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Molecular epidemiology of *Cryptosporidium* spp. in dairy cattle in Guangdong Province, South China

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Research Article

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Abstract

To determine the prevalence of *Cryptosporidium* in dairy cattle in Guangdong Province, South China, 1440 fecal samples were collected from 10 farms and screened for *Cryptosporidium* with PCR. The overall prevalence of *Cryptosporidium* was 4.38% (63/1440), and the infection rates in preweaned calves, postweaned calves, heifers and adults were 6.4% (19/297), 6.19% (33/533), 1.48% (4/271) and 2.06% (7/339), respectively. Three *Cryptosporidium* species, *Cryptosporidium andersoni* ($n = 33$), *Cryptosporidium bovis* ($n = 22$) and *Cryptosporidium ryanae* ($n = 8$) were detected by DNA sequence analysis of the 63 positive samples, and *C. andersoni* was identified as the most common species on the dairy cattle farms. In preweaned calves, *C. bovis* was the most prevalent species (9/19, 47.4%). In contrast, *C. andersoni* was the predominant species (19/33, 57.6%) in postweaned calves and the only species found in heifers and adults. The zoonotic species *Cryptosporidium parvum* was not detected in this study. Twenty-four *C. andersoni* isolates were successfully classified into three multilocus sequence typing (MLST) subtypes. MLST subtype A4,A4,A4,A1 was the predominant subtype, and MLST subtype A2,A5,A2,A1, previously found in sheep, was detected in cattle for the first time. A linkage disequilibrium analysis showed that the *C. andersoni* isolates had a clonal genetic population structure. However, further molecular studies are required to better understand the epidemiology of *Cryptosporidium* in Guangdong.

Introduction

Cryptosporidium is an important protozoan parasite, mainly causing gastrointestinal disease in humans and animals, including livestock, companion animals and wildlife (Fayer, 2010). Among domestic animals, cattle are recognized as the most common mammalian species to be infected by *Cryptosporidium*, and preweaned calves are considered an important reservoir for zoonotic *Cryptosporidium* infections (Xiao and Feng, 2008; Xiao, 2010; Imre *et al*., 2011). Contamination of cattle manure has led to several food-borne and water-borne outbreaks of human cryptosporidiosis (Blackburn *et al*., 2006; Baldursson and Karanis, 2011). *Cryptosporidium* infections frequently result in morbidity, weight loss and delayed growth, and sometimes mortality of young animals.

To date, 37 valid *Cryptosporidium* species and over 70 genotypes have been described (Ryan *et al*., 2014; Kváč *et al*., 2016; Zahedi *et al*., 2017; Čondlová *et al*., 2018; Kváč *et al*., 2018). Four of them, namely *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni* and *Cryptosporidium ryanae* are the most common species that can infect cattle and cause bovine cryptosporidiosis. Occasionally, *Cryptosporidium felis*, *Cryptosporidium hominis*, *Cryptosporidium suis*, *Cryptosporidium scrofarum* and *C. suis*-like have also been detected in cattle (Trout and Santín, 2008). Many studies conducted in industrialized nations demonstrate that the four common species have age-related distributions. *Cryptosporidium parvum* is mostly found in preweaned calves and is a significant cause of diarrhoea. *Cryptosporidium bovis* and *C. ryanae* usually infect postweaned calves and yearlings, although *C. bovis* is more prevalent than *C. ryanae*, and neither is associated with diarrhoea (Santín *et al*., 2008). In contrast, *C. andersoni* is commonly seen in adult cattle and has been associated with gastritis, reduced milk yield and poor weight gain (Esteban and Anderson, 1995).

In China, *Cryptosporidium* infections have been reported in dairy cattle in Xinjiang, Ningxia, Gansu, Shaanxi, Heilongjiang, Henan, Shandong, Hubei and other provinces (Liu *et al*., 2009; Wang *et al*., 2011*a*, 2011*b*; Zhang *et al*., 2013, 2015; Zhao *et al*., 2013, 2014; Cui *et al*., 2014; Huang *et al*., 2014; Ma *et al*., 2015; Qi *et al*., 2015*a*, 2015*b*; Fan *et al*., 2017), but no information is available on the prevalence or genotypes of *Cryptosporidium* infections in dairy cattle in Guangdong Province. In this study, we conducted the first

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Table 1. Infection rates and distribution of *Cryptosporidium* species in dairy cattle of different collection sites and age groups in Guangdong Province

