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Short Communication

A *Cryptosporidium parvum* genotype shift between week old and two week old calves following administration of a prophylactic antiprotozoal.

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Abstract

This study looked to assess the stability of *Cryptosporidium parvum* genotypes in calves between the final day of treatment with the antiprotozoal halofuginone lactate and seven days post-treatment. Paired faecal samples were collected on the final day of treatment and seven days later from 54 calves across seven farms in South-west England. The presence of *Cryptosporidium* species was detected using polymerase chain reaction targeting the 18s rDNA. The presence and genotype of *C. parvum* was determined by PCR and amplicon sequencing targeting the gp60 locus. On farms where *C. parvum* was detected at both sampling times there was a distinct genotype shift. Detection of gp60 genotype IIaA15G2R1 decreased from 40% to 7% while IIaA17G1R1 increased from 0% to 41%, supplemented by IIaA16G3R1 in one sample. A shift in *C. parvum* genotypes present in calves within a one week sampling timeframe has not been described prior to this study, indicating that the timeframe is likely suitable for observing variation in *C. parvum* populations and interactions with antiprotozoal control strategies.

Keywords: *Cryptosporidium parvum,* halofuginone lactate, calves, genotype, gp60.

Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60

Highlights

- A single *Cryptosporidium parvum* genotype was initially dominant on multiple farms
- gp60 genotypes became more diverse within a one week sampling timeframe
- First description of a *C. parvum* genotype shift in neonate calves
- Explanatory factors considered: drug interaction and maturation of calves

1. Introduction

Cryptosporidium parvum is a zoonotic parasite prevalent among UK calves, reported to occur at levels of 45.1% in individuals under one month of age (Smith et al., 2014). Infection largely results in morbidity in juveniles, associated with diarrhoea, anorexia and dehydration, although for immunocompromised individuals further complications can arise (Taylor et al., 2007, De Graaf et al., 1999). Halofuginone lactate is licensed for control and treatment of *C. parvum* in UK cattle; however the effects of treatment on *C. parvum* population structure has not been defined.

Cryptosporidium follows a lifecycle broadly in line with other members of the suborder Eimeriorina with three key phases: sporulation, schizogony, and gametogony. Notably, sporulation occurs within the host and not externally, meaning oocysts are capable of auto-infection of the same host as well as being immediately infectious upon shedding (Taylor et al., 2007). Following the initiation of infection it takes a minimum 72 hours for progeny oocysts to be produced (the pre-patent period; (Taylor et al., 2007)); with self-limiting infections lasting around two weeks (Olson et al., 1999). The tough oocyst wall permits significant longevity in the environment. Overall, oocysts can survive in soil and faecal matter for up to 12 weeks at below 25°C, and for over 12 weeks in water (Olson et al., 1999), making effective control and biosecurity a challenge for farmers. One option for control is the use of halofuginone lactate, licensed for treatment of *Cryptosporidium* in calves for both prophylactic (prevention of diarrhoea) and therapeutic purposes (reduction of diarrhoea). It is most commonly used prophylactically and is administered for seven days orally to calves starting from 24-48 hours old (European Medicines Agency, 2016). Field and trial studies have shown efficacy in reducing *Cryptosporidium* oocyst shedding when investigating faecal presence compared to placebo treatment. Commonly, data has been collected from the final day of treatment (~ 7-9 days) and thereafter at regular intervals up to ~28 days.

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Jarvie and colleagues showed that, in the first month of life, halofuginone treated calves excreted 70% fewer oocysts than placebo treated calves ($p<0.05$) (Jarvie et al., 2005); and Lefay observed that calves after seven days of halofuginone treatment excreted 44% fewer oocysts compared to a placebo ($p<0.05$) (Lefay et al., 2001). Conversely, Almawly observed only a delay in oocyst shedding by halofuginone treated calves but no overall significant difference in total numbers or the occurrence of diarrhoea (Almawly et al., 2013). Trotz-Williams noted a difference only in overall oocyst shedding, not in the occurrence of diarrhoea or body weight gain (Trotz-Williams et al., 2011). Finally, De Waele's study noted halofuginone was more successful (reduction in oocyst excretion and diarrhoea) when high-level farm hygiene was also present (De Waele et al., 2010). Overall, halofuginone is considered to reduce oocyst shedding and mitigate against diarrhoea and weight loss.

