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Abstract

The presence of *Cryptosporidium* in institutions such as veterinary teaching hospitals, where students and staff are in frequent contact with animals, could represent a serious public health risk. In this study the detection and quantification of the *Cryptosporidium* oocysts present on the environmental surfaces of an Equine Perinatology Unit (EPU) were investigated. During 3 foaling seasons 175 samples obtained by swabbing an area of the floor and walls of boxes and utility rooms of EPU with sterile gauze, in 3 different moments. Samples were collected at the end of foaling season (July), after washing procedures (September) and after washing and disinfecting procedures, at the beginning of a new foaling season (December). All the samples were subjected to nested-PCR, followed by genotyping and sub-typing methods and to qPCR, allowing the oocyst quantification. *Cryptosporidium* spp. was detected in 14 samples, of which 11 were from walls and three were from floors. The highest number of oocysts was found in a sample collected from the floor of one utility room used for setting up therapies and treatments. In most cases, oocyst numbers, estimated by qPCR, were reduced or eliminated after washing and disinfecting procedures. The genotyping and sub-typing methods allowed identification of 2 subtypes of *C. parvum* (IIaA15G2R1 and IIdA23G1) and 1 of *Cryptosporidium* horse genotype (VIaA15G4) that were described in foals hospitalized at the EPU in the same years. The results of the present study show that qPCR can be used to evaluate *Cryptosporidium* contamination of environmental surfaces of a veterinary teaching hospital and the efficacy of the disinfection procedures.

Key words: *Cryptosporidium*, nested-PCR, qPCR, environmental surfaces, equine.

1. Introduction

Cryptosporidium species are common food and water-borne protozoa affecting a wide variety of domestic and wild animals as well as humans. In horses, cryptosporidiosis was first described in immunodeficient Arabian foals by Snyder et al. (1978) and subsequently reported in immunocompetent horses worldwide (Santin et al., 2013). The latter study showed that foals were more susceptible to infection by *Cryptosporidium* spp. than older animals. Fully infectious, environmentally resistant oocysts are excreted in the host's faeces. *Cryptosporidium* oocysts have shown considerable resistance against the effects of most commercial disinfectants and those with greater efficacy needed prolonged exposure times, which is not always obtainable in the field. Commercial disinfectant containing hydrogen peroxide, chlorine dioxide and ammonia appear the most effective (Fayer et al., 2008).

The ability of *Cryptosporidium* to break through water treatment barriers and to cause large-scale outbreaks has had huge impact on the water industry and its regulation, so it has been used as a reference pathogen for the faecal-orally transmitted protozoan in the design and implementation of the WHO Guidelines for drinking Water Quality (Chalmers and Katzer, 2013).

While many studies focused on detection of *Cryptosporidium* spp. in water have been performed (Castro-Hermida *et al*., 2009; Smith and Nichols, 2010; Feng *et al*., 2011; Khaldi *et al*., 2011; Nolan *et al*., 2013), only two surveys were published on the detection of *Cryptosporidium* spp. on environmental surfaces, in particular in a newborn calves herd in California (USA) (Atwill *et al*., 1998) and in a swimming pool in Sicily (Italy) (Maida *et al*., 2008).

Quantitative real-time PCR (qPCR) has proven to be a user-friendly and fast approach to detect and enumerate microorganisms in various environmental samples (Haugland et al., 2005). Many studies proposed *Cryptosporidium*-specific qPCR as a technique for detecting *Cryptosporidium* oocysts in water samples (Fontaine and Guillot, 2002; Masago et al., 2006; Staggs et al., 2013) and recently in soil and vegetables (Hong et al., 2014). In addition, qPCR has the advantage that the level of oocyst contamination can be easily quantified. Environmental contamination with *Cryptosporidium* is of

particular concern in institutions such as veterinary teaching hospital, where students, staff and animals are in close contact to each other. This represents a risk for the possible transmission to both healthy animals and humans, as already described in other studies (Anderson et al., 1982; Pohiola et al., 1986; Levine et al 1988; Reif et al., 1989; Konkle et al., 1997; Preiser et al., 2003; Gait et al., 2008; Kinross 2015).

