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Research paper

A morphological and molecular comparison of *Eimeria bovis*-like oocysts (Apicomplexa: Eimeriidae) from European bison, *Bison bonasus* L., and cattle, *Bos taurus* L., and the development of two multiplex PCR assays for their identification

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ABSTRACT

The European bison, *Bison bonasus* is the largest terrestrial mammal in Europe; it is also on the red list, being recognized as vulnerable to extinction by the International Union for Conservation of Nature. The species suffers from low genetic variability, rendering it vulnerable to various environmental and biological threats.

This study presents the first molecular confirmation of *Eimeria bovis* infection in European bison, and details a 1708 bp nucleotide sequence of the 18S rRNA gene in European bison-derived *E. bovis* (GenBank: MK691697). It also describes two multiplex PCR assays based on 18S rRNA gene for identifying *Eimeria bovis* oocysts and developmental stages in European bison and cattle. These yielded DNA banding patterns common for those of *Eimeria* spp. (250 bp for the first assay and 305 bp for the second assay) and species-specific *E. bovis* DNA in positive samples (344 bp and 586 bp, respectively). Both multiplex PCRs yielded bands characteristic of *Eimeria* spp. and *E. bovis* in samples containing DNA of oocysts from both bison and cattle. Moreover, convergent results were obtained for the DNA of the wall of colon in both assays, indicating the presence of developmental stages of *Eimeria* spp. other than *E. bovis*. Despite displaying the same sporulation time (four days), and similar general morphological features, the *E. bovis* oocysts derived from European bison were significantly narrower than those obtained from cattle ($t = -6.19$, $p < 0.001$), with a significantly higher shape index (length/width ratio) ($t = 3.94$, $p < 0.001$).

The result provides further evidence for infection of European bison with a highly-pathogenic bovine protozoan, *E. bovis*.

1. Introduction

The European bison, *Bison bonasus*, is the largest terrestrial mammal in Europe, and one recognized as being vulnerable to extinction (IUCN, 2019). In fact, its population became extinct in the wild after the First World War, and the species was later restored from 12 founders of captive individuals (Krasińska and Krasiński, 2007). Despite its restoration in the wild, the modern European bison suffers from low genetic variability, rendering it vulnerable to various environmental and biological threats, including parasitic diseases (Kita and Anusz, 2006). Hence, constant monitoring of the population health status is required, including infection with parasites (Karbowiak et al., 2014).

Previous investigations have found that the European bison hosts eleven species of bovine eimerians: *Eimeria alabamensis*, *Eimeria auburnensis*, *Eimeria bovis*, *Eimeria brasiliensis*, *Eimeria bukidnonensis*, *Eimeria canadensis*, *Eimeria cylindrica*, *Eimeria ellipsoidalis*, *Eimeria pellita*, *Eimeria subspherica*, and *Eimeria zuernii* (Pyziel et al., 2014). Of these, *E. bovis* is the most prevalent in European bison (Pyziel et al., 2014) and cattle (Klockiewicz et al., 2007) in Poland, with an overall prevalence of 29.7% and 58.6%, respectively; it is also the predominant source of eimerians oocysts in American bison (Penzhorn et al., 1994). This highly-prevalent species is also a highly-pathogenic one known to be responsible for outbreaks of clinical coccidiosis among bison calves (Chibunda et al., 1997; Pandit, 2009).

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The aim of the study was to conduct a morphological, developmental and molecular comparison of *E. bovis*–like oocysts among European bison and cattle.

2. Material and methods

2.1. Coprological study

Fecal samples were collected from 20 free-roaming European bison inhabiting the Białowieża Forest (52° 41′ N, 23° 43′ E) and from 10 individual grazing female cattle from north-west Poland (53° 46′ N, 21° 26′ E). The samples were examined using the McMaster's quantitative method (Taylor et al., 2007) in sucrose solution (SG = 1.27) (Pyziel and Demiaszkiewicz, 2013) to identify those with the highest OPGs (oocysts per gram) of *E. bovis* for further morphological and molecular investigation.

