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PROTOZOOLOGY - ORIGINAL PAPER

Distribution of *Cryptosporidium parvum gp60* subtypes in calf herds of Saxony, Germany

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Abstract

Cryptosporidiosis is a common protozoan parasitic infection that causes diarrhoea in neonatal calves. The high shedding of environmentally resistant oocysts facilitates outbreaks of cryptosporidiosis in humans. In total, 58 farms (512 calves) in Germany (Saxony and Brandenburg) were visited three times each. Faecal samples of pre-weaned calves were microscopically examined for oocysts of *Cryptosporidium* spp. using Heine staining and were scored with regard to their consistency. Overall, 88.9% of calves tested microscopically positive for *Cryptosporidium* spp. in at least one sample, and the excretion of oocysts was significantly ($P < 0.01$) associated with a higher faecal score (diarrhoea). After DNA extraction from pooled farm isolates, 47 samples were successfully subtyped by sequence analysis of the 60 kDa glycoprotein gene (*gp60*). All isolates belonged to subtype family IIa. IIaA15G2R1 was the most common subtype (present on 66% of the farms), followed by IIaA16G3R1 (13%). Subtypes IIaA14G1R1, IIaA14G2R1, IIaA1612R1, IIaA16G2R1, IIaA17G1R1, IIaA17G2R1, IIaA17G4R1 and IIaA19G2R1 were found sporadically. This is the first description of *gp60* subtype IIaA17G4R1 in cattle in Germany.

Keywords *Cryptosporidium* · Calf · Germany · *gp60* subtypes

Introduction

Protozoan parasites of the genus *Cryptosporidium* infect all classes of vertebrates worldwide (Fayer and Xiao 2008). As merogony, gametogony, and sporogony are involved in the life cycle of the parasite, it was assigned to the subclass Coccidia (Plutzer and Karanis 2009). More recently, however, *Cryptosporidium* has been classified as a gregarine parasite of the subclass Cryptogregarina (Cavalier-Smith 2014). Currently, at least 39 *Cryptosporidium* species are considered valid (Ježková et al. 2016; Ryan et al. 2016; Zahedi et al. 2017a; Čondlová et al. 2018; Kváč et al. 2018; Horčičková et al. 2018). Cattle are frequently infected with four *Cryptosporidium* spp., including *C. parvum*, *C. bovis*, *C. andersoni* and *C. ryanae*. *C. andersoni* is mostly found in adult cattle, and *C. bovis* and *C. ryanae* are the dominant species in post-weaned calves (Fayer et al. 2006; Santín et al. 2008; Xiao 2010). *C. parvum* causes the majority of infections in suckling calves, with the first 3 weeks of life representing the period with the highest infection rates (Santín et al. 2004; Kváč et al. 2006; Fayer et al. 2007; Izzo et al. 2011).

By different methods of detection, prevalence of *Cryptosporidium* reported for herds was 50% (China; Li et al. 2016), 53% (Norway; Hamnes et al. 2006), 57% (Netherlands; Bartels et al. 2010), 76% (Canada; Trotz-Williams et al. 2005) and 93% (Germany; Göhring et al. 2014). Weber et al. (2016) detected *Cryptosporidium* in 54% of the calves (Switzerland), and Cho et al. (2013) found *C. parvum* in 15% of faecal samples from the calves (USA). These prevalence data illustrates the ubiquitous distribution of this parasite. *C. parvum* oocyst excretion was repeatedly shown to be associated with diarrhoea, and the potential of the parasite to act as a primary pathogen was emphasised (Trotz-Williams et al. 2005; Cho et al. 2013; Göhring et al.

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2014). Diarrhoea is a frequent clinical problem in neonatal calves and may lead to reduced body weight gain (Windeyer et al. 2014; Shivley et al. 2018) or to a later first calving as heifer (Waltner-Toews et al. 1986). In addition to the economic losses caused by the infection and its veterinary relevance, *Cryptosporidium* also plays an important role in human medicine (Kotloff et al. 2013), as well as in environmental issues ("one health"). Domestic livestock, predominantly cattle, is a major reservoir for *C. parvum* (Robertson et al. 2002; Hunter et al. 2004; Hunter and Thompson 2005). Cattle to human transmission can occur through direct contact with the animal, as well as indirectly due to the contamination of recreational water (Robertson et al. 2002; Roy et al. 2004; Hunter et al. 2004).

