Research Article

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Cryptosporidium meleagridis and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria

Abd Elkarim Laatamna¹, Nikola Holubová^{2,3}, Bohumil Sak², Martin Kváč^{2,3}

¹ Faculty of Natural Sciences and Life, University of Djelfa, Moudjbara, Djelfa, Algeria;

² Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic;

³ Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic

Abstract: A total of 345 faecal samples were collected from domestic, captive and wild birds in rural areas, urban areas and a Zoo in Algeria. Samples were screened for the presence of parasites belonging to the genus *Cryptosporidium* Tyzzer, 1910 by microscopy and PCR analysis of the small-subunit rRNA (SSU), actin and 60-kDa glycoprotein (gp60) genes. *Cryptosporidium* spp. were detected in 31 samples. Sequence analysis of SSU and actin genes revealed the presence of *C. baileyi* Current, Upton et Haynes, 1986 in domestic chicken broilers (n = 12), captive ostriches (n = 4) and a wild mallard (n = 1), and *C. meleagridis* Slavin, 1955 in a graylag goose (n = 1), chickens (n = 11) and turkeys (n = 2). Twenty-three chicken and two turkey broilers from five farms were positive for cryptosporidia, with an overall prevalence of 2% and 6%, respectively. Both *C. meleagridis* and *C. baileyi* were detected in farmed chicken broilers, with a prevalence ranging from 9% to 69%. Farmed turkey broilers were positive only for *C. meleagridis*, with a 13% prevalence at the animal level. Subtyping of *C. meleagridis* isolates at the gp60 locus showed the presence of subtype IIIgA22G3R1 in graylag goose and chicken broilers and IIIgA23G2R1 in chicken and turkey broilers. Infection with cryptosporidia was not associated with any clinical diseases. The results of the present study, which provides the first data on the prevalence of *Cryptosporidium* spp. in wild birds in Africa, demonstrate the presence of human pathogenic *C. meleagridis* in both domestic and wild birds in Algeria.

Keywords: avian cryptosporidia, PCR, epidemiology, Northern Africa

Birds are important to public and animal health because they carry various pathogens, including the zoonotic parasites of the genus *Cryptosporidium* Tyzzer, 1910 (see Reed et al. 2003, Graczyk et al. 2008). The genus *Cryptosporidium* comprises species of apicomplexan parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all vertebrate classes (O'Donoghue 1995). Species of genus *Cryptosporidium* additionally infect the bursa of Fabricius, respiratory system and other organs in birds (Nakamura and Meireles 2015).

So far, only four bird specific species of *Cryptosporidium* have been described: *Cryptosporidium meleagridis* Slavin, 1955; *Cryptosporidium baileyi* Current, Upton et Haynes, 1986; *Cryptosporidium galli* Pavlásek, 1999; and *Cryptosporidium avium* Holubová, Sak, Horčíčková, Hlásková, Květoňová, Menchaca, McEvoy et Kváč, 2016 (Slavin 1955, Current et al. 1986, Ryan et al. 2003a, Holubová et al. 2016). These species infect a broad spectrum of birds, although they differ in their host range and site of infection; *C. meleagridis* also causes disease in humans (McLauchlin et al. 2000, Cama et al. 2003). Besides these four species, 13 *Cryptosporidium* genotypes have been described in birds worldwide, including avian genotypes I–VI, black duck genotype, Eurasian woodcock genotype

and goose genotypes I–V (Nakamura and Meireles 2015, Chelladurai et al. 2016).

Additionally, the major human pathogenic species *C. hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson, Olson, Lal et Xiao, 2002 and *C. parvum* Tyzzer, 1912 and the artiodactyl and rodent species *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blagburn, 2000 and *C. muris* Tyzzer, 1907 have been detected in birds (Graczyk et al. 1998, Zhou et al. 2004, Ng et al. 2006, Qi et al. 2014).

Although cryptosporidiosis is one of the most prevalent parasitic infections in domestic, captive and wild birds worldwide, research on cryptosporidia in birds has lagged well behind that in mammals (Kváč et al. 2014). A major gap in our understanding of the diversity of cryptosporidia in birds is lack of prevalence and, in particular, genotyping data from the African continent (Bezuidenhout et al. 1993, Penrith et al. 1994, Soltane et al. 2007, Berrilli et al. 2012, Baroudi et al. 2013). In the present study, we determined the occurrence of *Cryptosporidium* spp. in domesticated and wild birds in Algeria using molecular tools, including subtype identification of *C. meleagridis*. Additionally, the role of wild birds as natural source of cryptosporidia for farmed birds was evaluated.

