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SHORT COMMUNICATION

Genetic diversity of *Cryptosporidium* spp. including novel identification of the *Cryptosporidium muris* and *Cryptosporidium tyzzeri* in horses in the Czech Republic and Poland

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Abstract Faecal samples were collected from 352 horses on 23 farms operating under six different management systems in the Czech Republic and Poland during 2011 and 2012. Farms were selected without previous knowledge of parasitological status. All faecal samples were screened for *Cryptosporidium* spp. presence using microscopy, following aniline-carbol-methyl violet staining and PCR analysis of the small-subunit (SSU) rRNA and the 60-kDa glycoprotein (gp60) genes. *Cryptosporidium muris*-positive samples were additionally genotyped at four minisatellite markers: MS1 (encoding a hypothetical protein), MS2 (encoding a 90-kDa heat shock protein), MS3 (encoding a hypothetical protein) and MS16 (encoding a leucine-rich repeat family protein). *Cryptosporidium* spp. was detected by PCR in 12/352 (3.4 %) samples from 4 out of 13 farms. None of the samples tested by microscopy was positive. There was no relationship between *Cryptosporidium* prevalence and age, sex, diarrhoea or management system; however, *Cryptosporidium* was found only on farms where horses were kept on pasture during the day and in a stable overnight. Sequence analyses of SSU and gp60 genes revealed the presence of *C. muris* RN66 (n=9), *Cryptosporidium parvum* IIaA15G2R1 (n=1), *Cryptosporidium tyzzeri* IXbA22R9 (n=1), and *Cryptosporidium* horse genotype VIaA15G4 (n=1). The *C. muris* subtypes were identified as MS1-M1, MS2-M4, novel MS2-M7 and MS16-M1 by multilocus sequence of three minisatellite loci. The MS3 locus was not amplified from any isolate. This is the first report of *C. tyzzeri* and *C. muris* subtypes from horses.

Keywords Horse · *Cryptosporidium* · SSU · gp60 · MLST

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Introduction

Cryptosporidium is among the most common parasites of domestic and wild animals and humans. Interest in *Cryptosporidium* has heightened in the veterinary field, not only because of the potential for zoonotic transmission, but also because of difficulties controlling economic losses in production animals (Ramirez et al. 2004). A large body of work has been published on *Cryptosporidium* and cryptosporidiosis in domestic and captive animals (Kváč et al. 2014a). Although cryptosporidiosis was initially considered to occur

rarely in horses and was associated with immunodeficiency (Snyder et al. 1978), more recent studies have shown that horses are frequently infected with *Cryptosporidium* (Olson et al. 1997; Xiao and Herd 1994). Natural equine cryptosporidiosis has been reported in many countries worldwide, including America (Cole et al. 1998; de Souza et al. 2009; Xiao and Herd 1994), Canada (Gajadhar et al. 1985; Olson et al. 1997), New Zealand (Grinberg et al. 2003, 2009), Africa (Laatamna et al. 2013) and a number of European countries (Majewska et al. 2004; Ryan et al. 2003; Sturdee et al. 2003; Veronesi et al. 2010). Horses appear susceptible to at least three *Cryptosporidium* spp.: *Cryptosporidium* horse genotype, *Cryptosporidium parvum* and *Cryptosporidium erinacei* (previously known as hedgehog genotype) (Grinberg et al. 2003; Laatamna et al. 2013; Ryan et al. 2003). All *Cryptosporidium* spp. detected in horses to date are also infectious for humans (Kváč et al. 2014b; Robinson et al. 2008; Xiao et al. 2009). The aim of this study was to determine the diversity of *Cryptosporidium* spp. in horses under various conditions in the Czech Republic and Poland and to determine any associations between infection occurrence and age, sex, housing systems and consistency of faeces.

Material and methods

During 2011 and 2012, faecal specimens were collected from 352 horses of different ages (5 days to 32 years) on 23 horse farms throughout the Czech Republic (CR; n=20) and Poland (P; n=3). The farms were screened without previous knowledge of parasitological status. The management systems in operation on the farms, which were exclusively horse farms were as follows: (i) year-round grazing with shelters (CR farm #1–4), (ii) year-round grazing with overnight housing in stables during winter (CR #5–7), (iii) year-round housing in stables on concrete floors (CR #8–10), (iv) year-round housing in the stables on the deep straw bedding (CR #11), (v) daytime grazing and overnight housing in stables on deep straw bedding (CR #12–16) and (vi) daytime grazing and overnight housing in stables on concrete floors (CR #17–20; P #21–23). Each sample was individually placed in a plastic dish without fixation, stored in at 4 °C and analysed within 48 h for the presence of *Cryptosporidium* using the aniline-carbol-methyl violet staining method (Miláček and Vítovec 1985). The faecal consistency (loose if it took the form of the container and solid if it maintained its original shape) was noted at the time of sampling. Repeated analyses of the same animals were excluded from the survey to avoid estimating cumulative prevalence.

