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Research paper

Longitudinal monitoring of *Cryptosporidium* species in pre-weaned dairy calves on five farms in Shanghai, China

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ABSTRACT

In pre-weaned dairy calves, the zoonotic and pathogenic species *Cryptosporidium parvum* is the dominant *Cryptosporidium* species in most industrialized nations. In several studies in China, however, *C. bovis* has been the dominant one. To further examine the distribution of *Cryptosporidium* species in pre-weaned dairy calves in China, 818 fecal specimens were collected from five farms in Shanghai, with repeated samplings (up to five times) on each farm. PCR-restriction fragment length polymorphism (RFLP) analysis of the small subunit rRNA gene was used to detect and genotype *Cryptosporidium* spp. *Cryptosporidium parvum* was subtyped by sequence analysis of the 60 kDa glycoprotein gene. *Cryptosporidium* occurrence on farms varied between 25.0% (Farm 2) and 55.0% (Farm 4), with a mean infection rate of 37.0%. Three *Cryptosporidium* species were detected, including *C. bovis* (193/303 or 63.7%), *C. parvum* (72/303 or 23.8%) and *C. ryanae* (32/303 or 10.6%). Concurrent infection of *C. bovis* and *C. ryanae* was detected in six (1.9%) animals. During the first two samplings, *C. bovis* was the dominant species on four farms and *C. parvum* was detected on only one farm (Farm 1). One of the study farms (Farm 3) started to have *C. parvum* at the third sampling. *C. parvum* was associated with the occurrence of moderate or watery diarrhea, while *C. bovis* was not. All *C. parvum* were subtype IIdA19G1, which is dominant in China but rare elsewhere. Genotyping and subtyping results indicated that the introduction of *C. parvum* to Farm 3 was caused by brief housing of several bull calves from another farm. Data from the study suggest that *C. parvum* is still uncommon in pre-weaned dairy calves in China and measures should be developed to prevent its spread in the country.

1. Introduction

Cryptosporidium spp. are important apicomplexan parasites, causing moderate to severe diarrhea in a wide variety of vertebrates, including humans (Checkley et al., 2015). These species are transmitted by the fecal-oral route. As *Cryptosporidium* spp. are common in domestic animals, these animals, especially calves, are considered major reservoir hosts. Many outbreaks of cryptosporidiosis due to calf contact have been reported in humans in industrialized nations (Xiao, 2010).

To date, more than 30 *Cryptosporidium* species are recognized (Ryan and Hijjawi, 2015). Among them, *C. parvum*, *C. bovis, C. ryanae* and *C. andersoni* are four common species in cattle (Xiao, 2010), although *C. parvum* is the only zoonotic species. *Cryptosporidium parvum* is also the only one considered to cause diarrhea (Santin, 2013), although *C. andersoni* is associated with poor weight gain and reduced milk yield (Esteban and Anderson, 1995). The distribution of *Cryptosporidium* species in dairy cattle is age-related; in industrialized nations, *C. parvum* is the dominant species in pre-weaned dairy calves, *C. bovis* and *C. ryanae* are common in post-weaned calves and yearlings, whereas *C. andersoni* is mostly seen in adult animals (Fayer et al., 2006; Santin et al., 2008; Santin et al., 2004).

The distribution of *Cryptosporidium* spp. in pre-weaned dairy calves in China is probably different from those in industrialized nations. In this country, *C. parvum* was the dominant species in pre-weaned dairy calves in only four of the nine studies conducted thus far, with *C. bovis* as the dominant species in the remaining ones (Cui et al., 2014; Huang

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et al., 2014; Li et al., 2016; Ma et al., 2015; Qi et al., 2015b,c; Wang et al., 2011; Zhang et al., 2013, 2015). Elsewhere, *C. bovis*, as a common *Cryptosporidium* species in pre-weaned dairy calves, has been reported in only a few studies in Sweden, Egypt and Malaysia (Amer et al., 2013; Muhid et al., 2011; Silverlas et al., 2010).

Sequence analysis of the 60 kDa glycoprotein (*gp60*) gene has identified IIa subtypes of *C. parvum* in dairy calves in most industrialized nations (Xiao, 2010). In some countries, such as Sweden, Egypt and Malaysia, however, IId subtypes have been identified in concurrence with IIa subtypes in dairy calves (Amer et al., 2013; Muhid et al., 2011; Silverlas et al., 2010). Elsewhere, IId subtypes are usually found in pre-weaned lambs and goat kids, sometimes in concurrence with IIa subtypes (Diaz et al., 2015; Geurden et al., 2008; Imre et al., 2013; Taylan-Ozkan et al., 2016; Tzanidakis et al., 2014). While in China, in addition to the lower occurrence of *C. parvum* in pre-weaned dairy calves, all *C. parvum* isolates subtyped belonged to the IId family, with the following four subtypes identified: IIdA14G1, IIdA15G1, IIdA17G1 and IIdA19G1 (Cui et al., 2014; Huang et al., 2014; Li et al., 2016; Qi et al., 2015b,c; Wang et al., 2011; Zhang et al., 2013, 2015). Among them, IIdA15G1 and IIdA19G1 are the dominant ones.

