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Widespread 5-methylcytosine in the genomes of avian Coccidia and other apicomplexan parasites detected by an ELISA-based method

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Abstract To date, little is known about cytosine methylation in the genomic DNA of apicomplexan parasites, although it has been confirmed that this important epigenetic modification exists in many lower eukaryotes, plants, and animals. In the present study, ELISA-based detection demonstrated that low levels of 5-methylcytosine (5-mC) are present in *Eimeria* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., and *Neospora caninum*. The proportions of 5-mC in genomic DNA were $0.18 \pm 0.02\%$ in *E tenella* sporulated oocysts, $0.19 \pm 0.01\%$ in *E. tenella* second-generation merozoites, $0.22 \pm 0.04\%$ in *T. gondii* tachyzoites, $0.28 \pm 0.03\%$ in *N. caninum* tachyzoites, and 0.06 ± 0.01, 0.11 ± 0.01, and $0.09 \pm 0.01\%$ in *C. andersoni*, *C. baileyi*, and *C. parvum* sporulated oocysts, respectively. In addition, we found that the percentages of 5-mC in *E. tenella* varied considerably at different life stages, with sporozoites having the highest percentage of 5-mC ($0.78 \pm 0.10\%$). Similar stage differences in 5-mC were also found in *E. maxima*, *E. necatrix*, and *E. acervulina*, the levels of 5-mC in their sporozoites being 4.3-, 1.8-, 2.5-, and 2.0-fold higher than that of sporulated oocysts, respectively ($p < 0.01$). Furthermore, a total DNA methyltransferase-like activity was detected in whole cell extracts prepared from *E. tenella* sporozoites. In conclusion, genomic DNA methylation is present in these apicomplexan parasites and may play a role in the stage conversion of *Eimeria*.

Introduction

DNA methylation, a biochemical process by which methyl groups are added to the cytosine or adenine of DNA, is one of the major epigenetic modifications involved in biological processes in mammalians, plants, insects, and fungi (Gao et al. 2012; Glastad et al. 2011; Pegoraro et al. 2016; Suzuki and Bird 2008; Yi 2012). This modification in DNA does not change the primary DNA sequence but can impact gene expression and activity in a heritable fashion (Lee et al. 2010).

Although DNA methylation is widespread in organisms, the quantity and patterns of modification appear highly variable among species, tissues/organs, and age/developmental stages (Feng et al. 2010). The percentages of 5-methylated cytosines (5mC) varies from 0 to 3% in insects, to 5% in mammals and birds, to 10% in fish and amphibians, and to more than 30% in some plants (Field et al. 2004). Notably, in a diverse group of model organisms, global DNA methylation levels have generally been found to be very low, but data have shown variable results. However, whole genome sequencing projects have repeatedly demonstrated that DNA methylation is far more widespread than one would expect based on lack of classical DNA methyltransferase (DNMT) 1 and/or 3 in these model organisms. For example, no detectable methylated cytosine was found in the embryos of *Drosophila melanogaster* in two reports (Liu et al. 2012; Raddatz et al. 2013). Other studies have indicated that methylation is most prevalent in

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embryonic stages, but is localized to specific regions, which may represent as much as 1% of the genome (Lyko et al. 2000; Takayama et al. 2014). Subsequent studies have demonstrated the presence of low levels (0.03%) of 5-methylcytosine (5-mC) in adult *D. melanogaster* (Capuano et al. 2014). In another model organism, *Aspergillus flavus*, no DNA methylation was observed using bisulfite sequencing (Liu et al. 2012; Raddatz et al. 2013), although it had been demonstrated in previous studies (Geyer et al. 2011; Gowher et al. 2001). Cytosine methylation in yeast is also equivocal because of the detection limit of bisulfite sequencing. Data for 19 genomes from 16 yeast species/strains analyzed by gas chromatography/mass spectrometry (GC/MS) showed that DNA methylation is common in these yeasts and that their genome-wide DNA methylation levels ranged from 0.01 to 0.36% (Tang et al. 2012). However, cytosine DNA methylation was never found in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and other yeast species using the liquid chromatography selective reaction monitoring (LC-SRM) method (Capuano et al. 2014).