The purpose of this study was to detect and quantify the oocysts on the environmental surfaces of the Equine Perinatology Unit "Stefano Belluzzi" (EPU), of the Department of Veterinary Medical Sciences, *Alma Mater Studiorum* -University of Bologna using nested-PCR and qPCR. In this research two molecular tools were compared and the risk associated with environmental contamination and the efficacy of cleaning and disinfection procedures were evaluated, in the building (EPU) where, in the same period, Galuppi et al. (2015) reported a prevalence of 37.8% for *Cryptosporidium* in faecal specimens collected from foals.

2. Material and Methods

2.1 Location

EPU houses 13 boxes, 5 of which are dedicated to neonatal intensive therapy. In addition, two paddocks and two utility rooms, one used for setting up therapies and treatments (A) and one for drug and medical supplies storage (B), complete the unit (Fig. 1). The floor of the unit is made of concrete. The walls of all the 13 boxes are made of plaster, 11 boxes (number: 3, 4, 5, 6, 7, 8, 9,10, 11, 12,13) present a wooden wall and 5 boxes (number: 1, 2, 3, 5,7) presented a rubber mat leaning on a wall.

At the EPU, during each foaling season (from January to July) pregnant mares and foals affected by one or more diseases were hospitalized for variable lengths of time and, at the discharge, routine disinfection procedures are performed as described by Galuppi et al. (2015). Briefly, feces are removed and straw replaced every day from the boxes; at animal discharge, the straw is completely removed and watering devices emptied; the organic debris is removed from walls and floors using a pressure washer and, once dried, the boxes are coated with 2% Steramine G solution left for 48 H;

finally Virkon-S 1% solution is sprayed and left for at least 1 h. Moreover, at the end of the foaling season, two more steps of disinfection practices are carried out. In September, the walls and floors of the boxes are cleaned with a pressure washer (about 100 °C and 100 bar); in December, after a new cleaning with a pressure washer, the dried boxes are wet with Steramine G® solution (10% quaternary ammonio compound and 2.5% nonionic surfactant) left for 48 hours; finally 1% Virkon-S® solution (21.4% potassium peroxymonosulfate and 1.50 % sodium chloride) was sprayed and left for at least one hour.

2.2 Sampling

From September 2011 to December 2013 (including 3 foaling seasons), samples were collected by rubbing a sterile gauze over an area of approximately 60×60 cm of the floor and wall of boxes and on the floor of utility rooms. Each year, sampling was performed as follow: in July (except 2011), at the end of the foaling season; in September, after washing procedure; in December, after washing and disinfecting procedure, before the beginning of the new foaling season.

One hundred seventy-five samples were collected: 70 from boxes floor, 10 from utility rooms floor and 95 from boxes wall (54 samples from plastered walls, 22 from walls covered with rubber mats and 19 from wooden walls).

2.3 Molecular analysis

Each gauze was placed into a 50 ml tube with 9 ml of PBS and left overnight. The gauze was hung up at the tube cap, outside the PBS, and then centrifuged at 900 x g for 30 min. The dry gauze was removed and the tube subject to a new centrifugation step (900 x g for 30 min) to obtain a pellet which was resuspended in 1 ml PBS, transferred in a 1.5 ml sterile microtube and centrifuged at 17,500 x g for 15 min. The obtained pellet was first subjected to 3 freeze-thawing and then to DNA extraction by NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) following the manufacturer's instruction (Galuppi et al., 2015). DNA samples were then subjected to nested-PCR, genotyping and sub-typing methods, performed by PCR-RFLP analysis and sequencing as described by Caffara

et al. (2013). Briefly, the 18S rDNA gene was amplified by nested PCR with primers C1F-C1R and C2F-C2R (Miller et al., 2006), following the PCR conditions reported by Xiao and Ryan (2008). PCR amplicons were genotyped by PCR-RFLP analysis after digestion with *SspI* and *VspI* endonucleases and restriction patterns were compared with the profiles reported by Xiao and Ryan (2008). The DNA of positive samples was subtyped by nested PCR of the 60 KDa glycoprotein (gp60) gene, using primers AL3532_f –AL3535_r (Alves et al., 2003) and AL3532_f-AL3534_r (Peng et al., 2001), following the conditions reported by Xiao and Ryan (2008). For sequencing (18S rDNA and gp60), the bands were excised, purified and sequenced with an ABI 3730 DNA analyzer at StarSEQ GmbH (Mainz, Germany). The DNA trace files were assembled by Vector NTI Advance 11 software (Invitrogen, Carlsbad,CA) and multiple sequence alignments were constructed using BioEdit.

