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The occurrence and genetic characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The Netherlands, Germany and Greece

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ABSTRACT

Faecal samples were collected from foals between the age of 1 week and 6 months in Belgium, The Netherlands, Germany and Greece. A quantitative direct immunofluorescence assay based on the commercial MERIFLUOR *Cryptosporidium*/*Giardia* kit was performed to evaluate the presence of (oo) cysts. Parasite positive samples were genotyped, based on the 18S ribosomal DNA gene and the heat shock protein (HSP70) gene for *Cryptosporidium* and on the β-giardin gene and the triose phosphate isomerase (TPI) gene for *Giardia*. In total, 134 foals from Belgium, 44 foals from The Netherlands, 30 foals from Germany and 190 foals from Greece were examined. No *Cryptosporidium* oocysts were identified in faecal samples from foals in Germany and The Netherlands. In Belgium and Greece, 4.5% and 1.1% of the foals examined were *Cryptosporidium* positive, respectively, all with a low oocyst excretion ranging from 100 to 2450 oocysts per gram of faeces. For *Giardia*, 14.2%, 11.4%, 10.0% and 11.6% of the foals in Belgium, The Netherlands, Germany and Greece, respectively, were found to excrete cysts, with a range of 50 up to 4,000,000 cysts per gram of faeces. Younger animals secreted significantly more *Giardia* cysts than older horses ($p < 0.05$), but no significant correlation between *Giardia* infection and diarrhoea was observed. Most *Giardia* positive samples belonged to assemblage AI and/or BIV, but also assemblage E was detected in two samples. Together with the identification of *Cryptosporidium* horse genotype, this suggests only a low risk for zoonotic transmission.

1. Introduction

Cryptosporidium spp. and *Giardia* spp. are protozoan parasites that have been reported worldwide in a wide range of hosts, including horses.

In horses, excretion of *Cryptosporidium* oocysts has been reported in different geographical areas, with infection rates ranging between 0.75% (De Souza et al., 2009) and 25% (Smith et al., 2010). In some studies, the prevalence was higher in foals (Veronesi et al., 2010), while in other studies, the peak prevalence was observed in adult animals (Majewska et al., 1999, 2004. Part of this variation may be due to differences in study design, the limited number of animals and/or farms in the study, and the diagnostic technique that was used. Although an impact of *Cryptosporidium* infection on horse health has been reported (Netherwood et al., 1996; Majewska et al., 2004; Grinberg et al., 2008; Frederick et al., 2009), it seems to be less important compared to ruminants and especially intensively reared calves, in which infection with *Cryptosporidium parvum* is an important cause of neonatal diarrhoea (de Graaf et al., 1999).

Similarly, the prevalence of *Giardia duodenalis* has been reported in horses from various locations with considerable variation (0.5–35%) (Pavlásek et al., 1995; Olson et al., 1997; Atwill et al., 2000; De Souza et al., 2009; Veronesi et al., 2010; Traversa et al., 2012; Santin et al., 2013), but the number of studies on the prevalence of *G. duodenalis* in foals is more limited compared to studies on *Cryptosporidium*. Moreover, the impact of *Giardia*

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infection on equine health remains undefined, in contrast to calves and lambs, where *Giardia* infections have been associated with a decreased growth and diarrhoea (Olson et al., 1995; Geurden et al., 2010).

The relevance of animal infections is not only limited to the impact on animal health or production, but should also be considered from a public health point of view as they may also be a source of infection, either by infecting people by direct contact or by contaminating water supplies, since many outbreaks of infections by both parasites are waterborne (Baldursson and Karanis, 2011). An increasing number of studies seems to indicate a public health relevance of equine *Cryptosporidium* infections. Horses were frequently infected with the zoonotic *C. parvum* (Ryan et al., 2003; Grinberg et al., 2008; Imhasly et al., 2009; Smith et al., 2010; Laatamna et al., 2015) and occasionally the human species *Cryptosporidium hominis* was detected in horses (Laatamna et al., 2015). Similarly for *G. duodenalis*, the assemblages A and B have been identified, although the zoonotic potential of *G. duodenalis* in horses remains largely unexplored (Traub et al., 2005; Traversa et al., 2012; Santin et al., 2013).

In Europe, with the exception of Italy – where extensive studies have been performed (Veronesi et al., 2010; Traversa et al., 2012; Caffara et al., 2013) – there are only limited data on the prevalence of both protozoal infections in horses as well as their zoonotic importance. The objective of the present study was to acquire additional data on the occurrence and genotypes of *Cryptosporidium* and *Giardia* in foals in regions of Europe where no records were available.