Genomic DNA of *Caenorhabditis elegans* reportedly lacks 5-mC at any time during development or age (Simpson et al. 1986); however, 5-methyl-2′-deoxycytidine is present in *C. elegans* genomic DNA as revealed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Hu et al. 2015). In contrast, in the parasitic nematode, *Trichinella spiralis*, DNA methylation not only exists but also dramatically increases during the transition from the new born larvae to mature stages, and the suggestion is that it is a mechanism for life cycle transition in this species (Gao et al. 2012). In another important zoonotic flatworm, *Schistosoma mansoni*, which only contains the DNMT2 coding gene in its genomic DNA, and not DNMT1 or DNMT3 genes, the data on DNA cytosine methylation are considered controversial (Geyer et al. 2013; Geyer et al. 2011; Raddatz et al. 2013). Methylated DNA has been detected in the parasitic protozoans *Trypanosoma brucei* and *Entamoeba histolytica* by mass spectrometry (Fisher et al. 2004; Militello et al. 2008). Although the genomes of *Toxoplasma*, *Plasmodium*, and *Cryptosporidium* species contain DNMT2-like methyltransferases as their only candidate DNA methyltransferase gene, no detectable cytosine methylation has been discovered using LC-MS/MS (Choi et al. 2006; Gissot et al. 2008). Interestingly, a recent whole genome analysis of DNA methylation in *P. falciparum* detected 5-mC and revealed strand specificity in this parasite genome using LC-MS/MS (Ponts et al. 2013).

In this study, we characterized the existence of genomic DNA methylation in important avian pathogenic coccidia (*E. tenella*, *E. acervulina*, *E. maxima*, and *E. necatrix*) and in other common veterinary apicomplexan parasites, *Toxoplasma gondii*, several *Cryptosporidium* species, and *Neospora caninum*, using an ELISA-based detection method. Genomic DNA methylation levels in different stages of

E. tenella and the presence of a total DNA methyltransferase-like activity were also investigated.

Material and methods

Parasites

The oocysts and second-generation merozoites of *Eimeria* spp. were obtained by propagation in coccidia-free chickens in two separate experiments. Animals were reared in wire cages with heat lamps for the first 2 weeks of their life, thereafter, at 21 ± 1 °C with a 12-h light/dark cycle with free access to food and water. All experiments were performed in accordance with the animal care guidelines and approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China (No. LVRIAEC2014–001, 2014.01.03).

Chickens were infected orally at 14-day-old with a specific number of sporulated oocysts for each of the following four avian *Eimeria* species studied: *E. acervulina* (Guangdong strain, 1×10^5 sporulated oocysts), *E. maxima* (Guangdong strain, 1×10^5), *E. necatrix* (Guangxi strain, 5×10^4), and *E. tenella* (Guangdong strain, 5×10^4). Fresh oocysts of *E. tenella* were harvested 7 days (168 h) post-infection from the ceca as described by Katrib et al. (2012). Unsporulated oocysts of *E. tenella* were harvested on ice, and procedures were performed as quickly as possible. Fresh oocysts of other three *Eimeria* spp. were collected from feces. Unsporulated oocysts, partially sporulated oocysts (sporulated 7 h), and fully sporulated oocysts were used for analyses. Sporulated 7 h oocysts were incubated at 28 °C for 7 h in 2.5% potassium dichromate ($K_2Cr_2O_7$). Sporulation of oocysts was carried out at vapor-bath incubator (28 °C, 120 rpm) for 48–72 h in 2.5% $K_2Cr_2O_7$ solution until the sporulation rates reached 95% or greater. Sporulation was monitored by light microscopy using a Neubauer's hemocytometer (Shirley 1995). Oocysts (including unsporulated oocysts, sporulated 7-h oocysts, and sporulated oocysts) were further purified by isopycnic centrifugation with a using a saturated sodium chloride solution then treated with 2% sodium hypochlorite. Sporozoites were isolated using enzymatic excystation methods as previously described (Tomley 1997). The second-generation merozoites (120 h post-infection) of *E. tenella* were isolated from infected chicken ceca (Xie et al. 1992). Sporozoites and second-generation merozoites were purified using DE-52 anion-exchange chromatography (Tomley 1997).