To further examine the transmission of *Cryptosporidium* spp. in pre-weaned dairy calves in China, we genotyped and subtyped the pathogens in fecal specimens from five dairy cattle farms in Shanghai. Longitudinal sampling was conducted to follow the occurrence of *C. parvum* on one of the study farms.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethic Committee of the East China University of Science and Technology. The dairy calves were handled in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. Permission was obtained from owners or managers of dairy farms before specimen collections.

2.2. Specimen collection

Between April 2015 and March 2016, 818 fresh fecal specimens were collected from pre-weaned Holstein calves on five dairy cattle farms in Shanghai, China (Table 1). These farms are in two neighboring districts, Fengxian (Farms 1–4) and Jinshan (Farm 5), and are owned by the same dairy enterprise. These farms were sampled 2–5 times at 2–3 months intervals to avoid the repeated sampling of the same animal, with Farm 3 being sampled five times, Farm 1 four times, and Farms 2, 4 and 5 twice (Table 1). For each specimen, approximately 25 *g* of feces were collected directly from the rectum using disposable gloves into a 50-ml centrifuge tube. Fecal specimens were stored in 2.5% potassium dichromate at 4 °C before DNA extraction.

The five farms were assessed for hygiene status, animal density and facility condition using a score of 1–5, with 1 representing "very poor" and 5 representing "excellent". The hygiene status was mainly based on of the cleanliness of the housing stalls for the calves, with 1 as having the presence of massive fecal accumulation, and 5 as very clean. For animal density, 1 represented animal density of > 60 animals per 100 m^2 of stalls, while 5 represented animal density of < 30 animals per 100 m^2. For facility conditions, 1 represented the absence of a paddock, while 5 represented the presence of a large paddock. An overall farm quality score was calculated based on the sum score of the three parameters. The fecal consistency of the collected specimens was assigned to three categories: formed feces with no diarrhea (n = 383), loose feces with moderate diarrhea (n = 350) and liquid feces with watery diarrhea (n = 83).

2.3. DNA extraction

Approximately 200 mg of each fecal specimen was washed three times with distilled water by centrifugation at 2000 *g* for 10 min. Genomic DNA was extracted from the washed fecal material by using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA). The extracted DNA was stored at −20 °C until PCR analysis.

2.4. Cryptosporidium detection, genotyping and subtyping

Cryptosporidium spp. were detected by nested PCR analysis of a ~830-bp fragment of the small subunit (SSU) rRNA gene and identified to species using restriction fragment length polymorphism (RFLP) analysis of the PCR products with restriction enzymes *Ssp*I and *Mbo*II

Table 1
Occurrence of *Cryptosporidium* spp. and *C. parvum* subtype in pre-weaned dairy calves on five farms in Shanghai, China.

Farm	Sampling point	Sample size	No. positive for Cryptosporidium (%)	*Cryptosporidium* species				C. parvum subtype (no.)
				C. bovis	*C. ryanae*	*C. parvum*	*C. bovis* + *C. ryanae*	
1	1st	37	14 (37.8)	6	0	8	0	IIdA19G1 (8)
	2nd	12	5 (41.7)	2	0	3	0	IIdA19G1 (3)
	3rd	46	29 (63.0)	8	0	21	0	IIdA19G1 (21)
	4th	25	8 (32.0)	3	0	5	0	IIdA19G1 (3)
	Subtotal	**120**	**56 (46.7)**	**19**	**0**	**37**	**0**	**IIdA19G1 (35)**
2	1st	47	9 (19.1)	6	3	0	0	–
	2nd	9	5 (55.6)	5	0	0	0	–
	Subtotal	**56**	**14 (25.0)**	**11**	**3**	**0**	**0**	–
3	1st	116	42 (36.2)	33	8	0	1	–
	2nd	43	19 (44.2)	12	5	0	2	–
	3rd	82	30 (36.6)	11	1	18	0	IIdA19G1 (15)
	4th	85	23 (27.1)	12	1	10	0	IIdA19G1 (9)
	5th	69	19 (27.5)	10	2	7	0	IIdA19G1 (7)
	Subtotal	**395**	**133 (33.7)**	**78**	**17**	**35**	**3**	**IIdA19G1 (29)**
4	1st	30	14 (46.7)	6	8	0	0	–
	2nd	10	8 (80)	6	1	0	1	–
	Subtotal	**40**	**22 (55.0)**	**12**	**9**	**0**	**1**	–
5	1st	109	48 (44.0)	46	1	0	1	–
	2nd	98	30 (30.6)	27	2	0	1	–
	Subtotal	**207**	**78 (37.7)**	**73**	**3**	**0**	**2**	–
Total	-	**818**	**303 (37.0)**	**193**	**32**	**72**	**6**	**IIdA19G1 (66)**

The bold values refers to the subtotal in each farm.