The DNA of each sample was also subjected to qPCR assay, amplifying ~159 bp of the 18S rRNA gene specific for *Cryptosporidium* spp. using JVAF (5'-ATGACGGGTAACGGGGAAT-3'), JVAR (5'- CCAATTACAAAACCAAAAA-3') primers and JVAP18S TaqMan probe (5'- Cy5-CGCGCCTGCTGCCTTCCTTAGATG-BHQ-3') (Jothikumar et al., 2008). An Internal Positive Control (TaqMan Exogenous Internal Positive Control Reagents) was included (IPC, Life Technologies, Milan, Italy) in every reaction mixture (Malorny et al., 2004).

Briefly, all reactions consisted of 1X TaqMan® Fast Universal PCR Master Mix (2X) (Applied Biosystems, Milan, Italy); 500 nM of each primer, 100 nM of the target probe, 2 µl of IPC Mix (10X), 0.4 µl of IPC DNA (50X) and 5 µl of DNA in 20 µl total volume. The cycling conditions were 95°C for 2 min, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 20 s. All DNA samples were tested in a Peltier based real-time PCR instrument (Applied Biosystem 7500 Fast real-time PCR system, Life Technologies).

In order to establish the Limit Of Detection (LOD), the amplicons were cloned into a pEX-A plasmid vector (Eurofins MWG Operon, Ebersberg, Germany). A standard curve based on Ct values obtained from nine 10-fold serial dilutions of the plasmid in DNase- and RNase- free water were analyzed,

with an initial concentration of 5 x 10^8 plasmid copies/PCR. Three replicates of each dilution were tested. Additionally, nine 2-fold serial dilutions of the plasmid were analyzed on a range of 400-0.78 gene copies/PCR. Six replicates of each dilution were tested. The LOD was identified as the lowest concentration with all the six replicates positives.

Environmental samples were tested in duplicate. Each sample was considered positive with target Ct values ≤ 36 for both replicates and negative with target Ct values > 36 and IAC Ct values < 40 for one or both replicates.

Enumeration of *Cryptosporidium* spp. oocysts

A standard curve was used to estimate *Cryptosporidium* numbers in positive samples. To prepare the standard curve, *Cryptosporidium* oocysts were purified from the faeces of an infected horse (*C. parvum* IIdA23G1) using a salt flotation method (Elwin et al., 2001). Purified oocysts were resuspended in deionized water, homogenized, and enumerated microscopically following staining with a modified Ziehl-Neelson method. Dilutions were prepared containing approximately 50, 25, 12, 6, 3 and 1 oocyst in a total volume of 50 ul and subjected to DNA extraction using using QIAamp DNA® Stool Mini Kit (Qiagen, Valencia, CA) and qPCR using the method described earlier.

The standard curve was linear in the range of 50 to 1 oocysts DNA/sample corresponding to a range of 100 to 2 gene copies per PCR reaction, due to the presence of multiple18S rRNA gene copies in *Cryptosporidium* genome (Le Blancq et al., 1997), with R^2 of 0.98 (Fig 3).

2.4 Statistical analyses

Agreement between the two tests (nested-PCR and qPCR) was assessed by Cohen's Kappa statistic, with K values of 0.00 to 0.20 indicating slight agreement, 0.21 to 0.40 indicating fair agreement, 0.41 to 0.60 indicating moderate agreement, 0.61 to 0.80 indicating substantial agreement and 0.81 to 1.00 indicating almost perfect agreement (Landis & Koch, 1977). McNemar's test was used to assess significance of differences between the two tests, while a χ^2 test was performed to evaluate different

positive rates among sampling sites (floor *vs* wall) and among different materials covering the walls (plaster, rubber or wood). Values of $p < 0.05$ were considered statistically significant.