Genomic DNA extraction

Eimeria spp. oocysts and second-generation merozoites, harvested from two separate experiments, representing two discrete biological samples, were used for preparation of

genomic DNA. The genomic DNA of *Eimeria* spp. above were extracted with the DNAzol genomic DNA Extraction Kit (Bai Taike Co. Ltd., China), according to the manufacturer's instructions. The genomic DNA from purified oocysts was homogenized mechanically using 1.4-mm glass beads with a fixed amount of DNAzol, as previously described (Blake et al. 2003; Zhao et al. 2001). Genomic DNA of sporozoites and second-generation merozoites were extracted directly using the DNAzol kit. Purified DNA was reprecipitated with isopropanol, washed with 70% ethanol, and resuspended in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA purity and concentration were determined using a spectrophotometer (AQ-07 Nucleic Acid Meter, USA) and evaluated using gel electrophoresis. Every batch of genomic DNA was divided into triplicates as replicates for experimental reproducibility.

Genomic DNA of *T. gondii* tachyzoites (RH strain), *N. caninum* tachyzoites, and *C. parvum*, *C. andersoni*, and *C. baileyi* were extracted separately using the same methods used above for the *Eimeria* spp. and provided by Prof. Xingquan Zhu's (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences), Qun Liu's (College of Veterinary Medicine, China Agricultural University), and Longxian Zhang's (College of Veterinary Medicine, Henan Agricultural University), respectively.

Methylated DNA assay

Parasite genomic DNA methylation was fluorescently detected using a commercially available ELISA-based detection method, the EpiSeeker methylated DNA Quantification Kit (Fluorometric; Abcam, USA), and used according to the manufacturer's instructions. Briefly, DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using a primary anti-5-mC antibody, followed by incubation with a secondary detection antibody, then quantified fluorometrically in relative fluorescence units (RFUs) using a fluorescence microplate reader (Molecular Devices SpectraMax M5, USA) at excitation/emission wavelengths of 530/590 nm. The absolute amount of methylated DNA was extrapolated using a standard curve prepared from the kit positive control (0 to 10 ng). A standard curve was generated at each detection time, and all controls and samples were tested in triplicate.

The control samples provided in the kit included a negative DNA control sample (ESM3), which is an unmethylated polynucleotide containing 50% cytosine, and a positive control sample (ESM4), a methylated polynucleotide containing 50% 5-mC.

Data analyses

The slope (RFU/ng) of the standard curve was determined using linear regression, and the most linear part of the standard curve was used for calculation of the optimal slope. The amount of methylated DNA is proportional to the fluorescence intensity measured; therefore, the amount and percentage of methylated DNA (5-mC) in the test samples were calculated using the following formulas:

$$5\text{-mC (ng)} = \left(\text{sample FRU} - \text{EMS3 FRU}\right) \Big/ \text{slope} \times 2^* \quad (1)$$

$$5\text{-mC}\% = 5\text{-mC amount (ng)} \times S^{-1} \times 100\% \quad (2)$$

where slope (Eq. 1) is RFU/ng on the standard curve using linear regression, S is the amount of input sample DNA in ng, 2* is a factor to normalize 5-mC in the positive control to 100% (as the positive control contains only 50% of 5-mC), and S (Eq. 2) is the amount of input sample DNA in ng. Data were expressed as means ± SEM and statistically compared by means of one-way ANOVA followed by the Bonferroni's post hoc test; probabilities of less than 5% ($p < 0.05$) were considered statistically significant.

Extraction of the whole cell proteins from *E. tenella* sporozoites

Whole sporozoite proteins were extracted according to Braun and Shirley's description (1995) with a few of modifications. In brief, the freshly isolated sporozoites were collected in a 1.5-mL tube by centrifugation at 3000 rpm for 10 min at 4 °C, and the supernatant was removed. Sporozoites were resuspended with ice cold CelLytic M Cell Lysis Reagent (10 μL 100 mM PMSF, 1 μL 2 M DTT, and 1 μL proteinase inhibitor cocktail were added per 1 mL lysis reagent) (Motohashi 2015) and incubated for 15 min on a shaker. The lysates were centrifuged for 15 min at 12,000×g to pellet cellular debris; the protein-containing supernatant was transferred to a new tube. The protein concentration of the whole cell extract was determined using the Bradford method.