Address for correspondence: M. Kváč, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, České Budějovice, 370 05, Czech Republic. Phone: (+420) 387775419; Fax: (+420) 385310388; E-mail: kvac@paru.cas.cz

MATERIALS AND METHODS

Samples

A total of 345 faecal samples from juvenile and adult domestic and wild birds were collected at poultry farms, rural and urban areas, and a zoo at the Bourdj Bou Arreridj Setif and Algiers province of Algeria (Table 1). At poultry farms, chicken and turkey broilers were kept on soil and laying hens were kept in hatcheries/cages. Birds from the zoo were housed in an aviary with a concrete floor or, in the case of ostriches, were kept on soil. Captive birds from urban areas were kept in bird cages. Faecal samples of wild and captive birds and poultry were obtained directly from the ground immediately after defecation. Cloacal contents of farmed birds were collected after necropsy. Each sample was placed into an individual sterile plastic container without fixative, transported to the laboratory in a cool box and stored at 4 °C until processing. All samples were screened for the presence of oocysts of cryptosporidia using the aniline-carbol-methyl violet staining method (Miláček and Vítvec 1985). Faecal consistency was noted at the time of sampling. The infection intensity was determined from the microscopic examination as number of oocysts per gram (OPG) according to Kváč et al. (2007).

Molecular study

DNA was extracted from 200 mg of faeces by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a Fast Prep® 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (PSP Spin stool DNA Kit, STRATEC Molecular GmbH, Birkenfeld, Germany). Purified DNA was stored at -20 °C prior to being used for PCR analysis. A nested PCR approach was used to amplify a region of the small subunit of rRNA gene (SSU; ~ 830 bp; Xiao et al. 1999, Jiang et al. 2005) and actin (~ 1,066 bp; Sulaiman et al. 2002) in all samples. The 60 kDa glycoprotein (gp60; ~ 830 bp; Alves et al. 2003, Li et al. 2014, Guo et al. 2015) was amplified in positive samples on the basis of results of SSU and actin genotyping. Negative and positive controls (*C. parvum*) were included in all PCR sets. Purified secondary products (QIAquick® Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA, USA) using the secondary PCR primers.

The nucleotide sequences of each gene obtained in this study were edited using ChromasPro 1.7.5 software (Technelysium Pty Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server with automatic selection of alignment mode (<http://mafft.cbrc.jp/alignment/software/>). Phylogenetic analyses were performed and best DNA phylogeny models were selected using the MEGA6 software (Tamura et al. 2013). Phylogenetic trees with bootstrap support were inferred by maximum likelihood (ML) and maximum parsimony methods. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KY352474–KY352489.

Statistical analyses

A two-sample z-test for proportions (independent groups) was used to assess relationships between parasite detection (*Crypto-*

sporidium spp.) and presence or absence of signs of diarrhoea. All computations were performed with R 2.15.1.

RESULTS

Microscopical examination revealed the presence of oocysts of cryptosporidia in 22 of 345 faecal samples, with an infection intensity ranging from 1,000–4,000 OPG. Targeting the SSU and actin genes, *Cryptosporidium*-specific DNA was detected in all microscopy-positive samples and in nine samples that were microscopy-negative (Table 1). The infection rate of cryptosporidia varied among regions and farms. The prevalence at commercial chicken and turkey farms reached up to 9–69% and 13%, respectively (Table 1). Twelve examined animals suffered from diarrhoea at the time of sampling. However, only one of them was *Cryptosporidium*-positive (Table 1); thus, the presence of diarrhoea was not associated with *Cryptosporidium* infection (p value = 0.1871).