Total DNA was extracted from 200 mg of faecal samples from each specimen by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep®24 Instrument (MP Biomedicals, CA, USA) using QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany).

The extracted DNA was kept frozen at −20 °C until used for genotyping. Nested PCR protocols amplifying a fragment of the small-subunit (SSU) rRNA, the 60-kDa glycoprotein (gp60) and four minisatellite markers, including the MS1 (encoding a hypothetical protein), MS2 (encoding a 90-kDa heat shock protein), MS3 (encoding a hypothetical protein) and MS16 (encoding a leucine-rich repeat family protein) genes of *Cryptosporidium* were performed in duplicate as previously described by Alves et al. (2003); Jiang et al. (2005); and Feng et al. (2011). Negative (PCR water) and positive controls (samples containing DNA of *Cryptosporidium suis* for SSU, *Cryptosporidium hominis* for gp60, *Cryptosporidium andersoni* for MS genes) were included with each PCR amplification. PCR products were visualized following electrophoresis on a 1 % agarose gel containing 0.2 g/ml ethidium bromide. All sequences were confirmed by sequencing amplicons from two independent DNA extractions. PCR products were sequenced in both directions on an ABI 3730XL sequence analyser (Applied Biosystems, Foster City, CA). Sequences were assembled, manually edited and aligned using the ChromasPro 1.7.4 (Technelysium, Pty, Ltd.), BioEdit v7.0.5.3 (Hall 1999 and MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/server/), and were compared with sequences in GenBank. Phylogenetic trees were inferred by the neighbour-joining method, with pairwise deletions, from distances estimated using the Kimura 2-parameter distance model (MEGA5) (Tamura et al. 2011). Bootstrap support for branching was based on 1000 pseudoreplicates. Phylograms were edited for style using CorelDrawX5. Sequences have been deposited in GenBank under the accession numbers KJ469983, KJ469985-KJ469989 and KP176787-KP176793.

Relationships between *Cryptosporidium* spp. presence and the age or sex of the animal or farm management practices were determined using a classical chi-squared test of independence without Yates' continuity correction. Statistical analyses were performed using R (version 2.15.0), a software environment for statistical computing.

Results

Cryptosporidium spp. was detected in 12/352 (3.4 %) samples tested by PCR and none of the samples was tested by microscopy. Positive samples were from 4/23 (17.4 %) of farms. Statistical analyses did not show any association between sex or age of the animal and *Cryptosporidium* prevalence (data not shown). *Cryptosporidium* was detected only on farms operating under management systems combining pasture grazing and housing in stables. None of horses showed signs of diarrhoea at the time of sampling or during the

2 weeks prior to sampling. Analysis of partial sequences of the SSU gene showed the presence of *C. parvum* ($n=1$), *Cryptosporidium* horse genotype ($n=1$) and *C. muris* ($n=9$) (Fig. 1a). Neighbour-joining trees constructed using on gp60 sequences obtained in this study and sequences published in GenBank revealed the presence of *C. parvum* ($n=1$), *C. tyzzeri* ($n=1$) and *Cryptosporidium* horse genotype ($n=1$) belonging to family IIa, IXb and VIa, respectively. Based on the established gp60 nomenclature (Sulaiman et al. 2005), the *Cryptosporidium* subtypes were named IIaA15G2R1 (*C. parvum*), IXbA22R9 (*C. tyzzeri*) and VIaA15G4 (horse genotype) (Fig. 1b). *Cryptosporidium parvum* and horse genotype were identified in two young stallions on the same farm (#15). At the four minisatellite loci (MS1, MS2, MS3