Factors		No. positive/sample size	Prevalence (%) (95% CI)	*Cryptosporidium* species (no.)
Collection site	HZ-1	12/82	14.63 (6.82–22.45)	*C. andersoni* (12)
	HZ-2	7/67	10.45 (2.93–17.97)	*C. bovis* (4), *C. ryanae* (1), *C. andersoni* (2)
	HZ-3	11/40	27.5 (13.04–41.96)	*C. bovis* (3), *C. ryanae* (2), *C. andersoni* (6)
	HZ-4	11/111	9.91 (4.26–15.56)	*C. bovis* (7), *C. ryanae* (3), *C. andersoni* (1)
	SZ-1	8/179	4.47 (1.41–7.53)	*C. bovis* (6), *C. ryanae* (2)
	GZ-1	5/138	3.62 (0.47–6.78)	*C. andersoni* (5)
	GZ-2	4/118	3.39 (0.08–6.70)	*C. andersoni* (4)
	GZ-3	5/374	1.34 (0.17–2.51)	*C. bovis* (2), *C. andersoni* (3)
	GZ-4	0/12	0	/
	QY-1	0/319	0	/
	Total	63/1440	4.38 (3.32–5.43)	*C. bovis* (22), *C. ryanae* (8), *C. andersoni* (33)
Age group	Preweaned	19/297	6.40 (3.60–9.20)	*C. bovis* (9), *C. ryanae* (7), *C. andersoni* (3)
	Postweaned	33/533	6.19 (4.14–8.24)	*C. bovis* (13), *C. ryanae* (1), *C. andersoni* (19)
	Heifer	4/271	1.48 (0.03–2.92)	*C. andersoni* (4)
	Adult	7/339	2.06 (0.54–3.59)	*C. andersoni* (7)

molecular epidemiological survey of dairy cattle in Guangdong Province to identify the infection rates and species distribution of *Cryptosporidium*.

Materials and methods

Study area and specimen collection

Guangdong Province is located at the southern end of mainland China. The annual average temperature is above 20 °C and rainfall is abundant in this region. In this study, 1440 fecal specimens (approximately 20 g each) were collected from eight large-scale dairy cattle farms and two small dairy cattle farms in the cities of Huizhou, Guangzhou, Shenzhen and Qingyuan in Guangdong Province in April 2016 (Table 1). The collected specimens represented 10–15% of the total cattle on each farm. A fresh fecal specimen was collected from each animal using a sterile disposable latex glove immediately after its defecation onto the ground, and the sample was then placed individually into a disposable plastic bag. The cattle were divided into age groups: preweaned calves (<2 months old), postweaned calves (2–6 months old), heifers (7 months to 2 years old) and adult cattle (>2 years old). All the specimens were stored in 2.5% potassium dichromate at 4 °C before DNA extraction.

DNA extraction and PCR amplification

All fecal specimens collected were washed three times with distilled water by centrifugation at 1500 × ***g*** for 10 min at room temperature. The genomic DNA was extracted from 200 mg of each specimen using the E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), according to the manufacturer's instructions. The eluted DNA was stored at −20 °C until PCR analysis.

All DNA samples were screened for *Cryptosporidium* using nested PCR amplification of an approximate 830-bp fragment of the small subunit rRNA (SSU rRNA) gene, as previously described (Alves *et al.*, 2003). Positive and negative controls were included in each PCR analysis. Then, the samples positive for *C. andersoni* at the SSU rRNA locus were subtyped by amplifying the four minisatellite/microsatellite targets including MS1 (coding for hypothetical protein), MS2 (coding for 90 kDa heat shock protein), MS3 (coding for hypothetical protein) and MS16 (coding for leucine-rich repeat family protein) according to the previously described nested PCR (polymerase chain reaction) protocols (Feng *et al.*, 2011; Zhao *et al.*, 2013). KOD-Plus amplification enzyme (Toyobo Co., Ltd., Osaka, Japan) was used for PCR amplification. The secondary PCR products were examined by agarose gel electrophoresis and visualized after GelRed™ staining (Biotium Inc., Hayward, CA, USA).