ML analysis of SSU and actin sequences revealed two distinct clusters among isolates of cryptosporidia from birds in the present study. Fourteen isolates clustered with *C. meleagridis*, sharing 100% sequence identity with *C. meleagridis* isolates with accession nos. AF112574 and AF382351. The remaining 17 isolates belonged to *C. baileyi*, sharing 100% sequence identity with accession nos. AF093495 and EU741853. Analysis of the gp60 gene of *C. meleagridis* isolates showed the presence of subtypes IIIgA22G3R1, in a wild goose ($n = 1$) and chicken broilers ($n = 10$), and IIIgA23G2R1 in turkey ($n = 2$) and chicken broilers ($n = 1$; Table 1). The gp60 gene of *C. baileyi* was not amplified by any of the used sets of primers. *Cryptosporidium meleagridis* and *C. baileyi* were found only on commercial farms and in wild birds. Cryptosporidia were not detected in traditionally bred poultry in rural areas.

DISCUSSION

In Algeria, only Baroudi et al. (2013) described the occurrence of *Cryptosporidium meleagridis* and *C. baileyi* in farmed poultry, whereas the occurrence of cryptosporidia in wild birds has not yet been studied. Therefore, the present study provides the first data on the prevalence of *Cryptosporidium* spp. in wild birds in Africa. *Cryptosporidium baileyi* is considered the most common avian species of *Cryptosporidium* worldwide and it has the broadest host range (Nakamura and Meireles 2015). In the present study, *C. baileyi* was detected in mallard, ostriches and chicken broilers. Whereas *C. baileyi* has been reported frequently in ducks and chickens worldwide (Morgan et al. 2001, Ryan et al. 2003b, Chvala et al. 2006, Huber et al. 2007), the only previous reports in ostriches have been from China and the Czech Republic (Ryan et al. 2003b, Wang et al. 2011). The overall prevalence of *C. baileyi* in ostriches in the present study reached 31%, which is comparable to the data by Wang et al. (2011) who reported prevalence of 1–29%, and lower than the 60% prevalence in farmed ostriches and rheas originating from Belgium, France, Netherlands, Portugal and Spain (Gordo et al. 2002). In contrast to the report of Wang et al. (2011), who did not detect

Table 1. A survey of *Cryptosporidium meleagridis* Slavin, 1955 and *C. baileyi* Current, Upton et Haynes, 1986 in faecal samples of domestic, captive and wild birds based on microscopic and molecular examination.

Area	Host	Age	Screened samples/MIC/PCR positive	Molecular characterisation			Diarrhoea			
				SSU	actin	gp60				
Wild birds	Rural	<i>Anser anser</i> (Linnaeus) (greylag goose)	4/0/0	-	-	-	-			
			2/1/1	<i>C. meleagridis</i>	<i>C. meleagridis</i>	IIIgA22G3R1	-			
			2/0/0	-	-	-	-			
			3/0/0	-	-	-	-			
		A	5/1/1	<i>C. baileyi</i>	<i>C. baileyi</i>	ND	1			
		<i>Anas platyrhynchos</i> Linnaeus (mallard)	A	4/0/0	-	-	-	-		
		A	14/0/0	-	-	-	-	-		
A	8/0/0	-	-	-	-	-				
A	<i>Ciconia ciconia</i> (Linnaeus) (white stork)	A	25/0/0	-	-	-	-			
Captive birds	Zoo	<i>Dromaius novaehollandiae</i> (Latham) (emu)	A	3/0/0	-	-	-	-		
				3/0/0	-	-	-	-		
		<i>Balearica regulorum</i> (Bennett) (grey crowned-crane)	A	4/0/0	-	-	-	-		
		<i>Numida meleagris</i> (Linnaeus) (helmeted guineafowl)	A	2/0/0	-	-	-	-		
		<i>Anas platyrhynchos</i> Linnaeus (mallard)	A	6/0/0	-	-	-	-		
				2/0/0	-	-	-	-		
		<i>Eclectus roratus</i> (Müller) (eclectus parrot)	A	5/0/0	-	-	-	-		
				1/0/0	-	-	-	-		
		<i>Pavo</i> sp. (peacock)	A	3/0/0	-	-	-	-		
				6/0/0	-	-	-	-		
		<i>Columba livia</i> f. <i>domestica</i> Gmelin (domestic pigeon)	A	2/0/0	-	-	-	-		
		<i>Phasianus colchicus</i> Linnaeus (ring-necked pheasant)	A	2/0/0	-	-	-	-		
				3/0/0	-	-	-	-		
		<i>Struthio camelus</i> Linnaeus (ostrich)	A	13/4/4	<i>C. baileyi</i>	<i>C. baileyi</i>	ND	-		
				2/0/0	-	-	-	-		
		<i>Melopsittacus undulatus</i> (shaw) (Budgerigar)	J	2/0/0	-	-	-	-		
		<i>Serinus canaria</i> (Linnaeus) (canary)	J	6/0/0	-	-	-	-		
				5/0/0	-	-	-	-		
		J	<i>Carduelis carduelis</i> (Linnaeus) (European goldfinch)	J	10/0/0	-	-	-	-	
Domestic birds	Rural	<i>Gallus gallus</i> f. <i>domestica</i> (Linnaeus) (hen)	chicken broiler	2–3 weeks	6/0/0	-	-	-	-	
				3–4 weeks	11/0/0	-	-	-	3	
				3–5 weeks	16/11/11	<i>C. baileyi</i>	<i>C. baileyi</i>	ND	-	
				4 weeks	7/0/0	-	-	-	7	
				5 weeks	8/1/1	<i>C. baileyi</i>	<i>C. baileyi</i>	ND	-	
				5–6 weeks	11/1/1	<i>C. meleagridis</i>	<i>C. meleagridis</i>	IIIgA23G2R1	-	
				5–6 weeks	8/0/0	-	-	-	-	
				5–7 weeks	9/0/0	-	-	-	-	
				8 weeks	16/2/10	<i>C. meleagridis</i>	<i>C. meleagridis</i>	IIIgA22G3R1	-	
			laying hen	2 weeks	16/0/0	-	-	-	-	
				A	13/0/0	-	-	-	-	
					12/0/0	-	-	-	-	
			broiler hen	A	8/0/0	-	-	-	-	
					7/0/0	-	-	-	-	
			traditional breed	hen	A	3/0/0	-	-	-	-
						2/0/0	-	-	-	-
						5/0/0	-	-	-	-
						2/0/0	-	-	-	1
		<i>Meleagris gallopavo</i> f. <i>domestica</i> Linnaeus (turkey)	commercial breed	turkey broiler	4–7 weeks	19/0/0	-	-	-	-
					14–16 weeks	15/1/2	<i>C. meleagridis</i>	<i>C. meleagridis</i>	IIIgA23G2R1	-
traditional breed	A		4/0/0	-	-	-	-			
Total			345/22/31				12			