In order to study sporulation time and morphology of *E. bovis*, one volume of feces was mixed with five volumes of 2.5% dichromate solution ($K_2Cr_2O_7$) and incubated at 23 °C for 96 h (Duszynski and Wilber, 1997). The degree of sporulation was measured daily using direct flotation in sucrose flotation solution (Taylor et al., 2007). All measurements of the length and width of oocysts and sporocysts, as well as observation of their morphological features were done using an Olympus BX50 (Olympus, Hamburg, Germany) light microscope at × 1000 magnification together with the CellˆD imaging software package (Olympus, Hamburg, Germany).

Following this, the length, width and shape index (length/width ratio) of 121 oocysts from European bison were compared with those of 30 from domestic cattle; in addition, 36 sporocysts from European bison were also compared with 27 from cattle. The normality of each variable was calculated with the use of the Kolmogorov-Smirnov test, and in all cases an unpaired *t*-test was then used to compare data. All statistical analyses were performed using SPSS software ver. 24.0 (IBM Corporation, Armonk, NY).

2.2. Extraction of genomic DNA from oocysts

Sporulated oocysts were separated from fecal debris by flotation in saturated sodium chloride solution (SG = 1.20) using McMaster chambers. They were then individually picked up with a 100 µl pipette with a capillary tip under × 100 magnification. More than 30 oocysts of *E. bovis* were obtained from each host species and suspended in Molecular Biology Reagent Water (Sigma-Aldrich, USA) in microcentrifuge tubes. The oocysts were then washed several times by centrifugation at 11,000 x *g* for 1 min and sedimented.

Following this, genomic DNA was extracted using a Nucleospin Tissue DNA Extraction Kit (Macherey-Nagel, Düren, Germany) with an initial breakage of oocyst and sporocyst walls according to Hnida and Duszynski (1999) and Zhao et al. (2001), with minor modifications. In the first step, 180 µl of lysis buffer was added to approximately 30 µl of suspended oocysts; together with 25 µl of proteinase K solution and 1 mm glass beads (MP Biomedicals Europe). The tubes were then vortexed at maximum speed for 10 min, incubated overnight at 56 °C, and centrifuged to separate the glass beads. The supernatant was removed and used for further procedures according to the user manual.

2.3. Amplification and sequencing of partial SSU rDNA of E. bovisfrom wisent

Primers for the PCR amplification and sequencing of *E. bovis* from European bison (Table 1) were designed using FastPCR, version 5.4 (PrimerDigital, Helsinki, Finland) based on the base sequences of the small subunit ribosomal DNA (SSU) (Table 2) aligned with the use of GeneDoc-Multiple Sequence Alignment Editor (Nicholas et al., 1997). To amplify the *E. bovis* SSU, forward primer F2 was used together with a reverse R6 primer (Table 1). The PCR was performed in a Techne TC-512 thermal cycler (Bibby Scientific Limited, UK) in a total reaction volume of 50 µl comprising 70 mM Tris−HCl at pH 8.3, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 1 µl of each primer at 20 pM/µl, 1 unit HiFiTaq polymerase (5 units/µl) (Novazym Polska) and 1 µl of template DNA. The thermal profile involved an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 90 °C for 30 s, primer annealing at 60 °C for 180 s, and extension at 72 °C for 180 s, this was followed by prolongation of final extension for nine min. The PCR product was purified with the use of the Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany), eluted with 30 µl of Molecular Biology Reagent Water (Sigma-Aldrich, USA) and sequenced in both directions by Genomed S.A. (Warsaw, Poland). The SSU rDNA of *E. bovis* was sequenced using four forward primers and three reverse primers, including those used for amplification (Table 1). The sequences were assembled into contigs using Contig-Express software (New York, USA). The sequence alignment was performed using GeneDoc-Multiple Sequence Alignment Editor (Nicholas et al., 1997).

Table 1
Primers for PCR amplification and sequencing of *Eimeria bovis* from European bison.

