NSW.

[* denotes identical subtypes identified in calves and humans on these farms. # denotes *C. bovis* identified in humans]



Research Highlights

- One of the 1st studies to directly track transmission between cattle and humans
- High prevalence of *Cryptosporidium* in cattle (75%)
- Identification of *C. bovis* in humans (only second report worldwide)
- Identification of identical *C. parvum* subtypes in cattle and humans from the same farms