Infection and oocyst shedding is expected to resume post-treatment (Trotz-Williams et al., 2011, Jarvie et al., 2005) but, considering Zambriski's work, it is still beneficial to treat since the onset of shedding can be delayed and the overall yield reduced (Zambriski et al., 2013). Delaying the onset of oocyst shedding can reduce environmental contamination, decrease the severity of subsequent disease and the consequential economic burden. While the influence of parasite occurrence has been considered, the impact of genetic variation within *C. parvum* populations has not been assessed. For example, the efficacy of halofuginone for treatment of *C. parvum* in calves may be influenced by underlying genetic variation and parasite population structure. *C. parvum* has historically been distinguished from other parasite species morphologically and later by zoonotic capacity. Now, the accessibility of molecular-based discrimination has enabled accurate sub-species genotyping based on markers such as the gp60 coding sequence (Chako et al., 2010). This study aimed to define variation in *C. parvum* genotype occurrence using the sampling timeframe of one to two weeks as determined in previous studies of halofuginone efficacy. The hypothesis followed that the diversity of *C. parvum* genotypes in calf faecal samples would vary between the final day of treatment with the antiprotozoal halofuginone and seven days post-treatment.

2. Materials and methods

2.1 Sampling

Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60

To evaluate temporal genetic variation in *C. parvum* paired faecal samples were required from calves. The differential excretion of *Cryptosporidium* oocysts at the final day of halofuginone treatment and seven days post-treatment has previously been used to indicate treatment efficacy, as observed in the studies referenced above. On this basis, the same sampling timeframe was adopted to assess variation in genotype occurrence. Sampling before treatment was not viable since neonatal prophylaxis commonly begins at 24/48 hours old (European Medicines Agency, 2003), while oocyst shedding begins a minimum of 72 hours after infection (the pre-patent period; (Taylor et al., 2007)).

Sampling packs were distributed to farms in Dorset, UK, between April 26th and June 1st, 2018. Sample packs consisted of: 50ml Falcon polypropylene tubes preloaded with 10ml of 2.5% (w/v) potassium dichromate to prevent bacterial proliferation and degradation of any oocysts (Olson et al., 1999), instructions, farm information questionnaire and consent form. The questionnaire was designed to identify herd size and breed, type of production system, and history of halofuginone use. Ethical review was undertaken by the Royal Veterinary College Clinical Research Ethical Review Board and approved under reference M2017 0124.

Farmers non-invasively collected approximately 20ml faeces during voiding at the final day of prophylactic halofuginone treatment and seven days post-treatment. Calf ID, age and collection date were all recorded. Samples were stored at 4 °C and then returned to the farms' veterinary practice and thence to the Royal Veterinary College (RVC) within two weeks. The questionnaire recorded the management system adopted by each farm. The questionnaire and instructions were tested by the RVC farm administrator for clarity prior to application.

2.2 Sample Processing

Faecal samples were analysed on receipt in no specific order. A modified protocol for a QIAamp Fast DNA Stool Extraction Kit (Qiagen, Hilden, Germany) was used to extract whole genomic DNA (gDNA) from *Cryptosporidium* present in faecal samples. Briefly, a faecal sample was mixed and sub-sampled for ~0.2 g solid matter, centrifuged at 10,000 *g* for 1 minute and the supernatant discarded, leaving the pellet. Each sample was combined with glass beads (0.4 - 0.6 mm; Sigma, UK) equal to 0.5× the pellet's volume and 1 ml of InhibitEX buffer (Qiagen, Hilden, Germany), and homogenised using a BeadBeater

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137 at 3,000 × oscillations/min for 1 minute. The rest of the protocol followed the manufacturer's instructions.
138 Samples were stored at -20 °C prior to PCR.