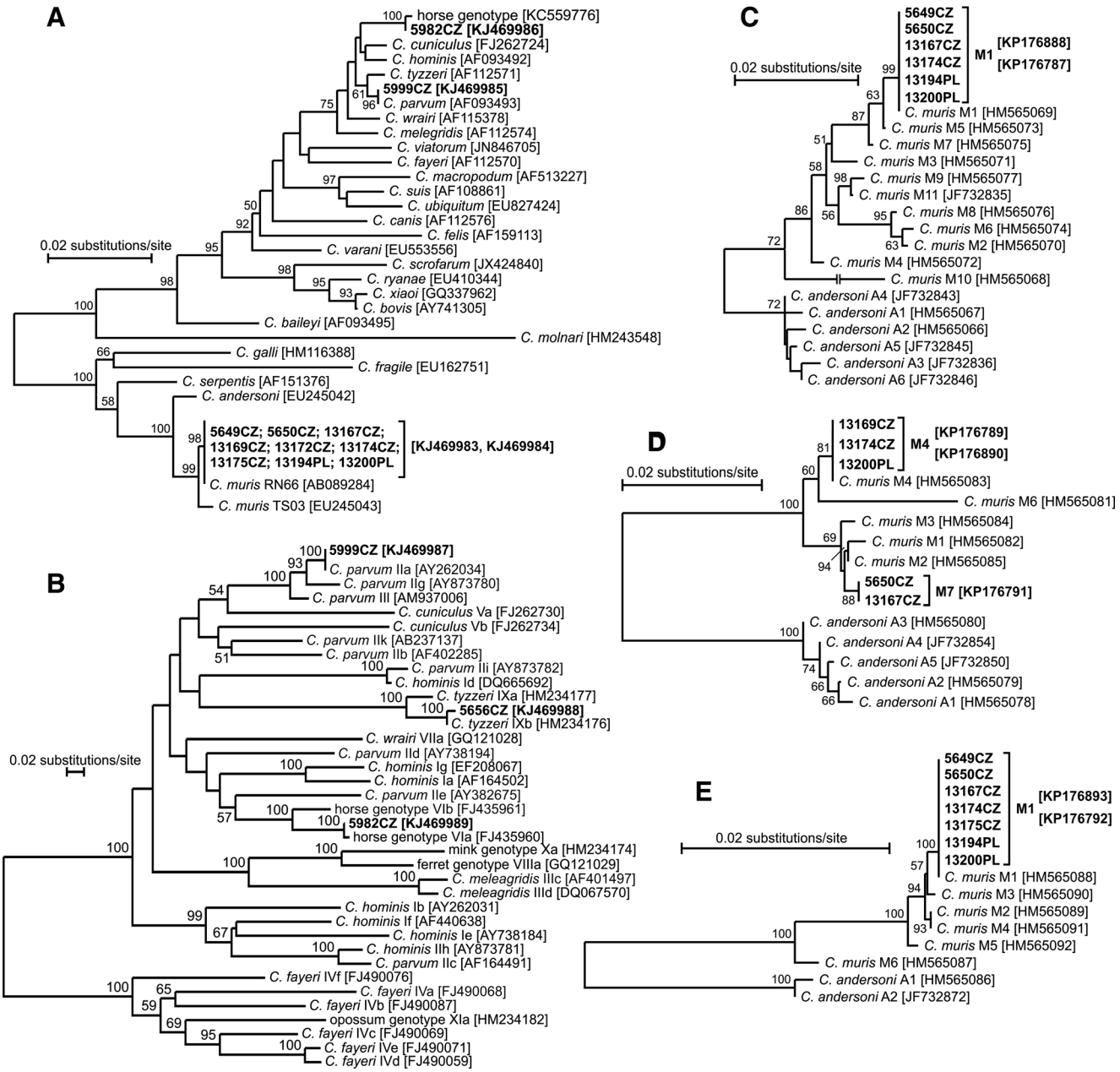

Fig. 1 Phylogenetic relationships between *Cryptosporidium* spp. found in present study (*highlighted*) and other *Cryptosporidium* spp. as inferred by a neighbour-joining analysis of **a** SSU (820 base positions in the final dataset), **b** gp60 (692 base positions in the final dataset), **c** MS1 (encoding a hypothetical protein; 436 base positions in the final dataset), **d** MS2 (encoding a 90-kDa heat-shock protein; 406 base positions in the final dataset) and **e** MS16 (encoding a leucine-rich repeat family protein; 549 base positions in the final dataset) genes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). Numbers at the nodes represent bootstrap values for the nodes gaining more than 50 % support. A *scale bar* is included in each tree. Interrupted branch has been shortened fivefold. *CZ* Czech Republic, *PL* Poland

and MS16), six, five, zero and seven *C. muris*-positive isolates were sequenced, respectively (Table 1). A total of four out nine *C. muris* isolates were subtyped at three loci.