Epidemiological investigations using consistent genetic markers are necessary to better explore the zoonotic potential of *Cryptosporidium*. The genomic sequence of *Cpgp40/15* appears highly polymorphic, even within the same *Cryptosporidium* species (Strong et al. 2000; Leav et al. 2002). Therefore, the sequence analysis of this gene is a frequently applied subtyping method (Khan et al. 2018). Among the nearly 20 *C. parvum* subtype families that have been described so far, subtype family IIa is mostly found in cattle, while IId is very common in sheep and goats (Xiao and Feng 2017; Feng et al. 2018).

Apart from a study by Broglia et al. (2008), little is known about the molecular characterisation of *C. parvum* in Germany. Therefore, the present study was conducted to spot subtyping of *C. parvum* isolates from calf herds in Saxony, Germany, based on the sequence analysis of the surface glycoprotein gene *gp60*.

Materials and methods

Faecal sample collection

A total of 583 calves on 61 calf farms located in Saxony ($N = 60$) and Brandenburg ($N = 1$), Germany, were included in the current investigation. To cover a wide time range of the period with the highest rates of *C. parvum* infection (pre-weaning period), each calf was visited three times at an interval of 1 week. At each visit, rectally extracted faecal samples from 5 to 13 calves per farm were collected irrespective of their clinical condition (diarrhoeic or non-diarrhoeic). At the first visit, calves were between 1 and 28 days old. The consistency of individual faecal samples was scored (faecal score (FS): 1 = firm; 2 = pasty; 3 = mushy; 4 = soupy; 5 = fluid). Diarrhoea was defined as an FS > 2. The diagnosis of cryptosporidiosis was performed microscopically at × 200 magnification using carbolfuchsin-stained faecal smears (Heine 1982). Individual samples were homogenised mechanically. Based on the average number of oocysts in 10 randomly selected fields of vision, the intensity of excretion was evaluated semi-quantitatively by a six-tier score system (excretion score (ES)) according to Rieux et al. (2013b): 0 = negative; 1 = 0.1–1 oocysts per field; 2 = 1.1–10 oocysts per field; 3 = 10.1–20 oocysts per field; 4 = 20.1–30 oocysts per field; 5 = more than 30 oocysts per field. Due to missing data, 58 farms and 512 calves were ultimately included in the statistical analyses (see Statistics).

Purification of oocysts and DNA isolation

To achieve the best quantitative yield of oocysts per farm for cell culture experiments (data not shown), positive samples originating from the same farm were pooled. The oocysts were purified according to Joachim et al. (2003a) and stored at 4 °C in phosphate buffered saline (PBS) supplemented with penicillin/streptomycin and amphotericin B until DNA extraction.

The oocyst walls were disrupted by ultrasonic treatment for 8 min on ice. DNA extraction was performed using the NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Darmstadt, Germany) according to the manufacturer's recommendations. Eluted DNA samples were stored at − 20 °C until further analysis.

Genotyping and subtyping

Amplification of the SSU-rRNA gene in order to identify *Cryptosporidium* spp. was conducted by nested PCR with primers and under the reaction conditions previously described (Xiao et al. 1999). The secondary PCR product was subjected to digestion with *SspI, MboII* and *VspI* restriction endonuclease enzyme (Thermo Fisher Scientific, Waltham, USA) per the conditions suggested by the manufacturer. The restriction digestion products were separated by electrophoresis on 1.5% agarose gels and visualised by ethidium bromide staining under UV illumination. Banding patterns were used to identify *C. parvum* in field specimens according to Feng et al. (2007).

Subtyping was done by sequence analysis of the *gp60* gene. The primers and reaction conditions of the *gp60* nested PCR were previously described by Alves et al. (2003) and Glaberman et al. (2002) in order to amplify a fragment of the *gp60* gene (approximately 920 bp) with Taq polymerase. Nested PCR products were subjected to polyethylene glycol precipitation (26% PEG4000 (AppliChem GmbH, Darmstadt, Germany), 6.6 mM $MgCl_2$, 0.6 M NaOAc, pH 0.5) and unidirectionally sequenced with the same *gp60* forward primer used for nested PCR.