Measurement of DNA methyltransferase-like activity

DNA methyltransferase activity in the whole sporozoite protein extract was measured using the EpiQuik DNA Methyltransferase Activity/Inhibition Kit (Epigentek, USA) (Geyer et al. 2011; Ponts et al. 2013), following the manufacturer's instructions. This kit measures total DNMT activity (de novo and maintenance activity) and includes a positive control and all reagents for enzymatic transfer of methyl groups (substrate and methyl donor) and detection of the methylated DNA. Briefly, the kit utilizes a unique cytosine-rich DNA substrate, which is stably coated on the strip wells. DNMT enzymes, if present in the protein extracts (the test sample), transfer a methyl group to the cytosine substrate from the

methyl donor, S-adenosyl methionine (Adomet). Methylated DNA is detected using a 5-mC specific antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, is then fluorometrically quantified using ELISA-based detection.

Results

5-Methylcytosine is present across apicomplexan parasites

In contrast to previous data (Choi et al. 2006; Gissot et al. 2008), our data show that, similar to other single-cell eukaryotes (Fisher et al. 2004; Militello et al. 2008; Ponts et al. 2013), low levels of 5-mC were detected in the genomic DNA of all the apicomplexan parasites we examined and there were significant differences between species. This ELISA-based detection method identified cytosine DNA methylation at levels of $0.18 \pm 0.02\%$ in *E. tenella* sporulated oocysts, $0.19 \pm 0.01\%$ in *E. tenella* second-generation merozoites, $0.22 \pm 0.04\%$ in *T. gondii* tachyzoites, $0.28 \pm 0.03\%$ in *N. caninum* tachyzoites, and 0.06 ± 0.01, 0.11 ± 0.01, and $0.09 \pm 0.01\%$ in *C. andersoni*, *C. baileyi*, and *C. parvum* sporulated oocysts, respectively (Fig. 1).

Methylated DNA content in *E. tenella* varies considerably by life stage

Our results also demonstrated that there are distinct dynamic changes in the 5-mC content in genomic DNA during different stages of *E. tenella*. The percentage of cytosine DNA methylation was the highest in sporozoites. In *E. tenella*, the proportion in sporozoites ($0.78 \pm 0.10\%$) was 4.3-fold higher ($p < 0.01$) than in sporulated oocysts ($0.18 \pm 0.02\%$). In
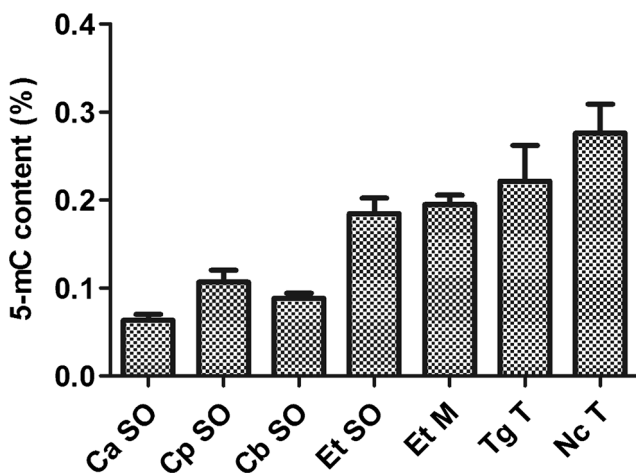
contrast, 5-mC levels in unsporulated oocysts and 7-h sporulated oocysts were only 0.11 ± 0.01 and $0.09 \pm 0.01\%$, respectively. The percentage of 5-mC in the *E. tenella* second-generation merozoites was $0.19 \pm 0.01\%$ (Fig. 2a). Moreover, 5-mC contents varied significantly between sporozoites and sporulated oocysts in three other *Eimeria* spp. as well. Specifically, 5-mC percentages in *E. maxima*, *E. necatrix*, and *E. acervulina* sporozoites were 1.8-fold (0.88 ± 0.05 vs. $0.48 \pm 0.03\%$), 2.5-fold (1.69 ± 0.19 vs. $0.67 \pm 0.06\%$), and 2.0-fold (1.62 ± 0.15 vs. $0.83 \pm 0.06\%$) greater than that in sporulated oocysts ($p < 0.01$) (Fig. 2b). The level of methylation in *E. tenella* was lowest among the four *Eimeria* spp. examined. Levels in *E. necatrix* and