3. Results

Cryptosporidium spp. was detected in 14 out of 175 environmental samples. Seven positive samples were detected by both methods, 6 by qPCR only and 1 by nested-PCR only. The K Cohen test show substantial agreement (K = 0.65; 95% CL) between the two tests. No statistical difference was observed between the two tests (McNemar's $\chi^2 = 2.28$; $p = 0.065$).

In order to establish the Limit Of Detection (LOD) of the real-time PCR assay, a standard curve was prepared from 10-fold serial dilutions of a plasmid containing the target 18S rDNA sequence. Ct values showed a linear correlation (R2 of 0.9989) with a linear range of detection from 8.7 to 0.7 $\log_{10}$ gene copies/PCR (Fig 2). The PCR efficiency was 95.81%. Additionally 2-fold serial dilutions of the plasmid were tested in six replicates per dilution in the range 400-0.78 gene copies/PCR. The LOD, calculated as the lowest gene copy number for which all six PCR replicates were positive, was 1.56 and this corresponded to a mean Ct of 36.86 with a 95% CI of ±0.58 (true mean range of 36.28 to 37.44) (Table 2).

The lowest dilution (1 oocyst/sample) had a mean Ct value of 34.21 and a 95% CI of ±0.66. The range for the true mean was from 33.55 to 34.87 (Fig 3). The Ct values and the number of oocysts, ranged from 29.67 to 36 and from < 1 to 11 respectively. Out of the 161 negative samples, 159 had undetermined Ct value and two had Ct value >36. All the duplicate reactions had undetermined Ct value. For the two samples with a Ct value >36, this value was obtained in only one of the duplicate reactions. Both of these samples were negative by the nested PCR assay.

Eight out of 13 boxes (61.5%) had at least one sample that was positive for *Cryptosporidium*. Eleven out of 95 (11.6%) samples from walls were positive, while only 2 out of 70 (2.8%) samples from floor were positive. This difference was statistically significant ($\chi^2 = 4.22$; $p = 0.039$). Furthermore,

one (utility room A) out of two utility rooms was positive for *Cryptosporidium* spp. from the floor, with the lowest Ct value and the highest number of oocysts.

Data from the eight boxes and utility room that had at least one *Cryptosporidium* positive sample during the three foaling seasons are presented in Table 1.

No statistical differences were observed between the samples from plastered walls (7 positive out of 54, 13%) and rubber mats (3 out of 22, 13.6%) or wooden ones (1 out of 19, 5.3%).

PCR-RFLP analysis of the 18S rRNA gene allowed identification of *Cryptosporidium parvum* in 3 samples (box 2 in September and December 2011; box 9 in July 2013) and *Cryptosporidium* horse genotype in 4 different samples (box 6, 7, 8 in July 2012 and box 8 in September 2012). All the samples were successfully subtyped at the gp60 locus by sequencing ~800 bp of the PCR product. Among *C. parvum*, 2 subtype families have been detected: IIaA15G2R1 and IIdA23G1, whereas *Cryptosporidium* horse genotype belongs to subtype family VIa (VIaA15G4).

4. Discussion

Cryptosporidium spp. is a protozoan parasite of medical and veterinary significance and the dissemination of resistant oocysts in the environment plays an important role in the epidemiology of cryptosporidiosis (Fayer et al., 2008). In horses, cryptosporidiosis was first described in 1978 (Snyder et al., 1978) and subsequently was reported in some surveys (Gajadhar et al., 1985; Xiao and Herd, 1994; Grinberg et al., 2003; Grinberg et al., 2009; Imhasly et al., 2009; Veronesi et al., 2010; Perrucci et al., 2011; Caffara et al., 2013; Galuppi et al., 2015; Kostopoulou et al. 2015). The present research focused on *Cryptosporidium* environmental contamination of the Equine Perinatology Unit EPU where cryptosporidiosis in foals was already reported (Galuppi et al., 2015).