To confirm the results of unidirectional sequencing, an additional PCR reaction was performed. With the aim of comparing the *Cpgp40/15* gene sequence (cgd6_1080; 975 bp) irrespective of the polymorphic region, a new primer pair was designed (forward primer: 5′ - ATGAGATTGTCGCTCATTATCGTATTACTCTCC - 3′; reverse primer: 5′ - TTACAACACGAATAAGGCTGCAAAGATTGCAAAAC - 3′) based on the published sequence of chromosome 6 (NC_006985.1) using Geneious 7.0 ® (Biomatters Ltd., Auckland, New Zealand). A Phusion High-Fidelity (HF) DNA polymerase (Thermo Scientific, Waltham, USA) was used with an annealing temperature of 56 °C. The reaction mixture contained 5 μl 5xHF Buffer, 0.5 μl NTPs, 0.5 μl forward primer (25 μM), 0.5 μl reverse primer (25 μM), 0.25 μl Phusion HF DNA Polymerase and 13.25 μl DEPC-water. PCR products were visualised by gel electrophoresis on a 2.5% agarose gel with ethidium bromide staining and were purified using a GeneJET PCR Purification Kit (Thermo Scientific).

Purified PCR products were sequenced bidirectionally ($N = 17$), applying the same primers used for Phusion PCR. If direct sequencing of Phusion PCR products was not successful, amplicons were cloned ($N = 30$) into a pJET1.2/blunt Cloning Vector with CloneJET PCR Cloning Kit (Thermo Scientific). Ligations were transformed in XL-1 Blue Competent Cells (Stratagene, La Jolla, USA). Colony PCRs were performed using sequencing primers of the CloneJET PCR Cloning Kit. One positive clone per amplicon was selected for plasmid preparation and subsequent sequencing. Plasmids were purified using a GeneJET Plasmid Miniprep Kit (Thermo Scientific) and subjected to bidirectional Sanger sequencing (Microsynth Seqlab AG, Göttingen, Germany) by the same primers used in the colony PCR.

Sequences were analysed with Geneious 7.0®. Consensus sequences were assembled from forward and reverse reads (Phusion PCR products or plasmids, respectively) and edited manually. The derived sequences were aligned to the sequence of the nested PCR. According to the number of TCA and TCG repeats in the 5′ region of the gene and the subsequent ACATCA sequence (poly-serine tract), subtypes were matched using the nomenclature of Sulaiman et al. (2005). DNA sequences from different isolates determined to be the same *gp60* subtype were compared with each other. BLAST analysis was performed using the NCBI BLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative sequences of every detected *C. parvum gp60* subtype were deposited in GenBank, and accession numbers were provided (MK034687 - MK034697, Table 2).

Statistics

Statistical analysis was conducted using SPSS statistics 22® (IBM, Armond, USA).

Data sets from animals were regarded as evaluable if FS and ES for at least two consecutive faecal samples were available. Only farms with evaluable data sets for at least five animals were considered for descriptive statistics.

Calves that shed oocysts on more than one visit were considered only once for the calculation of infection rates. For these animals, FS values recorded during the visit when the highest ES was seen were selected for calculation of the median per farm. Animals were regarded as not infected with *Cryptosporidium* spp. if faecal samples from all three visits were available for Heine staining and were diagnosed as negative. For uninfected animals, the median of the three FS values was used for data analysis.

As a consequence, data sets from 512 calves (87.8% of the initial 583 calves) in 58 herds (95.1% of the initial 61 herds) were included in the statistical evaluation.

Results

From a total of 512 calves analysed, 455 calves (88.9%) were microscopically positive for *Cryptosporidium* spp. in at least one sample. One of the three farms, which was excluded from the descriptive statistics (farm 32), was negative on all faecal samples. Data for all three visits were available only in two (out of seven) examined animals in this herd. As a consequence, in all herds included in the data analysis, oocyst excretion was microscopically diagnosed in at least one calf. The infection rate on farms ranged from 12.5 (1/8 calves, farm 52) to 100% of sampled animals. In 31 of 58 (53.5%) analysed herds, all animals shed oocysts at least once. The infection rates at individual farms, where *gp60* subtyping was successfully performed ($N = 47$), are listed in Table 1.

FS values are illustrated in Fig. 1. Although 64.9% of the *Cryptosporidium*-negative calves suffered from diarrhoea ($FS > 2$), the proportion of positive calves with diarrhoea was distinctly higher (88.4%). Group comparisons using a Mann-Whitney *U* test detected a significantly higher FS value for positive animals ($P < 0.01$). The median values of FS and ES per farm are listed in Table 1.