Fig. 1 5-mC levels of genomic DNA in different Apicomplexan parasites. *Ca C. andersoni*, *Cb C. baileyi*, *Cp C. parvum*, *Et E. tenella*, *Tg T. gondii*, *Nc N. caninum*, *SO* sporulated oocysts, *M* second-generation merozoites, *T* tachyzoites
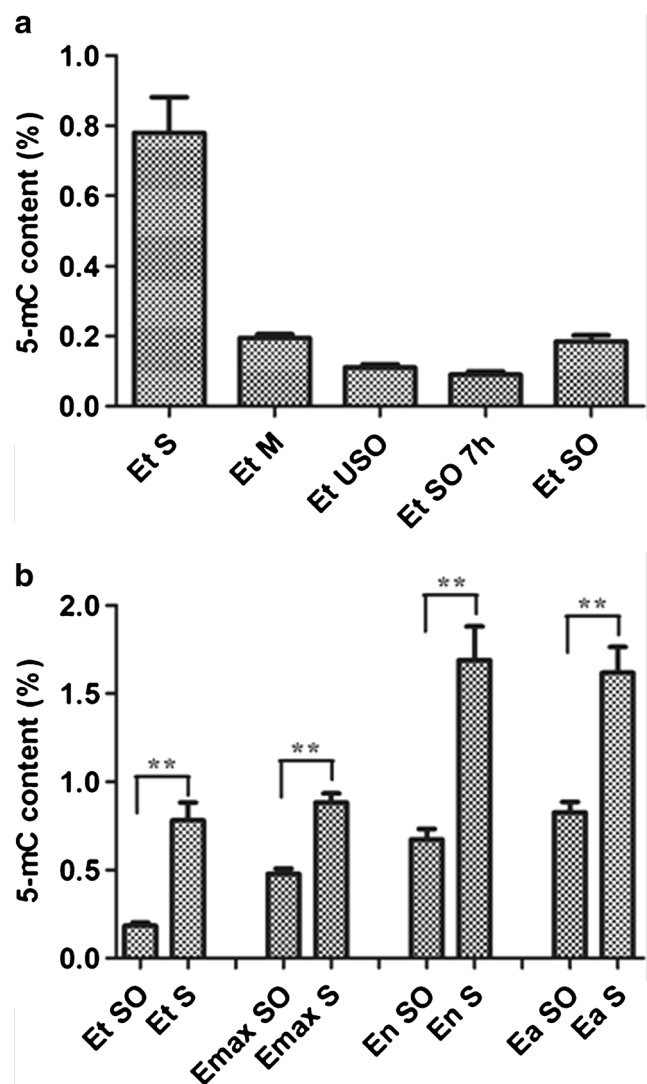
Fig. 2 5-mC contents in *Eimeria* spp. varied significantly by life stage. **a** Changes in 5-mC content of genomic DNA at different *E. tenella* stages. *S* sporozoites, *M* second-generation merozoites, *USO* unsporulated oocysts, *SO 7 h* 7-h sporulated oocysts, *SO* sporulated oocysts. **b** Changes in 5-mC content of genomic DNA between sporulated oocysts and sporozoites of *E. tenella* (Et), *E. maxima* (Emax), *E. necatrix* (En), and *E. acervulina* (Ea). **Significance at $p < 0.01$

E. acervulina were similar to each other and were higher ($p < 0.05$) than that seen in *E. tenella* and *E. maxima*.

A weak DNA methyltransferase-like activity detected in *E. tenella*

Given that 5-mC was observed in the genomic DNA of *E. tenella*, we hypothesized that there might be a measureable DNA methyltransferase-like activity in *E. tenella*. To test this hypothesis, total DNA methyltransferase-like activity was measured using an *E. tenella* sporozoite whole cell protein extract. Data showed that a weak DNA methyltransferase activity was detected and the amount of product increased with the amount of extract increased (Table 1), indicating the possible existence of active DNMTs in *E. tenella*.