Forward/Reverse	Name	Sequence
F	F2	5' – AAC CTG GTT GAT CCT GCC AGT AGT – 3'
F	F17*	5' – CAA GCA GGC TTG TCG CCC TGA A – 3'
F	F18*	5' – TGG AGG GCA AGT CTG GTG CCA – 3'
F	F22*	5' – TCA ACA CGG GGA AAC TCA CCA GGT C – 3'
R	R6	5' – GAC TTT TGC ATC CTT TAG AGG GCT – 3'
R	R17*	5' – CAC TCC ACC AAC TAA GAA CGG CCA – 3'
R	R24*	5' – ACG AAT GCC CCC AAC TGT CC – 3'

* Primers used exclusively for sequencing.

Table 2
The nuclear small subunit ribosomal DNA sequences (SSU) of *Eimeria* spp. used for alignment.

GenBank no.	Species	Host	Country	Reference
AB769587	*Eimeria bovis*	*Bos taurus*	Japan	Unpublished
AF291427	*E. alabamensis*	*Bos taurus*	Australia	Ellis et al., 2000
U77084	*E. bovis*	*Bos taurus*	Canada	Barta et al., 1997
U67115	*E. acervulina*	*Gallus gallus*	Canada	Barta et al., 1997
U67116	*E. brunetti*	*Gallus gallus*	Canada	Barta et al., 1997
U67117	*E. maxima*	*Gallus gallus*	Canada	Barta et al., 1997
U67118	*E. mutis*	*Gallus gallus*	Canada	Barta et al., 1997
U67119	*E. necatrix*	*Gallus gallus*	Canada	Barta et al., 1997
U67120	*E. praecox*	*Gallus gallus*	Canada	Barta et al., 1997
U67121	*E. tenella*	*Gallus gallus*	Canada	Barta et al., 1997
U76748	*E. mivati*	*Gallus gallus*	Canada	Barta et al., 1997

2.4. Development of two multiplex PCR assays

Based on the alignment of the *Eimeria* spp. SSU sequences (Table 2), two individual multiplex PCR assays were developed. Both multiplex PCR assays were intended to detect *E. bovis* and *Eimeria* spp. in faecal oocysts of European bison and cattle, as well as in the heart muscle tissue (negative control) and the colon wall (detection of developmental stages of *Eimeria* spp.) of European bison. The DNA of the heart muscle tissue and of the colon wall was extracted using Nucleospin Tissue DNA Extraction Kit (Macherey-Nagel) according to the manufacturer's instructions.

Each assay was performed with two forward primers (*E. bovis* specific primer and genus *Eimeria* specific primer) combined with one common reverse primer (genus *Eimeria* specific primer) (Table 3). The expected size of the *E. bovis*-positive and *Eimeria* spp.-positive fragments were 344 bp and 250 bp for the first assay and 586 bp and 305 bp for the second assay (Fig. 1).

Both multiplex PCRs were performed in a Techne TC-512 thermal

Table 3
Primers designed for two multiplex PCR assays.

TEST	PRIMERS		
	Forward/Reverse	Name	Sequence
Multiplex PCR no. 1	F	F5	5' – TTT CGA CGG TAG GGT ATT GGC CT – 3'
	F	F4	5' – CCA CTT CTG TGG AGT CCT GGT – 3'
	R	R45	5' – CTG GCA CCA GAC TTG CCC TCC A – 3'
Multiplex PCR no. 2	F	F19	5' – GAA GAC GAT TAG ATA CCG TCG T – 3'
	F	F14	5' – GCG CTT AAT TGC GTG TAC TGT G – 3'
	R	R17	5' – CAC TCC ACC AAC TAA GAA CGG CCA – 3'