Cryptosporidium DNA was successfully prepared from pooled farm isolates originating from 47 of 60 microscopically positive farms (70%). PCR products were amplified from all DNA preparations by SSU-rRNA nested PCR, *gp60* nested PCR (Taq polymerase) and Phusion PCR. By RFLP analysis, the restriction patterns typical of *C. parvum* (Chalmers et al. 2002; Feng et al. 2007) were identified in all of the amplified samples. *Gp60* gene sequence analysis revealed ten subtypes, all belonging to allele family IIa. The distribution of subtypes according to their geographical origin is illustrated in Fig. 2. For seven isolates, the *gp60* subtypes of nested PCR products were not in agreement with the subtypes from the same farm derived using the Phusion PCR products. Detailed information about *gp60* subtyping for these isolates is given in Table 1.

IIaA15G2R1 was the most common subtype found in 31 of 47 farms (66%) without a clear geographical pattern of

Table 1 *Gp60* subtypes of *C. parvum* positive calf herds in Saxony and Brandenburg. The infection rate and median values (first–third quartile) of the faecal score (FS) and excretion score (ES) of infected animals per farm. Only farms are listed in which *gp60* subtyping was successfully performed ($N = 47$). The percentage of infected calves was determined by Heine staining. Score range FS: 1 = firm; 2 = pasty; 3 = mushy; 4 = soupy; 5 = fluid. Score range ES according to Rieux et al. (2013b): 0 = negative; 1 = 0.1–1 oocysts; 2 = 1.1–10 oocysts; 3 = 10.1–20 oocysts; 4 = 20.1–30 oocysts; 5 = more than 30 oocysts per field of vision

Farm ID	*gp60* subtypes	Percentage of infected calves	Faecal score	Excretion score
1	IIaA15G2R1	71.4% (5/7)	4 (2.5–4.5)	2 (2–4.5)
4	IIaA16G3R1	100.0% (10/10)	3 (3–4)	3 (2–5)
5	IIaA15G2R1	100.0% (10/10)	4 (2.75–4)	3.5 (2–5)
6	IIaA15G2R1	100.0% (9/9)	4 (3–4.5)	2 (2–3)
7	IIaA17G2R1	100.0% (10/10)	3 (3–5)	2 (2–4.25)
8	IIaA15G2R1	87.5% (7/8)	3 (3–4)	2 (2–2)
9	IIaA16G2R1	88.9% (8/9)	5 (2.25–5)	2 (1.25–2)
10	IIaA15G2R1	25.0% (2/8)	3.5 (2–)[a]	3 (2–)[b]
11	IIaA16G1R1	75.0% (9/12)	4 (3–5)	3 (2–3.5)
12	IIaA15G2R1	100.0% (11/11)	3 (2–4)	2 (2–4)
13	IIaA17G2R1	87.5% (7/8)	3 (3–4)	3 (2–3)
14	IIaA15G2R1	100.0% (7/7)	3 (3–4)	2 (2–2)
15	IIaA15G2R1	100.0% (9/9)	3 (3–3)	3 (2–5)
16	IIaA16G1R1	100.0% (8/8)	3 (3–4)	4.5 (2.25–5)
17	IIaA15G2R1	88.9% (8/9)	3.5 (3–4.75)	2 (1.25–2)
18	IIaA17G4R1	100.0% (10/10)	4 (3.75–5)	2 (2–3.25)
19	IIaA15G2R1	100.0% (10/10)	3 (2–3)	4 (2.75–5)
21	IIaA19G2R1	90.0% (9/10)	3 (2.5–4.5)	2 (1.5–5)
22	IIaA16G3R1, IIa A15G2R1[c]	100.0% (10/10)	4 (3–4)	2 (2–3)
23	IIaA15G2R1, IIaA14G2R1[c]	100.0% (10/10)	3 (3–4)	3 (1.75–3.25)
24	IIaA15G2R1, IIaA14G2R1[c]	100.0% (10/10)	3 (2–5)	2.5 (2–4.25)
25	IIaA15G2R1	100.0% (10/10)	3.5 (2.75–4.25)	2 (1.75–3)
26	IIaA15G2R1	90.0% (9/10)	4 (3.5–5)	2 (2–3)
28	IIaA15G2R1	100.0% (10/10)	3 (2–4)	5 (2.75–5)
29	IIaA15G2R1, IIaA14G1R1[c]	100.0% (9/9)	3 (2.5–3)	3 (2–4)
31	IIaA16G3R1, IIaA17G2R1[c]	100.0% (10/10)	3 (3–4)	2 (1–3.5)
34	IIaA15G2R1	90.0% (9/10)	3 (3–3.5)	2 (1.5–2.5)
35	IIaA16G2R1	100.0% (10/10)	3 (3–4)	2 (1.75–2)
36	IIaA15G2R1	100.0% (7/7)	4 (3–5)	3 (2–5)
37	IIaA15G2R1	77.8% (7/9)	3 (3–3)	2 (1–2)
38	IIaA15G2R1	90.0% (9/10)	4 (3–4)	2 (1.5–4)
39	IIaA15G2R1	100.0% (10/10)	4 (3–5)	1 (1–2)
40	IIaA17G1R1	75.0% (6/8)	4 (3.75–5)	2.5 (1.75–4.25)
41	IIaA16G2R1	77.8% (7/9)	4 (3–5)	2 (2–3)
42	IIaA17G2R1, IIaA15G2R1[c]	100.0% (9/9)	4 (2–4)	2 (2–4)
43	IIaA15G2R1	100.0% (8/8)	3 (3–3.75)	2 (2–4.5)
44	IIaA15G2R1	100.0% (6/6)	3.5 (2.75–4.25)	2 (1.75–2.25)
45	IIaA15G2R1	66.7% (6/9)	3 (3–4)	1.5 (1–2)
46	IIaA15G2R1, IIaA14G2R1[c]	100.0% (6/6)	3 (2–4)	2.5 (1.75–5)
47	IIaA16G3R1	80.0% (7/9)	3 (3–4)	2 (2–2)
48	IIaA16G3R1	90.0% (9/10)	4 (3–4)	2 (2–2)
49	IIaA15G2R1	100.0% (9/9)	3 (2.5–4)	3 (1–3.5)
50	IIaA15G2R1	100.0% (6/6)	3.5 (2.75–4.25)	2 (1–3)
53	IIaA14R2R1	100.0% (10/10)	4 (3–4)	1 (1.75–3)
54	IIaA15G2R1	100.0% (8/8)	3 (3–3)	2 (1–2.75)
55	IIaA15G2R1	90.0% (9/10)	4 (3–4)	2 (1–2.5)
56	IIaA16G3R1	100.0% (10/10)	3.5 (3–4)	2 (1–3)