Discussion

Results of this study are consistent with the reported low worldwide prevalence of *Cryptosporidium* infection in horses (e.g. de Souza et al. 2009; Majewska et al. 2004; Olson et al. 1997; Veronesi et al. 2010; Xiao and Herd 1994). Unlike pigs or cattle, we found no association between management system or sex and *Cryptosporidium* infection (Maddox-Hyttel et al. 2006; Mohammed et al. 1999). However, this may be due to the very low prevalence of *Cryptosporidium* in horse populations. Most studies, including the present one, have found no association between *Cryptosporidium* infection and clinical signs in horses and foals, suggesting that cryptosporidiosis is frequently subclinical in healthy horses (e.g. McKenzie and Diffay 2000; Majewska et al. 2004; Veronesi et al. 2010; Xiao and Herd 1994). However, Grinberg et al. (2009), Perrucci et al. (2011) and Díaz et al. (2012) reported an association between infection by *C. parvum* and diarrhoea primarily in foals. The *C. parvum* subtypes, IIa A15G2R1 and IIaA18G3R1, associated with clinical cryptosporidiosis in foals (Díaz et al. 2012; Grinberg et al. 2008) also cause diarrhoea in humans and livestock (Glaberman et al. 2002; Trotz-Williams et al. 2006; Wielinga et al. 2008). Absence of clinical disease in horses infected with IIa A15G2R1 in this study could be due to the age of infected horses (older than 3 years). In addition, the failure to detect *C. parvum* oocysts by microscopy suggests that the infection intensity was low. It is possible that asymptomatically infected adult horses are sources of *C. parvum* causing cryptosporidiosis in foals.

The *Cryptosporidium* horse genotype detected was identical to an isolate found in a foal in Italy (VIaA15G4; Caffara et al. 2013) and belonged to the same subtype family as a horse genotype previously found in Prezewalski's and domestic horses (Burton et al. 2010; Ryan et al. 2003). In contrast, two human isolates from England (Robinson et al. 2008) and New Mexico (Xiao et al. 2009) belonged to the *Cryptosporidium* horse genotype VIb family, which has never been found in horses. Consistent with previous studies, we found a low frequency of *Cryptosporidium* horse genotype occurrence (e.g. Grinberg et al. 2003, 2008, 2009; Veronesi et al. 2010). Unexpectedly, we also found rodent-specific *C. muris* and *C. tyzzeri* in horses in this study. Both rodent-specific *Cryptosporidium* species have been found previously in non-specific hosts, including humans and domestic animals such as pigs, cattle and camels (Kváč et al. 2014a). It is not known whether the presence of *C. muris* and *C. tyzzeri* DNA in horse faeces was due to an active infection or mechanical passage of oocysts through the digestive tract. Previous studies suggested that the presence of *C. muris* and *C. tyzzeri* in faecal samples of snakes, lizards, raptors and pigs was due to the ingestion of mice or contamination from the environment. The association of these rodent species with horses kept in stables with straw bedding supports the hypothesis of passive transport. However, the *C. tyzzeri* subtype (IXbA22R9) was previously found only in *Mus musculus domesticus*, and all positive horses were bred within an area where mice (*Mus musculus musculus*) were infected with IXa family only (Kváč et al. 2013). The presence of *C. tyzzeri* IXb in horses kept in an area of IXa distribution could be explained by an ongoing infection in incoming horses, as the farm

Table 1 Specimens detected in the study and their species/subtype identity at the SSU, gp60 and four minisatellite MS loci

Specimen ID	#farm	Country	SSU	gp60	MLST minisatelite			
					MS1	MS2	MS3	MS16
5949CZ	#16	Czech Republic	*C. muris*	–	M1	–	–	M1
5650CZ			*C. muris*	–	M1	M7	–	M1
5656CZ	#21		–	IXbA22R9	–		–	
5982CZ			Horse genotype	VIaA15G4	–	–	–	–
5999CZ			*C. parvum*	IIaA15G2R1	–	–	–	–
9687CZ			*C. muris*	–	–	–	–	–
13167CZ			*C. muris*	–	M1	M7	–	M1
13169CZ			*C. muris*	–		M4	–	–
13174CZ			*C. muris*	–	M1	M4	–	M1
13175CZ			*C. muris*	–	–	–	–	M1
13194PL	#22	Poland	*C. muris*	–	M1	–	–	M1
13200PL			*C. muris*	–	M1	M4	–	M1

served as breeding centre for young horses, which could be more susceptible to infection.

MLST subtyping provided a data on the occurrence of *C. muris* subtypes on the monitored farms. The *C. muris* subtypes obtained from horses in the Czech Republic and Poland included variants of MS1-M1, previously found in a camel, Tawny frogmouth, laboratory mouse and human; MS2-M4, found in an ostrich, Siberian chipmunk, hamster, mara, laboratory mouse and human; and MS16-M1, found in a siberian chipmunk, hamster, mara, cat, domestic and laboratory mouse (Feng et al. 2011; Wang et al. 2012). While isolates originating from horses kept in Poland had the same subtypes at MS1, MS2 and MS16 loci as isolates from Czech Republic, novel variant at the MS2 locus, named M7, were found in horses in the Czech Republic. Although it is likely that various subtypes of *C. muris* could cause infections in various hosts, the susceptibility of horses to *C. muris* and *C. tyzzeri* remains unclear. Understanding of the epidemiology of *Cryptosporidium* infections in horses is gradually improving with an increasing number of studies supported by molecular analyses, but there remains much to discover.

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

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