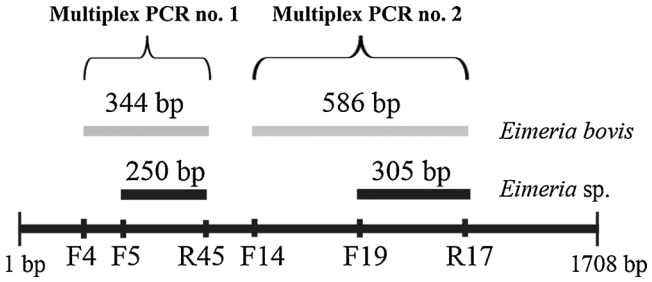

Fig. 1. Expected products of two multiplex PCR assays assigned within SSU (small subunit) rDNA of the genus *Eimeria*. *Eimeria bovis* – specific forward primers: F4 (assay no. 1) and F14 (test no. 2); *Eimeria* spp. forward primers: F5 (assay no. 1) and F19 (assay no. 2); *Eimeria* spp. reverse primers: R45 (assay no. 1) and R17 (assay no. 2). The sequences of the primers are presented in Table 3. Black rectangles: *Eimeria* genus-specific products; grey rectangles: *E. bovis* species-specific products.

cycler (Bibby Scientific Limited, UK) in a total volume of 25 µl. Each reaction mixture contained 70 mM Tris – HCl at pH 8.3, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 4 µl of DNA template, 1.5 unit HiFiTaq polymerase (5 units/µl) and set of primers at 20 pM/µl: 1 µl F4, 0.8 µl F5, 1.4 µl R45 (multiplex PCR no. 1) or 1 µl F14, 0.6 µl F19, 1.3 µl R17 (multiplex PCR no. 2). The reactions were run with the following parameters: an initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 40 s, and extension at 72 °C for 45 s with a prolongation of the final extension for 10 min. The amplicons were verified on 2% agarose gels containing ethidium bromide (0.5 µg/ml), with a Nova 100 bp DNA Ladder (Novazym) loaded as a reference on the gels. The results of electrophoresis were analysed by exposure to UV light from a Gel Logic 200 Imaging System (Kodak) equipped with Kodak 1D ver. 3.6 software.

3. Results

3.1. Morphology and sporulation of oocysts

Sporulation of the *E. bovis*-like morphotype oocysts from European bison and cattle took four days. In both cases, the oocysts were ovoid and pale brown in colour; all presented a bilayer wall which was entirely smooth with a distinct micropyle at the narrow end (Fig. 2). The Kolmogorov-Smirnov test found 11 of the 12 groups to have a normal distribution. Although oocysts from European bison were found to have a non-normal distribution, this was nonetheless very close to normality, and as the *T*-test shows great robustness to non-normality in large sample sizes (Ferenci and Kotosz, 2009) no non-parametric test was required.

Despite being generally very similar, the oocysts shed by the European bison were significantly narrower (t = -6.19, p < 0.001) (Table 4). As no statistical difference was observed with regard to the length (t = -0.94, p = 0.347), the European bison oocysts displayed a significantly higher shape index (length/width ratio) (t = 3.94, p < 0.001). In addition, no statistically significant difference was found between European bison and cattle *E. bovis* with regard to the size of the sporocysts (Table 4).