Discussion

To successfully control protozoan infections using novel vaccines and anticoccidials, it is helpful to understand the mechanisms involved in developmental stage conversion and gene expression regulation in these parasites. Unfortunately, relatively few mechanistic and regulatory processes are fully understood in the Apicomplexa. The absence of large families of recognizable transcription factors typically found in other eukaryotic organisms suggests that epigenetic mechanisms may play important roles in the regulation of gene expression in apicomplexan parasites (Gissot et al. 2007; Hakimi and Deitsch 2007). Moreover, although an important epigenetic factor, little is known about DNA methylation in apicomplexan parasites. Cytosine methylation, which is a widespread and important epigenetic regulator in plants and animals, was considered once to be only a minor regulator of epigenetic processes in apicomplexans (Gissot et al. 2008). Indeed, several studies reported no detectable 5-mC in *P. falciparum*, *T. gondii*, and *C. parvum* (Choi et al. 2006; Gissot et al. 2008), but the genome-wide analysis of DNA methylation in *P. falciparum* confirmed that the genome of *P. falciparum* is indeed methylated and that the methylation levels vary in the ring, trophozoite, and schizont stages

(1.16 ± 0.11, 1.31 ± 0.04, and $0.36 \pm 0.08\%$, respectively) (Ponts et al. 2013). Consistent with the findings for *P. falciparum*, our data show that the genomes of some common pathogenic avian coccidia species and other apicomplexans are, also in fact, methylated. The genomic DNA methylation levels measured in this study represent an indirect indicator of 5-mC activity in the genome. In the future, the absolute number of modified bases could be determined using whole genome sequencing techniques, such as bisulfite sequencing.

DNA methylation levels are not only different between organisms but are also known to vary depending on tissue type, developmental stage, organismal age, and environment stress. The extent of DNA methylation may reflect changes in both intrinsic and environmental exposure (Jaenisch and Bird 2003). Many studies reveal that DNA methylation has an important role in development. Except in humans, where total genomic DNA methylation has been found to typically decrease during aging; DNA methylation levels rise as organisms age or as tissues mature in a majority of animals and plants (Bollati et al. 2009; Jaenisch and Bird 2003). For example, the 5-mC content in yeasts varied considerably at different growth stages (Tang et al. 2012), and cytosine DNA methylation was found in *D. melanogaster* adults but absent in embryos (Capuano et al. 2014; Raddatz et al. 2013). In *T. spiralis*, a dramatic increase in DNA methylation is observed during the transition from new born larvae to mature stages, indicating that changes in DNA methylation might play an important role in regulating such conversions (Gao et al. 2012). Changes in methylated DNA contents between developmental stages or tissues could indicate the involvement of epigenetic regulation that maybe important in creating phenotypic diversity in cotton (Osabe et al. 2014). Even though the life cycle of *E. tenella* is quite different from the above organisms, similar changes in genomic DNA methylation were found across its life stages, suggesting that DNA methylation may be one of the developmental and transitional mechanisms used in the life cycle of these economically important eukaryotic pathogens.

DNA methylation is established and maintained by a family of enzymes termed DNMTs, including DNMT1, DNMT2, and DNMT3 (Goll and Bestor 2005). In eukaryotes, DNMT2

Table 1 DNA methyltransferase-like activity in whole cell extracts of *E. tenella* sporozoites

Amount of extract added	RFU1	RFU2	RFU3	Activity (RFU/h[a]/mg)
Positive (1 μL)	1991.0	1733.8	–	$902.3 \pm 37.1 \times 10^3$
Blank	59.0	59.7	54.4	0
4 μg	82.6	81.1	88.3	$3.29 \pm 0.28 \times 10^3$
8 μg	220.9	203.4	151.6	$8.39 \pm 1.3 \times 10^3$
12 μg	300.2	266.9	279.3	$9.35 \pm 0.4 \times 10^3$

RFU relative fluorescence units

[a] Incubation time was 2 h

is used to methylate transfer RNAs (tRNAs) and is the most widely conserved DNMT. There are diverse groups of animal species that have retained DNMT2 as their only DNA methyltransferase. Interestingly, the DNA methylation levels are very low in these species, such as in *D. melanogaster*. Genome methylation in *D. melanogaster* does not require DNMT2 suggesting the presence of a novel DNMT (Takayama et al. 2014). In *E. histolytica*, a DNMT2-like methyltransferase, which is responsible for DNA cytosine methylation, appears to methylate both specific DNA and tRNA targets (Fisher et al. 2006; Schulz et al. 2012).