DNA sequence analysis

Positive secondary PCR products were sequenced on an ABI Prism™ 3730 XL DNA Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA). Sequence accuracy was confirmed with bi-directional sequencing and by sequencing a new PCR product if necessary. The sequences were identified by alignment with reference sequences downloaded from GenBank (http://www.ncbi.nlm.nih.gov) using the ClustalX2.13. The *C. andersoni* subtypes were named according to the repeat characteristics of minisatellite repeats in four genetic loci by Feng *et al.* (2011).

Statistical analysis

The *Cryptosporidium* infection rates were evaluated using Regression Analysis in Statistic Package for Social Science (SPSS) for Windows with 95% confidence intervals (CI). Probability level (P) of <0.05 were considered as statistically significant. The linkage disequilibrium (LD) across all four loci was assessed with the standardized index of association (I_A^S) proposed by Haubold and Hudson (2000). LD was tested with LIAN version 3.7 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl) using a parametric method for the four microsatellite/minisatellite loci.

Results

Prevalence of Cryptosporidium

Eight of the 10 farms tested were positive for *Cryptosporidium*, with infection rates of 1.34–27.5% ($P < 0.01$; Table 1). Among the *Cryptosporidium*-positive farms, farm HZ-3 had the highest

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prevalence and farms GZ-4 and QY-1 had no *Cryptosporidium* infections. The infection rates of the different age groups were 6.4% (95% CI 3.60–9.20) in preweaned calves, 6.19% (95% CI 4.41–8.24) in postweaned calves, 1.48% (95% CI 0.03–2.92) in heifers and 2.06% (95% CI 0.54–3.59) in adult cattle ($P < 0.01$, Table 1).

Distribution of Cryptosporidium *species and subtypes*

Sequence analysis of the 18S rRNA gene fragment revealed the presence of three *Cryptosporidium* species: *C. andersoni* ($n = 33$) on seven farms, *C. bovis* ($n = 22$) on five farms and *C. ryanae* ($n = 8$) on four farms (Table 1). Except for farm SZ-1, *C. andersoni* was detected on all the *Cryptosporidium*-positive farms and was the only species found on three farms.

Cryptosporidium andersoni was found in all age groups of dairy cattle, responsible for 52.4% (33/63) of all *Cryptosporidium* infections in this study. The prevalence of *C. andersoni* in preweaned calves, postweaned calves, heifers and adult cattle was 1.01% (95% CI 0–2.15), 3.56% (95% CI 1.99–5.14), 1.48% (95% CI 0.03–2.92) and 2.06% (95% CI 0.54–3.59), respectively. In contrast, *C. bovis* and *C. ryanae* were only found in dairy calves, with infection rates of 2.65% (95% CI 1.56–3.75) and 0.96% (95% CI 0.30–1.63), respectively (Table 1).

At the four microsatellite/minisatellite loci (MS1, MS2, MS3 and MS16), 24, 25, 24 and 25 DNA preparations were sequenced successfully, respectively, and three, two, two and one haplotypes were identified, respectively. In total, 24 of 33 *C. andersoni* isolates were successfully subtyped at all four loci, and three *C. andersoni* MLST subtypes were identified. Of these, MLST subtype A4,A4, A4,A1 was most prevalent (87.5%, 21/24) in the dairy cattle, appearing on all *C. andersoni*-positive farms, demonstrating an extensive distribution in the investigated area. However, the other two MLST subtypes, A2,A5,A2,A1 ($n = 1$) and A1, A4,A4,A1 ($n = 2$), were only detected on farms GZ-2 and GZ-3, respectively. MLST subtype A4,A4,A4,A1 was predominant in all age groups of dairy cattle and was the only subtype detected in dairy calves. MLST subtypes A2,A5,A2,A1 and A1, A4,A4,A1 were only found in heifers and adult cattle, respectively (Table 2).

LD analysis of C. andersoni

The 24 *C. andersoni* isolates successfully subtyped at all the four loci were included in the LD analysis. The standardized index of association ($I_A^S = 0.4109$) was >0 and the pairwise variance ($V_D = 0.7431$) was greater than the 95% confidence limit ($L = 0.4527$), indicating the presence of LD and the clonal population structure of *C. andersoni* in Guangdong Province.