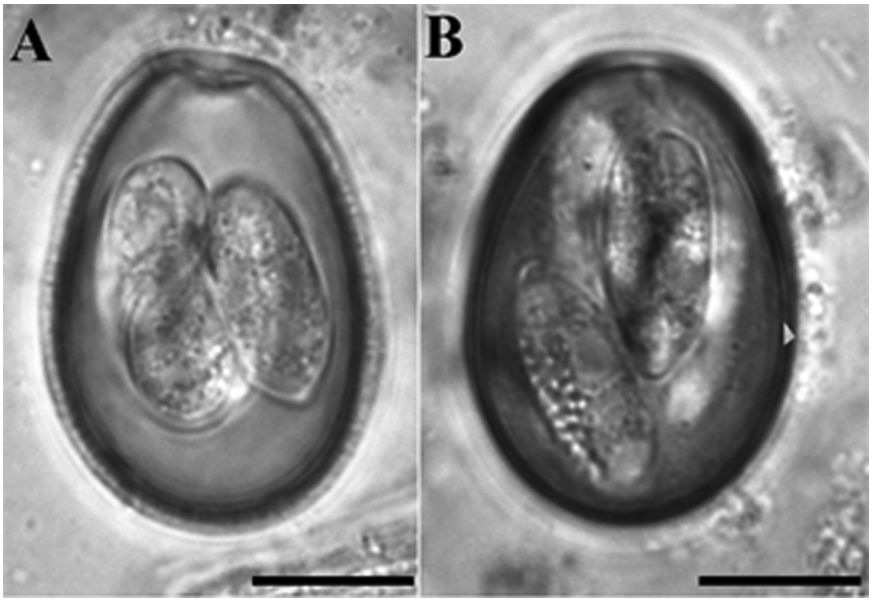

Fig. 2. Sporulated oocysts of *Eimeria bovis* (× 1000 magnification). **(A)** Oocysts derived from European bison. **(B)** Oocysts derived from cattle. Bar - 10 µm.

Table 4
Dimensions (in μm) of oocysts and sporocysts of *Eimeria bovis* from European bison and cattle with the results of their statistical analysis; mean value (*X*), standard deviation (*SD*) and range values are provided.

Host	Feature (μm)	Oocysts European bison (n = 121) + cattle (n = 31)			Sporocysts European bison (n = 36) + cattle (n = 27)		
		Length	Width	Shape index	Length	Width	Shape index
European bison (*Bison bonasus*)	*X* ± *SD*	28.83 ± 1.56	19.48 ± 1.01	1.48 ± 0.10	15.00 ± 0.51	6.84 ± 0.37	2.20 ± 0.11
	Range	25.52-32.79	17.58-22.88	1.27-1.77	14.18-15.97	6.31-7.94	1.87-2.45
Cattle (*Bos taurus*)	*X* ± *SD*	29.12 ± 1.20	20.75 ± 0.96	1.41 ± 0.07	15.19 ± 0.51	6.97 ± 0.39	2.18 ± 0.09
	Range	26.45-31.98	18.54-22.69	1.26-1.56	14.22-15.95	6.29-7.71	2.04-2.38
European bison vs.cattle	*T-value*	−0.94	−6.19	3.94	−1.41	−1.33	−0.56
	P-value	0.347	< 0.001	< 0.001	0.164	0.187	0.559

Sample size (n), mean (X), standard deviation (*SD*), *T*-test (*T*-value); probability (*P*-value).

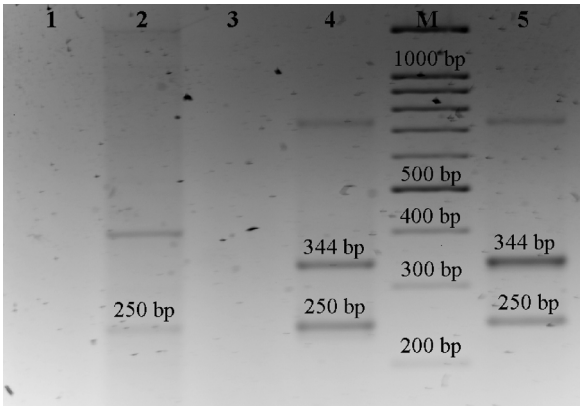

Fig. 3. Result of the multiplex PCR assay no. 1. M: Nova 100 bp DNA Ladder (Novazym); lane 1: European bison (*Bison bonasus*) heart muscle tissue - negative control; lane 2: European bison colon wall tissue; lane 3: Molecular Biology Reagent Water (Sigma-Aldrich, USA) - negative control; lane 4: *E. bovis* oocysts of European bison; lane 5: *E. bovis* oocysts of cattle (*Bos taurus*). *Eimeria* genus-specific product: ca. 340 bp; *E. bovis* species-specific product: ca. 250 bp.