[a] For one of the three infected animals from farm 10, no FS value was available at the time of oocyst excretion. Therefore, no third quartile could be calculated

[b] For two of the three infected animals from farm 10, the same ES value was evaluated. Therefore, no third quartile could be calculated

[c] Subtypes in italics were identified by sequence analysis using the Phusion PCR product or plasmid

distribution. In samples from six farms (13%) located mainly in Western Saxony and one farm in Southern Brandenburg, IIaA16G3R1 was detected. Subtype IIaA17G2R1 was identified on four farms, with three of them located more or less closely to each other in the southwest of Saxony. The same detection frequency applies to subtype IIaA14G2R1. IIaA16G2R1 was found in two herds close to the border to Poland and one cohort in Southwest Saxony, and

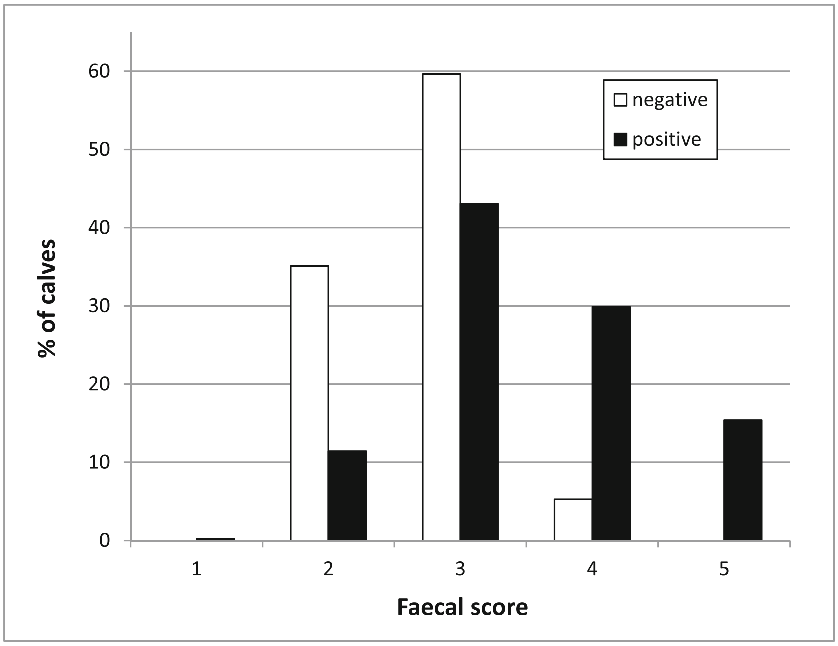

Fig. 1 Faecal score (FS) of calves that microscopically tested negative ($N = 57$) or positive ($N = 455$) for *Cryptosporidium*. The group comparison using a Mann-Whitney *U* test detected a significantly higher FS ($P < 0.01$) value for positive animals

IIaA16G1R1 was found in two herds in Central Saxony. Subtypes IIaA14G1R1, IIaA17G1R1, IIaA17G4R1 and IIaA19G2R1 were diagnosed at one location each.

Except for IIaA16G1R1 (farm isolates 11 and 16, GenBank accession numbers MK034690 and MK034691), all isolates from the same *gp60* subtype have the same nucleotide sequence. In the nucleotide sequence from the isolate from farm 11, the TCG trinucleotide is located after the second TCA trinucleotide. In the nucleotide sequence from farm 16 isolate, the TCG trinucleotide follows three TCA repeats. As representatives of their respective *gp60* subtype, nucleotide