Out of the 175 samples tested, only 14 positives were identified. This result is one of the first indications on the low level of *Cryptosporodium* contamination in equine perinatology Unit environment; this could be due to the biosecurity measures applied to reduce the spread of infections

220 such as dedicated attire, disposable exam gloves and footbaths at every entry point to the stables, and
221 the cleaning of the boxes after every discharge/death.
222 In the boxes of this facility, a greater number of positive samples were obtained from walls than
223 floors, this could be due to the greater attention provided to cleaning of the floor of boxes, more
224 prominently contaminated by animal feces. In contrast, walls that probably are cleaned in a less
225 accurate way could represent a niche of fecal contamination.
226 No statistical differences were observed between the different wall material, even if some materials
227 can be more difficult to clean and favor the persistence of fecal debris.
228 Plastered walls are characterized by porosity, fragility and by the presence of bumps that make the
229 procedures of cleaning and disinfection more difficult, however the walls covered by rubber mats,
230 certainly easier to clean, were also positive. This could be explained by the fact that the latter were in
231 boxes housing critically ill foals often immunodeficient who shed high number of oocysts and are
232 frequently positive during the foaling seasons. The wooden walls are characterized by cracks,
233 representing a suitable place for *Cryptosporidium* oocysts. Atwill et al. (1998), in a study performed
234 for the evaluation of periparturient dairy cows and contact surfaces as a reservoir of *C. parvum*
235 infection in calves, detected *C. parvum* mainly in walls and floors of wooden calf hutches. In our
236 study, only one wooden wall sample was positive. It was collected in a box that housed a foal with
237 diarrhea, positive for *Cryptosporidium* spp. (personal communication), while the other samples from
238 wooden walls were from boxes housing asymptomatic animals, that excrete only minor number of
239 oocysts (Veronesi *et al*., 2010; Perrucci *et al*., 2011).
240 Regarding the comparison between nested-PCR and qPCR, both targeting the 18S rRNA gene for
241 *Cryptosporidium* spp., qPCR was able to detect the highest number of positive samples, however the
242 difference between the two tests was not statistically significant. De Waele et al. (2011) in a study
243 performed to compare four diagnostic tests (microscopic examination of smears stained with either
244 phenol-auramine, or fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium*

245 monoclonal antibody, nested-PCR and qPCR) for detection of *Cryptosporidium* oocysts, identifying
246 the 18S rRNA qPCR as the most sensitive and specific test.
247 Others have found that qPCR methods targeting the 18S rRNA gene perform better than those
248 targeting protein coding loci, including actin gene, for the detection of *C. parvum*/*C. hominis* in sheep
249 (Yang *et al*. 2009) and *C. parvum* in calves (Homem *et al*. 2012).
250 In our research all the samples negative by nested-PCR showed high Ct values (>33.68), which
251 equated to ≤ 1 oocyst. Yang *et al*. (2009) and Homem *et al*. (2012) similarly found that samples
252 negative by nested-PCR but positive by qPCR had high Ct values.
253 The qPCR protocol used in the present study allows the detection of DNA from less than 1 oocyst,
254 which is consistent with the 0.5 oocysts per reaction detection limit reported by Jothikumar et al.,
255 (2008). This can be explained by the presence of multiple 18S rRNA gene copies in *Cryptosporidium*
256 genome. Le Blance et al. (1997) showed that *C. parvum* has five 18S rRNA gene copies per sporozoite
257 genome, which, given that there are four sporozoites in an oocyst, translates to 20 18 S rRNA gene
258 copies per oocyst. A limitation of qPCR assays targeting the 18S rRNA gene is that they are only
259 genus specific. Species specific qPCR assays have been described, but these target single copy genes
260 and therefore have reduced sensitivity compared to assays targeting the 18S rRNA gene (Fontaine
261 and Guillot, 2002; Guy et al., 2003; De Waele et al., 2012). Moreover a study aimed to determine the
262 applicability of ten TaqMan–based qPCR assay for detecting C. *hominis*, C. *parvum* or
263 *Cryptosporidium* spp. oocysts in water matrix, revealed that genus-qPCR target 18S rRNA can detect
264 the lowest number of oocysts (Staggs et al., 2013). Therefore, even if it is not possible to define the
265 species, the use of *Cryptosporidium* spp. qPCR targeting the 18S rRNA gene allows to detect a very
266 low number of oocysts in environmental samples where the parasite load is generally low. Moreover,
267 this gene contains polymorphic regions permitting, by sequencing, the identification of new species
268 or genotypes that can cause disease in humans, like the recent identification of *C. cuniculus* or horse
269 and skunk genotypes (Robinson et al., 2008; Chalmers et al., 2011).
270 All the positive samples in nested PCR were confirmed by qPCR, except one.