139

140 *2.3 PCR and Gel Electrophoresis*

141 Diagnostic nested PCR targeting a fragment of the *Cryptosporidium* 18S rRNA was carried out using
142 gDNA with the primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' (forward) and 5'-
143 AGGAGTAAGGAACAACCTCCA-3' (reverse) in the first round (Xiao et al., 19990, and 5'-
144 AGTGACAAGAAATAACAATACAGG-3' (forward) and 5'-CCTGCTTTAAGCACTCTAATTTTC-3'
145 (reverse) in the second (Abe et al., 2002), employing reagents and conditions as described previously
146 (Nolan et al., 2017). Subsequently, all samples that had been positive for *Cryptosporidium* in the 18S
147 rRNA assay were also subjected to PCR targeting a fragment of the 60 kDa glycoprotein (gp60) coding
148 sequence using the primers 5′-ATAGTCTCCGCTGTATTC-3′ (forward) and 5′-
149 GGAAGGAACGATGTATCT-3′ (reverse) as described previously (Nolan et al., 2017). Amplicons were
150 resolved using a 1.5% (w/v) agarose (ThermoFisher Scientific, Hemel Hempstead, UK) gel made with
151 0.5× TBE and stained with 0.01% (v/v) SafeView Nucleic Acid Stain (Novel Biological Solutions,
152 Huntingdon, UK). Electrophoresis was carried out at 40 V for 40 mins in 0.5× TBE buffer.

153

154 *2.4 Sequencing*

155 Amplicons for gp60 from all putatively *Cryptosporidium* positive samples were purified using a MinElute
156 PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. Concentrations
157 were standardised to 30 ng/µl using a spectrophotometer (DeNovix, Wilmington, USA) and then
158 sequenced using the primers employed in the original reaction by GATC Biotech (GATC Biotech,
159 Cologne, Germany). Sequences were assembled against the reference KY499051 (Genbank®) with
160 default parameters on CLC Main Workbench v6.9.1 (CLC bio, Aarhus, Denmark) and gp60 genotypes
161 annotated to confirm the presence of *C. parvum* following the conventional nomenclature (Xiao, 2010).
162 All sequences have been deposited with GenBank under the accession numbers LR594827-LR594829.

163

164 *2.5 Statistical Analysis*

165 Analysis was carried out using IBM SPSS Statistics version 25. The significance of variation in paired
166 genotype occurrence was assessed between days 7 and 14 for no genotype present or genotypes

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167 IIaA15G2R1, IIaA17G1R1 or IIaA16G3R1 using the McNemar test. Results were considered to be
168 significant when $p<0.05$. Additionally, the Kappa statistic of agreement was calculated, testing whether
169 the results were in agreement between the two sampling days.
170

171 ## 3. Results

172 The sample size comprised 54 calves from seven farms in South-west England, including between 5
173 and 10 calves per farm (Table 1). All farms were commercial dairy systems, reported a herd size of
174 over 100 head and either had Holstein or Holstein-Friesian cattle. All farms tested routinely used
175 halofuginone prophylactically and, at a minimum, had been using halofuginone for two years.
176

177 Sampling on the final day of halofuginone treatment found three of the seven farms tested to include
178 calves positive for *Cryptosporidium* based upon a positive 18S rRNA PCR (43%; Table 1). Between
179 two and seven calves were positive on each farm. Repeat sampling seven days post-treatment detected
180 *Cryptosporidium* on all farms (100%), with two to six calves positive per farm. It should be noted that
181 the PCR test was used was qualitative, and the level of *Cryptosporidium* excretion was not determined
182 per individual. Targeted sequencing of a *gp60* fragment revealed a total of three *C. parvum gp60*
183 genotypes, all of which had been described previously (Smith et al., 2014). IIaA15G2R1 was the only
184 *gp60* genotype detected at the conclusion of halofuginone treatment (Table 1). Sampling seven days
185 later identified IIaA15G2R1, IIaA17G1R1 and IIaA16G3R1 *gp60* genotypes; including four farms that
186 were host to more than one genotype.
187

188 Pairwise comparison of *Cryptosporidium gp60* genotype occurrence between sampling days 7 and 14
189 using the McNemar test was not statistically significant, likely influenced by the low sample size.
190 However, comparison using the Kappa statistic produced a Kappa value of 0.136 (standard error 0.076),
191 suggesting that the strength of association was 'poor' and indicating notable variation. By considering
192 paired samples at an individual calf level (Table 2), it was possible to differentiate persistent and
193 apparently varied infections.
194