sequences of isolates originating from farm 1 (IIaA15G2R1), farm 4 (IIaA16G3R1), farm 7 (IIaA17G2R1), farm 9 (IIaA16G2R1) and farm 53 (IIaA14G2R1) were deposited in GenBank with the accession numbers MK034689, MK034693, MK034695, MK034692 and MK034688, respectively. The nucleotide sequence of isolates originating from farm 18 (IIaA17G4R1), farm 21 (IIaA19G2R1) and farm 40 (IIaA17G1R1) was submitted to GenBank with the accession numbers MK034696, MK034697 and MK034694, respectively. Subtype IIaA14G1R1 was only identified by direct sequencing of the Phusion PCR product of *C. parvum* from farm 29 (GenBank accession number MK034687). The results of the BLAST analysis are shown in Table 2. In addition to the modified order of TCA and TCG trinucleotide repeats described for farm 16 (see above), the trinucleotide

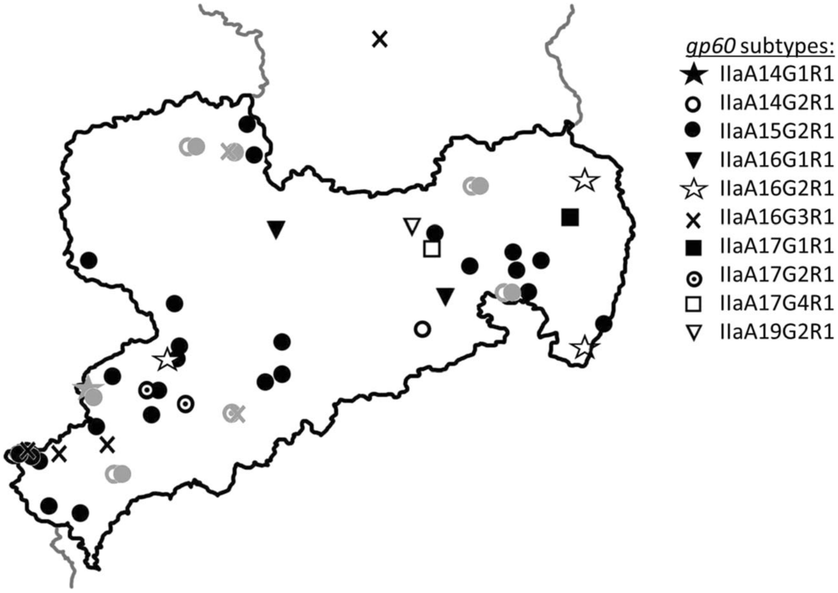

Fig. 2 Geographical distribution of *C. parvum* isolates with *gp60* subtypes in Saxony and Brandenburg detected in mixed faecal samples of up to 12 animals per farm. For seven farms, more than one subtype was identified by gene sequence analysis of *gp60* nested PCR (illustrated as two connected symbols in grey)

sequence of the farm 18 isolate (IIaA17G4R1) is also different from the previous records of this subtype (JF727757.1, JF727777.1 and JF727799). The four TCG trinucleotides in the GenBank sequences are located at position 3, 5, 6 and 7. The IIaA17G4R1 sequence obtained in the current study has TCG trinucleotides at position 3, 5, 7 and 9 (alternating with TCA). Sequences generated from the current study showed several nucleotide changes in the semi-conserved region of the gene subsequent to the poly-serine tract compared with the GenBank sequences (Table 2).