No genes encoding classical DNMT1 or DNMT3 were found in genome databases for *Eimeria* spp. (http://toxodb.org/toxo/), yet our results using the whole protein extracts of sporozoites show DNA methyltransferase-like activity and suggest the possible existence of an active DNA methylation mechanism in *E. tenella*. A single functional DNA methyltransferase belonging to the DNMT2 family has been identified in *P. falciparum* (Ponts et al. 2013). The same putative genes can also be found in the *Toxoplasma* and *Cryptosporidium* genome databases, ToxoDB and CryptoDB. In fact, when the keyword "DNA methyltransferase" was used to search these databases, the only predicted DNMT genes found for the species tested in this study encode DNMT2-like homologs; TgDNMT2 (*T. gondii*, TGME49_227660), NcDNMT2 (*N. caninum*, NCLIV_045620), EnDNMT2 (*E. necatrix*, ENH_00011710) , EaDNMT2 (*E. acervulina*, EAH_00045370), EmaDNMT2 (*E. maxima*, EMWEY_00019780), EtDNMT2 (*E. tenella*, ETH_00005900), and CpDNMT (*C. parvum*, cgd5_2100) can be found in both ToxoDB and CryptoDB. The functions of these proteins remain to be determined, and the DNA methylation patterns, as well as their regulation in gene expression, of avian *Eimeria* spp. and other apicomplexans still need to be confirmed in future research.

Different methodological approaches are currently being used for methylation analyses on a genome-wide scale. These include methods based on methylation-sensitive restriction enzymes, sodium bisulfite conversion of cytosine, and antibodies or proteins that bind to methylated DNA (Hahn and Pfeifer 2010). In this study, we employed a commercially available methylated DNA quantification kit to demonstrate the existence of 5-mC in important pathogenic avian coccidia and other apicomplexans of veterinary importance. This ELISA-based method has been widely used in other studies with other species for the detection of genomic DNA methylation (Geyer et al. 2013; Geyer et al. 2011; Lou et al. 2013; Padmanabhan et al. 2013). The kit is a highly sensitive and specific method that can detect as little as 50 pg of methylated DNA. The optimized antibody and enhancer solutions in this kit are specific for 5-mC, with no cross-reactivity to unmethylated cytosine and no or negligible cross-reactivity to hydroxylmethylcytosine within the indicated concentration range of the sample DNA. Therefore, we contend that the global DNA methylation levels that are reported in this study represent accurate percentages of genomic 5-mC. In summary, we used a highly sensitive and specific method to measure the genomic DNA methylation of cytosine in several species of veterinary apicomplexans and that DNA methylation is present in these veterinary/zoonotic protozoan parasites. The 5-mC content of *E. tenella* and other *Eimeria* spp. varied considerably between stages, suggesting a role in regulating parasite maturation. Furthermore, active DNMT-like activity was observed indicating active DMNTs, possibly a DNMT2 homolog, in *E. tenella*.

5-mC, 5-methylcytosine; Cp, *Cryptosporidium parvum*; DNMT, DNA methyltransferase; DNA, deoxyribonucleic acid; Ea, *Eimeria acervulina*; ELISA, enzyme-linked immunosorbent assay; Ema, *Eimeria maxima*; En, *Eimeria necatrix*; Et, *Eimeria tenella*; Nc, *Neospora caninum*; RFUs, relative fluorescence units; Tg, *Toxoplasma gondii*; RNA, transfer ribonucleic acid

Authors' contributions ZXG, HY, and XTM performed the experiments. BHL and ZLH analyzed the data. LQG contributed the reagents/materials. ZXG and HY wrote the paper. JPC designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Consent for publication Not applicable.

Availability of data and materials The dataset supporting the conclusions of this article is included within the article.

Conflict of interest The authors declare that they have no competing interests.

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