Although nested-PCR was less sensitive than qPCR, RFLP analysis of the 18S rRNA nested-PCR amplicons allowed isolates to be identified to the species level and the gp60 sequencing permitted the identification of the samples under study as *C. parvum* with two different gp60 subtypes (IIaA15G2R1 and IIdA23G1) and *Cryptosporidium* horse genotype (gp60 subtype VIaA15G4).

The subtypes detected on the surfaces are the same affecting hospitalized animals during the considered time period. *C. parvum* IIaA15G2R1, which was detected in the environment at the end of the 2011foaling seasons, was also detected in foals housed at the facility during the same period (Diaz et al., 2012). Similarly, the horse genotype was detected in the environment and in foals during the 2012 foaling season (Caffara et al., 2013). *C. parvum* was the only species detected in the environment in 2013 and was also diagnosed in two foals and seven humans of the same facility during the same period (Galuppi et al., 2016)

Even if some authors reported that in humans the mean infectious dose for some isolate of *C. parvum* can be as low as 12 or as high as 2066 oocysts (Messner et al., 2001; Okhuysen and Chappel, 2002), Pereira et al. (2002) reported that a single oocyst is sufficient to cause infection and disease in an animal model. In this study, *Cryptosporidium* DNA of less than 1 oocyst was detected, testing only a small area of the floor or wall, suggesting that probably a higher number of oocysts was present in the entire box.

Even if the disinfectants used in the EPU have not always proved to be effective against *Cryptosporidium* oocysts (Ares-Mazas et al., 1997; Weir et al., 2002), quantification of oocysts by qPCR showed a probable reduction or negativity of parasite load of the environmental surfaces after washing and disinfecting procedure as observed in box 2 in 2011, in boxes 6, 8, 9, 13 in 2012 and in box 9 in 2013 (see table 1).

In 2013 boxes 1, 2 and 11 were negative in July, the end of foaling season, but were positive, even if at very low amount (Ct 36 and Ct 34.15 from floor and wall of box 1; Ct 36 from wall of box 2; Ct 35.9 from floor of box 11, with < 1 oocyst for all samples) in September after the washing procedures.

We could hypotized that the negativity observed in box 1 and 11 in July 2013 could be due to the

297 deep disinfection procedures applied in these boxes after the death/discharge of two foals, positive
298 for *Cryptosporidium* (Galuppi et al., 2016), allowing a decrease of parasitic load in the environment.
299 Since the variability of the results due to the randomness of sampling is a well-known event when the
300 contamination is low and thus not uniformly distributed on the surfaces, the low amount of oocysts
301 detected in December 2013 could be explained by the presence of minimal faeces residual. Even if
302 none of the positive foals has been hosted in box 2, the very low positivity observed could be due to
303 its close proximity to the utility room, where the highest oocysts amount has been detected.
304 The high number of oocysts found in the utility room at the entrance of box 1, the highest among all
305 samples, highlighted the importance of disinfection procedures that have to be performed with
306 attention in all the areas of EPU that could represent a source of infection.
307

308 **5. Conclusion**

309 In the present study, *Cryptosporidium* DNA was detected on environmental surfaces of an Equine
310 Perinatology Unit, suggesting that EPU could represent a potential reservoir of *Cryptosporidium*
311 oocysts that might be transmitted to humans and animals. Due to the low number of oocysts detected
312 in the environment, qPCR should be selected as a highly sensitive molecular technique. In order to
313 reduce the risk of transmission, more efficient cleaning and disinfecting procedures should be
314 considered and extended to all rooms of the EPU also those not directly hosting animals.
315