Discussion

Cryptosporidia are widespread parasites in cattle, with *C. parvum* being a major cause of neonatal calf diarrhoea (Thomson et al. 2017). Based on retrospective analysis of laboratory data, Joachim et al. (2003b) and Raue et al. (2017) reported detection frequencies of *Cryptosporidium* spp. in Germany of approximately 25% and 40%, respectively. Gillhuber et al. (2014) demonstrated a *Cryptosporidium* spp. prevalence of more than 47%, including only diarrhoeic calves younger than 6 months. Göhring et al. (2014) focused on young calves (up to 4 weeks of age) and detected *Cryptosporidium* spp. by coproantigen ELISA in 61% of the cases. However, these studies are based on the examination of non-recurrent faecal samplings and may underestimate the prevalence of *Cryptosporidium* (Thomson et al. 2017). In the current study, the infection status was calculated based on the examination of three faecal samples collected weekly from the same animal. Thus, the percentage of calves that tested positive for oocyst excretion is considerably high. An investigation of neonatal calves with an even higher frequency (e.g. daily) might reveal that all calves actually experience cryptosporidiosis at some time during their life, as demonstrated by a longitudinal study on 30 calves that were followed up to an age of 24 months (Santín et al. 2008). This appears very likely because on half of all farms, each animal excreted oocysts at least once and considering the fact that at all farms at least one animal was tested once during the observation period, which is in line with previous observations (Göhring et al. 2014).

The association between *Cryptosporidium* infection and diarrhoea was previously reported (Bartels et al. 2010; Cho et al. 2013; Díaz et al. 2018). Although the clinical status of an infected animal is influenced by various factors (e.g. the immune status of the host, farm management, infection with other enteropathogens) that were not considered in the current study, our data confirm that neonatal calf diarrhoea is significantly related to the detection of *Cryptosporidium* oocysts.

In the current work, farm isolates from a mixture of up to 12 animals were subtyped at the *gp60* locus using Sanger sequencing with two different PCR products. The first was previously described (Taha et al. 2017). For the second PCR product, a new primer pair has been designed in order to have the opportunity to detect nucleotide differences beside the poly-serine tract of the gene. In addition to the subtyping of isolates from a single round PCR instead of nested PCR approaches, the new pair of primers enabled the detection of additional nucleotide acid alterations, some of which lead to changes in the amino acid sequence of the potential protein. This was not surprising, as prominent substitutions in the non-trinucleotide repeat region of the *gp60* gene have already been shown before (Amer et al. 2010; Díaz et al. 2018).

Mixed infections of different *gp60* subtypes occur within the same subtype in cattle (Smith et al. 2014; Heckler et al.

Table 2 Results of BLAST analysis of representative *gp60* subtype sequences

gp60 subtype	Accession number	GenBank sequence	% Identity (nucleotides)	Host (specified in GenBank)	Position of substitution[b]
IIaA14G1R1	MK034687	KP997144.1	99.9%	Cattle	184
IIaA14G2R1	MK034688	JF727773.1	99.9%	Mouse	529
IIaA15G2R1	MK034689	MF142042.1	100.0%	Dairy calf	–
IIaA16G1R1, farm 11	MK034690	EF073051	99.9%	Cattle	436
IIaA16G1R1, farm16	MK034691	EF073051	99.6%	Cattle	117[a], 120[a], 513
IIaA16G2R1	MK034692	MF142044.1	100.0%	Dairy calf	–
IIaA16G3R1	MK034693	JQ362492.1	100.0%	Cattle	–
IIaA17G1R1	MK034694	EF073050.1	100.0%	Cattle	–
IIaA17G2R1	MK034695	MG516783.1	100.0%	Cattle	–
IIaA17G4R1	MK034696	JF727757.1	98.99%	Human	126[a], 135[a], 619, 776, 787, 808, 918, 933, 957, 960
IIaA19G2R1	MK034697	JQ362495.1	100.0%	Cattle	–