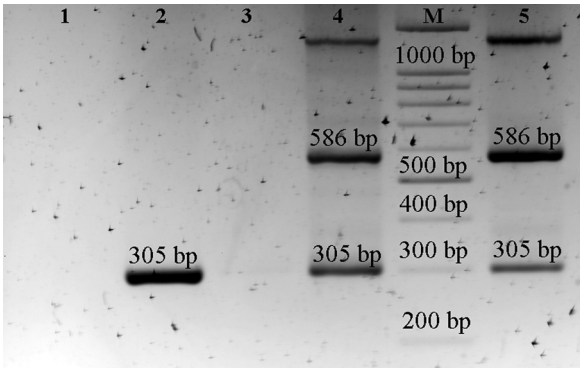

Fig. 4. Results of the multiplex PCR assay no. 2. M: Nova 100 bp DNA Ladder (Novazym); line 1: European bison (*Bison bonasus*) heart muscle tissue - negative control; lane 2: European bison colon wall tissue; lane 3: Molecular Biology Reagent Water (Sigma-Aldrich, USA) - negative control; lane 4: *E. bovis* oocysts of European bison; lane 5: *E. bovis* oocysts of cattle (*Bos taurus*). *Eimeria* genus-specific product: ca. 300 bp; *E. bovis* species-specific product: ca. 586 bp.

3.2. Sequence analysis and the multiplex PCR assays

A 1,708 bp long sequence of the SSU rRNA gene of *E. bovis*-like morphotype was obtained from European bison (GenBank: MK691697). The BLAST search (https://blast.ncbi.nlm.nih.gov/) found the homology between our sequence and the cattle-derived sequences of *E. bovis* to range from 98.8% to 99.9% (GenBank: U77084, AB769587, respectively).

Both multiplex PCR assays gave a similar negative outcome for the heart muscle-derived samples and Molecular Biology Reagent Water (Sigma-Aldrich, USA) (Figs. 3 and 4). Additionally, both the samples of the colon and the oocysts from European bison and cattle were found to be positive for *Eimeria* genus products. Moreover, only the samples containing the DNA of oocysts from European bison and cattle were found to display *E. bovis*-specific sequences in both multiplex PCRs. Additionally, extra products were obtained in the first multiplex PCR assay besides the expected ones: one around 400 bp (Fig. 3, line 2: the colon wall) and another of around 740 bp (Fig. 3, line 4 and line 5: oocysts from bison and cattle). Moreover, an extra 1350 bp fragment was also obtained in the second multiplex PCR assay (Fig. 4, line 4 and line 5: oocysts from both hosts).

4. Discussion

This study is the first to attempt to confirm *E. bovis* infection in red-listed European bison using molecular methods. A high degree of homology was found between our sequence obtained from European bison and those of cattle-derived *E. bovis*. The samples also displayed similar sporulation times and general morphological features to those given in earlier descriptions of *E. bovis* (Pyziel and Demiaszkiewicz, 2015; Golemansky, 2003; Rind et al., 2000; Sommer, 1998); however, the *E. bovis* oocysts derived from European bison were significantly narrower than those derived from cattle.

The study yielded two multiplex PCR assays for identifying *E. bovis* oocysts of bison and cattle. In each, a unique electrophorectic DNA banding pattern characterized by a single DNA fragment for *E. bovis* and *Eimeria* spp. was identified: this characteristic pattern can serve as an internal control for the integrity of the reaction, as recommended by Zarlenga and Higgins (2001).

Both multiplex PCRs yielded band characteristic of *Eimeria* spp. and *E. bovis* in samples containing DNA of oocysts from both bison and cattle. Moreover, convergent results were obtained for the DNA of the colon wall in both assays, indicating the presence of developmental stages of *Eimeria* spp. other than *E. bovis*.

Our findings provide further evidence of infection of European bison with a highly-pathogenic bovine protozoan, *E. bovis*. Hence, further investigation of histopathological lesions associated with *E. bovis* infection are needed in protected European bison.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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