Discussion

The overall *Cryptosporidium* prevalence in this study was 4.38%, which is lower than most rates reported in dairy cattle in Heilongjiang (17.33%, 257/1483), Anhui (14.9%, 52/350), Shanghai (12.5%, 55/440), Jiangsu (20.7%, 251/1215), Henan (13.0%, 276/2116), Xinjiang (16.0%, 82/514) and Shaanxi (5.24%, 122/2329) (Liu *et al.*, 2009; Wang *et al.*, 2011*a*, 2011*b*; Chen and Huang, 2012; Zhang *et al.*, 2013; Zhao *et al.*, 2014; Qi *et al.*, 2015*a*, 2015*b*; Zhang *et al.*, 2015), but higher than that in Ningxia (3.76%, 115/3054) (Zhao *et al.*, 2013; Huang *et al.*, 2014; Cui *et al.*, 2014; Qi *et al.*, 2015*b*). The infection rate of 6.4% in preweaned calves was lower than all previously reported studies, in which the prevalence was 10.22–47.68% (Wang *et al.*, 2011*b*; Zhang *et al.*, 2013; Huang *et al.*, 2014; Qi *et al.*, 2015*a*, 2015*b*; Fan *et al.*, 2017). However, the infection rate in postweaned calves (6.19%) was higher than that in Heilongjiang (5.5%) (Liu *et al.*, 2009), but lower than that in Henan (11.3%) and Xinjiang (16.2%) (Wang *et al.*, 2011*a*; Qi *et al.*, 2015*a*). Many factors, including specimen size, diagnostic technique, management system, season and geographic area, may be responsible for the differences in the prevalence of *Cryptosporidium* observed in different areas of China. These results were also consistent with previous studies, which showed that the prevalence of *Cryptosporidium* was higher in preweaned calves than in any other age group (Wang *et al.*, 2011*a*, 2011*b*; Huang *et al.*, 2014; Zhang *et al.*, 2015) (Table 1).

Table 2. The distribution of MLST subtypes of *Cryptosporidium andersoni* in dairy cattle of different collection sites and age groups in Guangdong Province

Factors		Subtyped no./ amplified no.[a]	MLST subtypes (n)[b]
Collection site	HZ-1	9/12	$A_4A_4A_4A_1$ (9)
	HZ-2	½	$A_4A_4A_4A_1$ (1)
	HZ-3	6/6	$A_4A_4A_4A_1$ (6)
	HZ-4	0/1	/
	GZ-1	5/5	$A_4A_4A_4A_1$ (5)
	GZ-2	¼	$A_2A_5A_2A_1$ (1)
	GZ-3	2/3	$A_1A_4A_4A_1$ (2)
	Total	24/33	$A_4A_4A_4A_1$ (21); $A_2A_5A_2A_1$ (1); $A_1A_4A_4A_1$ (2)
Age group	Preweaned	3/3	$A_4A_4A_4A_1$ (3)
	Postweaned	14/19	$A_4A_4A_4A_1$ (14)
	Heifer	1/4	$A_2A_5A_2A_1$ (1)
	Adult	6/7	$A_4A_4A_4A_1$ (4); $A_1A_4A_4A_1$ (2)
	Total	24/33	$A_4A_4A_4A_1$ (21); $A_2A_5A_2A_1$ (1); $A_1A_4A_4A_1$ (2)

[a]Subtyped no. indicates the number of *C. andersoni* isolates subtyped successfully at all the four loci (MS1, MS2, MS3 and MS16) with PCR; amplified no. indicates the number of *C. andersoni* isolates analysed with PCR at all the four loci (MS1, MS2, MS3 and MS16).
[b]Haplotypes are arranged in the order of the gene loci amplified: MS1, MS2, MS3 and MS16.

Cryptosporidium andersoni is the predominant species in postweaned calves and adult cattle, which had been confirmed in China, Mongolia, Egypt and some European countries (Burenbaatar *et al.*, 2008; Ondráčková *et al.*, 2009; Wang *et al.*, 2011*a*; Amer *et al.*, 2013; Zhao *et al.*, 2013; Ma *et al.*, 2015; Qi *et al.*, 2015*a*, 2015*b*). In contrast, in other countries, *C. bovis was* considered the predominant species in postweaned calves (Enemark *et al.*, 2002; Feng *et al.*, 2007). *Cryptosporidium andersoni* was the only species detected in heifers and adult cattle in this study, which was identical to previous studies conducted in Heilongjiang, Shaanxi and Henan Provinces (Liu *et al.*, 2009; Wang *et al.*, 2011*a*; Zhao *et al.*, 2013). The potential zoonotic transmission of *C. andersoni* is unknown, but the species has been isolated from humans with diarrhoea (Leoni *et al.*, 2006; Jiang *et al.*, 2014).