[a] Numbers in italics refer to positions of nucleotide acid distinctions in the poly-serine tract of the *gp60* gene

[b] Positions are described accordingly to the sequences generated in the current study (accession numbers in the second column from left)

2015; Taylan-Ozkan et al. 2016; Díaz et al. 2018). Thus, it is not surprising that in some cases more than one *gp60* subtype was identified per farm using different methods. Possibly, PCR polymerase slippage artefacts could contribute to the imparity of subtype identification. This could be the case for farm 31, since *gp60* subtypes IIaA16G3R1 and IIaA17G2R1 differ by a single nucleotide. However, if the annealing of one or two additional TCA trinucleotides is needed to switch between subtypes, polymerase slippage artefacts are very unlikely due to the stability of imperfect repeat regions (Bacon et al. 2000; Klintschar and Wiegand 2003). The sequence analysis of isolates derived from individual faecal samples could have avoided this situation, and the application of next-generation sequencing is the best-suited method to uncover the diversity of *C. parvum* within a herd and within the same host (Zahedi et al. 2017b). However, Sanger sequencing is a common tool (Khan et al. 2018) to identify the main subtype of a mixed infection more simply and expeditiously (Zahedi et al. 2017b). All *Cryptosporidium* isolates belong to the allele family IIa, which is the most common subtype of zoonotic *C. parvum* worldwide (Garcia-R et al. 2017).

In agreement with previous studies performed in Germany (Broglia et al. 2008; Göhring personal communication) and the neighbouring European countries of France (Follet et al. 2011; Rieux et al. 2013a), Belgium (Geurden et al. 2007), the Netherlands (Wielinga et al. 2008) and the Czech Republic (Kváč et al. 2011), IIaA15G2R1 was the most prevalent *gp60* subtype. This *C. parvum* subtype is widely distributed with varying frequencies in cattle (Alves et al. 2006; Trotz-Williams et al. 2006; Xiao et al. 2007; Amer et al. 2010; Imre et al. 2011; Heckler et al. 2015; Zahedi et al. 2018) and plays an important role in cases of human cryptosporidiosis in many parts of the world (Adamu et al. 2014; Valenzuela et al. 2014; McKerr et al. 2015; DeSilva et al. 2016; Hijjawi et al. 2016; Berahmat et al. 2017; Gargala et al. 2017; Naguib et al. 2018).

In 13% of farms, *C. parvum* was allocated to subtype IIaA16G3R1, which was previously reported in cattle in European countries (Rieux et al. 2013a; Smith et al. 2014; Kaupke and Rzeżutka 2015; Díaz et al. 2018) and other regions (Trotz-Williams et al. 2006; Ng et al. 2008). It is worth noting that the two most frequently detected subtypes in this study are the same as that Stensvold et al. (2015) observed in a relatively high proportion of human cryptosporidiosis cases in Denmark. This finding supports the possibility of zoonotic transmission in the investigated area.

Subtypes IIaA17G2R1 and IIaA14G2R1 were detected in four herds in this work and at similar frequencies elsewhere (Trotz-Williams et al. 2006; Broglia et al. 2008; Wielinga et al. 2008). Previous reports of *gp60* subtypes with a more limited distribution (IIa A14G1R1, IIaA16G1R1, IIaA16G2R1, IIaA17G1R1, IIaA19G2R1 in the current study) were published for Europe (Wielinga et al. 2008; Brook et al. 2009; Follet et al. 2011; Rieux et al. 2013a; Kaupke and Rzeżutka 2015), including Germany (Broglia et al. 2008). To the best of our knowledge, this is not the case for IIaA17G4R1. BLAST analysis for this subtype gave matches to sequences that were generated from *C. parvum* DNA from human and bovine faecal samples in the east of Australia (Waldron et al. 2011a, b).

Conclusion

In conclusion, this study provides new data regarding the molecular characterisation of *C. parvum* in pre-weaned calves in Saxony, Germany. We detected 100% of farms as microscopically positive for *Cryptosporidium* and a significant association between the shedding of *Cryptosporidium* oocysts and diarrhoea, emphasising the importance of the parasite in conventional calf husbandry. All reported *gp60* subtypes belong to the potentially zoonotic subtype family IIa, with IIaA15G2R1 being the most abundant subtype. To the best of our knowledge, this is the first report of subtype IIaA17G4R1 in cattle in Germany.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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