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Figure Caption

Fig.1: Planimetry of EPU

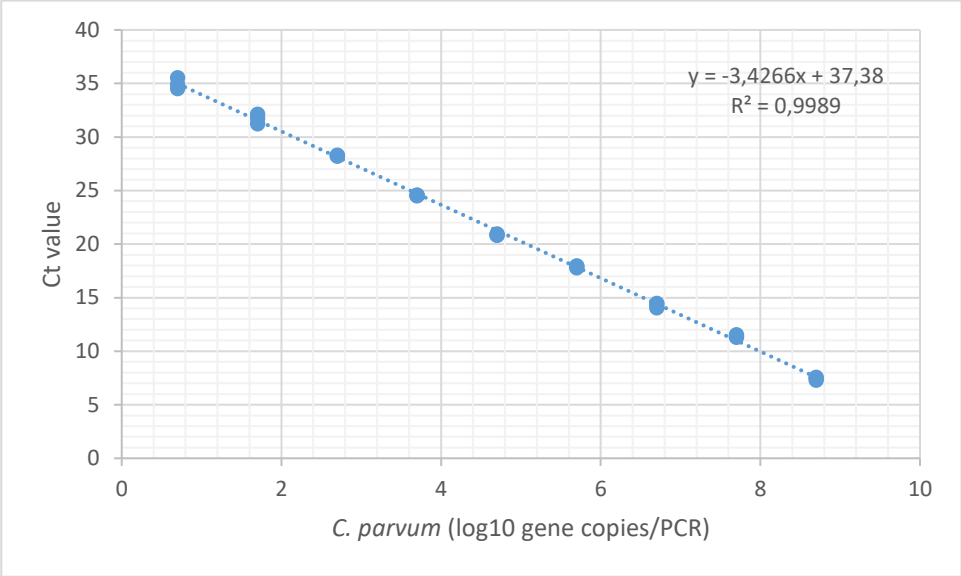

Fig 2: Standard curve based on Ct values obtained from nine 10-fold serially diluted C. parvum 18S rDNA target gene cloned in pEX-A plasmid vector starting from an initial concentration of 5 x 10^8 gene copies/PCR.

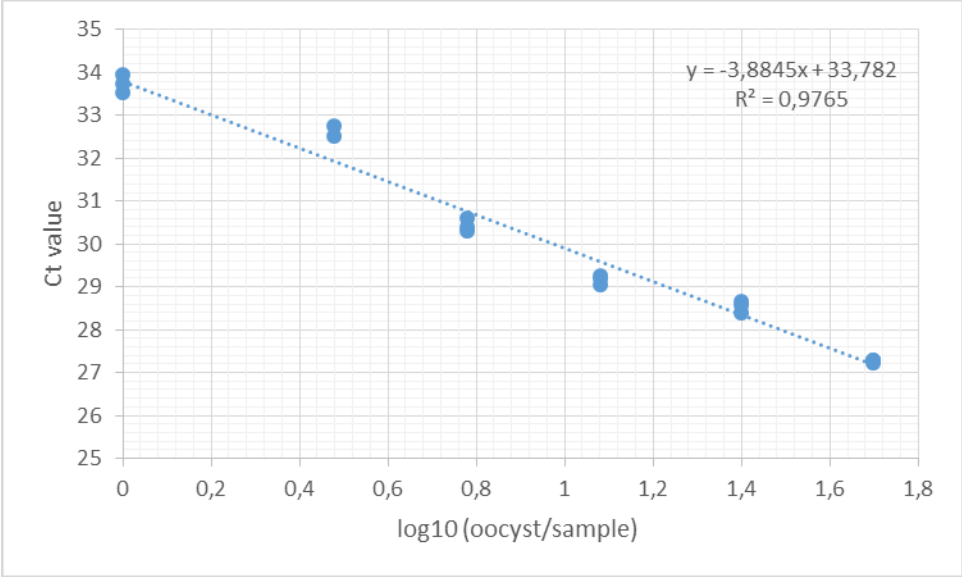

Fig 3: Standard curve based on Ct values of 50, 25, 12, 6, 3, 1 oocyst/sample (corresponding to a range of 100-2 gene copies/PCR).

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