Several studies have reported that zoonotic *C. parvum* is responsible for the majority of *Cryptosporidium* infections in preweaned calves (Santín *et al.*, 2004; Fayer *et al.*, 2006; Santín *et al.*, 2008; Trout and Santín, 2008; Amer *et al.*, 2013). However, other studies have shown that *C. bovis* is the species most commonly found in preweaned calves (Feng *et al.*, 2007; Silverlas *et al.*, 2010; Rieux *et al.*, 2013). In the present study, *C. bovis*, rather

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than *C. parvum*, was the most abundant species in preweaned calves, which was in concordance with the results from Shaanxi, Heilongjiang, Henan and Hubei Provinces, as well as Shanghai (Wang *et al.*, 2011*b*; Zhang *et al.*, 2013; Qi *et al.*, 2015*b*; Fan *et al.*, 2017), but differed from Xinjiang and Ningxia (Cui *et al.*, 2014; Huang *et al.*, 2014; Qi *et al.*, 2015*a*, 2015*b*).

However, *C. parvum* was not detected in the present study, which was also reported in early studies from China and abroad (Maikai *et al.*, 2011; Feng *et al.*, 2012; Murakoshi *et al.*, 2012; Nguyen *et al.*, 2012; Abeywardena *et al.*, 2014; Wegayehu *et al.*, 2016; Fan *et al.*, 2017). The reason for the absence of *C. parvum* remains unclear. The failure to detect *C. parvum* in preweaned calves in Guangdong Province suggests that the dairy cattle in this province have low zoonotic potential for the transmission of *Cryptosporidium* to humans. However, larger sample of dairy cattle from this province should be analysed by PCR to confirm the findings of the present study.

To date, 21 MLST subtypes have been identified in *C. andersoni* isolates from animals, 17 of which occur in cattle (Feng *et al.*, 2011; Wang *et al.*, 2012; Zhao *et al.*, 2013; Qi *et al.*, 2016). In the present study, the MLST subtype A4,A4,A4,A1 was the most prevalent subtype in dairy cattle in Guangdong, which was in agreement with the finding in Heilongjiang and other areas of China (Wang *et al.*, 2012; Zhao *et al.*, 2014), whereas subtype A2,A4,A2,A1 was predominant in dairy cattle in Xinjiang and A1,A4,A4,A1 in Shaanxi (Zhao *et al.*, 2013; Qi *et al.*, 2016). These differences may be related to the number of samples examined and geographic segregation. MLST subtype A2,A5,A2,A1 was found for the first time in cattle in this study, which was also identified in sheep, and further suggested that *C. andersoni* might circulate between cattle and sheep (Wang *et al.*, 2012).

In this study, the samples successfully amplified at all four loci were included in the LD analysis, which showed that *C. andersoni* isolated from dairy cattle in Guangdong Province had a clonal genetic population structure. The result differed from previous findings that *C. andersoni* population in cattle from Xinjiang and other geographical regions of China had an epidemic genetic structure (Wang *et al.*, 2012; Qi *et al.*, 2016). However, it was consistent with *C. andersoni* isolates in cattle from Shaanxi and Heilongjing Provinces (Zhao *et al.*, 2013; Zhao *et al.*, 2014). A clonal genetic population structure indicated that the prevalence of *C. andersoni* in cattle in Guangdong Province was not attributable to the introduction of cattle.

In conclusion, *Cryptosporidium* is common in dairy cattle in Guangdong province. Sequence analysis revealed the presence of *C. andersoni*, *C. bovis* and *C. ryanae* infection, with *C. andersoni* as the most prevalent species. Three MLST subtypes of *C. andersoni* were identified, and subtype A2,A5,A2,A1 was found for the first time in cattle. *Cryptosporidium andersoni* in dairy cattle in Guangdong presented a clonal genetic structure. The findings in this study provided valuable basic data for developing strategies and measures to control *Cryptosporidium* infection in dairy cattle and evaluate the risk of *Cryptosporidium* infection to humans.

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Conflict of interest. None.

Ethical standards. This study was performed in accordance with the Chinese Laboratory Animal Administration Act of 1988. Before the experiments, the protocol of the study was reviewed and approved by the Research Ethics Committee of Henan Agricultural University. All the fecal samples were collected from animals with the permission of the farm owners.

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