195 ## 4. Discussion

Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60

196 This study aimed to define the occurrence of *C. parvum* genotypes immediately following seven days
197 routine halofuginone prophylaxis and one week post-treatment. The results showed that the single
198 subtype IIaA15G2R1 was initially dominant on multiple farms in Dorset, UK, and that over the short
199 experimental timeframe genotypes became identifiably more diverse with the emergence of
200 IIaA17G1R1 and IIaA16G3R1. It is not possible to conclusively determine the cause of the change in
201 genotype complexity, however likely reasons can be explored. The first consideration is that the shift
202 was a result of the drug treatment. In the absence of a functional association between gp60 and the
203 outcome of halofuginone treatment, the genotype was used as a genetic marker. A genotype change
204 during the seven days after halofuginone withdrawal might suggest greater resistance defined
205 IIaA15G2R1, and greater susceptibility IIaA17G1R1, although it is possible that drug dosing was
206 inefficient. Unfortunately, a no-treatment control was not available to this study so causality cannot be
207 concluded. Despite extensive effort through the veterinary practice associated with the study, and
208 others in the region, we were unable to identify farmers who did not routinely medicate their calves.
209 Asking farmers to stop medicating their calves was not considered on ethical grounds.

210

211 Alternatively, the change in *C. parvum* genotypes could have been associated with calf maturation
212 rather than drug treatment. This may include an age-associated infection where, as calves become
213 more immunocompetent with increasing age, they become more resistant to certain genotypes.
214 Previous studies have suggested that *C. parvum* genotypes remain stable between week old and 2-4
215 week old calves in the absence of halofuginone prophylaxis (Thomson, 2015). It is also possible that
216 one or more calves may have moved from their pens during the experimental period and become
217 exposed to *C. parvum* genotypes in other environments, although no movement was reported.
218 Irrespective of the reason for the early dominance of IIaA15G2R1; the expansion of genotype
219 complexity within a short timeframe could provide an explanation why farms experience repeated bouts
220 of *C. parvum* infections; with immunity only covering individual genotypes. Notably, multiple farms have
221 shown the same pattern of early dominance by IIaA15G2R1, followed by the emergence of
222 IIaA17G1R1. This might be linked to geo-regional clustering which is common with *C. parvum*
223 genotypes (Brook et al., 2009) and might be associated with wildlife, livestock, human and/or machinery
224 movements.

225

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5. Conclusion

This study indicates that the collection of paired samples from one and two week old calves is appropriate to detect variation in *C. parvum* genotypes with relevance to the assessment of prophylactic drug efficacy. A distinct genotype shift was recorded in neonatal calves for the first time, with possible drivers including drug resistance or host maturation. Evidence for geo-regional clustering and repeat bouts of infection have also been considered. Further research is needed to analyse genotype association with the outcome of halofuginone treatment in a larger sample set, including a broader genome wide genetic analysis with additional sampling points during and after treatment.

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Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60

304 Tables

305 **Table 1.** Summary of *Cryptosporidium parvum* genotypes detected in one and two week old calves.

306 The numbers in brackets indicate the number of calf samples submitted per farm.

gp60 genotype	Farm	Number of calves positive for *C. parvum* (total calves)	
		Final day of treatment	7 days post-treatment
IIaA15G2R1	A	0 (8)	2(7)
	B	3(10)	1(9)
	C	7(10)	1(10)
	D	0(6)	0(6)
	E	0(5)	2(5)
	F	0(5)	0(5)
	G	2(10)	0(10)
	Total	**12 (54)**	**5 (52)**
IIaA17G1R1	A	0(8)	0(7)
	B	0(10)	5(9)
	C	0(10)	3(10)
	D	0(6)	2(6)
	E	0(5)	2(5)
	F	0(5)	1(5)
	G	0(10)	4(10)
	Total	**0 (54)**	**16 (52)**
IIaA16G3R1	G	0(10)	1(10)
	Total	**0 (54)**	**1 (52)**

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308

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Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60

310 **Table 2.** *gp60* genotypes for calves found to be *Cryptosporidium* positive at the conclusion of

311 halofuginone treatment and seven days later. Calves positive at a single time point are not shown.

Farm	Calf ID	Final day of treatment	7 days post-treatment
B	2.7	IIaA15G2R1	IIaA17G1R1
B	2.8	IIaA15G2R1	IIaA15G2R1
C	3.2	IIaA15G2R1	IIaA15G2R1
C	3.4	IIaA15G2R1	IIaA17G1R1
C	3.8	IIaA15G2R1	IIaA17G1R1
G	7.9	IIaA15G2R1	IIaA17G1R1

312

Abbreviations: Halofuginone lactate: Halofuginone, 60 kDa glycoprotein: gp60