2. Materials and methods

2.1. Sampling

Convenience samples were collected from animals that belonged either to individual owners or to larger farms/properties in four different countries, 3 countries situated in Western Europe (i.e. Belgium, The Netherlands and Germany) and one Mediterranean country, i.e. Greece. The samples were collected from 51 sites in Belgium, 30 in The Netherlands, 2 sites in Germany and 82 different sites from all over Greece. Sampling was performed in spring and summer, i.e. shortly after the foaling season. Individual faecal samples were collected from foals between 1 week and 6 months of age. The faecal samples were collected directly from the rectum and immediately transported to the participating lab in each country where they were stored at 4 °C and examined within 4 days of sampling. When a sample was found to be positive by coproscopic analysis for one of the two parasites, the sample was withheld for DNA extraction and molecular genotyping.

For every horse/sample, a data form was completed by interviewing the owner, providing information on age, breed, sex and presence or absence of diarrhoea (up to a maximum of 15 days before sampling).

2.2. Detection of Cryptosporidium and Giardia

A quantitative direct immunofluorescence assay (IFA) based on the commercial MERIFLUOR *Cryptosporidium*/*Giardia* kit (Meridian Diagnostics Inc., Cincinnati, Ohio) was performed. Briefly, one gram of the faecal material was suspended in 100 ml of distilled water and strained through a layer of surgical gauze. After sedimentation for 1 h and centrifugation at 3000 × *g* for 5 min, the sediment was resuspended in distilled water up to a volume of 1 ml. After thorough vortexing, an aliquot of 20 μl was pippeted onto a treated IFA-slide. After staining of the slide, as instructed by the manufacturer, the entire smear was examined at a 400× magnification under a fluorescence microscope. The number of *Cryptosporidium* oocysts per gram of faeces (OPG) and *Giardia* cysts per gram of faeces (CPG) was obtained by multiplying the total number of cysts on the smear by 50.

2.3. Molecular identification

DNA was extracted from the 1 ml faecal sample using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, incorporating an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95°C for 5 min) in the protocol to maximise disruption of (oo) cysts. For the amplification of the *Cryptosporidium* 18S ribosomal DNA gene (18S rDNA) and HSP70 gene, previously described PCR protocols were used (Morgan et al., 2001; Xiao et al., 2001). For the identification of *Giardia* DNA, the β-giardin gene (Lalle et al., 2005) and the triose phosphate isomerase (TPI) gene (Geurden et al., 2008) were used. In all above-mentioned PCR reactions, bovine serum albumin (BSA) was added to a final concentration of 0.1 μg BSA/μl reaction mixture. Amplification products were visualised on 1.5% agarose gels with ethidium bromide. A positive (genomic DNA from a positive faecal sample) and negative (PCR water) control sample were included in each PCR reaction. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and fully sequenced using the Big Dye Terminator v.3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with the program Seqman II (DNASTAR, Madison WI, USA). To determine the genotypes and subgenotypes, the fragments were aligned with the homologous sequences available in the GenBank database, using MegAlign (DNASTAR, Madison WI, USA). The β-giardin, TPI, 18S rDNA and HSP70 nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KM926502–KM926526, KM926527–KM926548, KM926549 and KM926550–KM926551, respectively.

2.4. Statistical analysis

Univariate analysis was carried out by descriptive statistics and results were expressed as mean (M), standard deviation (SD), median (Mdn) and minimum (min) and maximum (max) values. Within the *Giardia* infected animals, the association between age and CPG values was analysed by the non-parametric Spearman's rho correlation coefficient. In addition, the non-parametric Mann–Whitney test was utilised to compare the age of foals with and without diarrhoea. Finally, a multivariable logistic regression model was constructed to assess the main effects of age and CPG values and their interaction on the status (presence/absence of diarrhoea) of foals. Data were analysed using the SPSS statistics software (version 19.0).

3. Results

3.1. Occurrence of Cryptosporidium and Giardia

In total, 398 foals from 4 different countries were examined in the present study, i.e. 134 in Belgium, 44 in The Netherlands, 30 in Germany and 190 in Greece. The mean age was 64 days (SD ± 56.24), the median age was 31 days with a range of 5–180 days. Of the foals, 163 were male and 191 were female. For 44 foals, gender was not recorded (Table 1).

Eight foals were found positive for *Cryptosporidium* (2%) and the oocyst excretion ranged from 50 to 2450OPG with a median excretion of 750OPG. For *Giardia*, 49 foals (12.3%) were found to excrete cysts, with a range of 50–4,000,000CPG, and a median excretion