J – juvenile; A – adult; MIC – microscopy; PCR – Polymerase Chain Reaction; ND – not detected.

Fig. 1. Concatenated maximum likelihood tree (model Tamura 3-parameter G + I) based on partial small subunit ribosomal RNA and actin gene sequences of species and genotypes of *Cryptosporidium* Tyzzer, 1910, including those detected in the present study. Newly obtained sequences are bolded. Numbers at the nodes represent the bootstrap values (maximum likelihood/maximum parsimony; 1,000 replicates) gaining more than 50% support. Branch length scale bar indicates number of substitution per site.

Cryptosporidium in ostriches older than 12 months of age, we reported a high prevalence in adult ostriches. Although there have been many reports concerning *Cryptosporidium* infections in ostriches, the knowledge of species identity and their host and age specificity is far from clear. In contrast, the biological properties of *C. baileyi* in chicken is well known (Robertson et al. 2014). In Algeria, we detected a high prevalence of this species on a commercial farm, where 69% of screened birds were positive, and the overall prevalence of *C. baileyi* in this study varied from 9 to 69%. Our findings are consistent with the reported prevalence of *C. baileyi* in flocks of chicken broilers in the USA (10–60%) (Goodwin et al. 1996), Morocco (14–100%) (Kichou et al. 1996) and China (7%) (Wang et al. 2014).

Cryptosporidium meleagridis has been found at a high prevalence (29%) in turkeys aged over 4 weeks in Algeria (Baroudi et al. 2013). In contrast, we detected the same species in only 13% of 14–16 weeks old turkey broilers kept on the commercial farms, which is similar to the data reported by McEvoy and Giddings (2009), who found 3–11% of 4–9 week-old poults infected with *C. meleagridis*. Similarly to turkey flocks, infection of chickens with *C. meleagridis* varied. In the present study, *C. meleagridis* was detected in 9–63% of chicken broilers. Wang et al. (2014) reported a much lower prevalence (less than 2%) of *C. meleagridis* in chickens. In contrast, other authors reported *C. meleagridis* in 35–44% of turkeys (Pavlašek

1994, Gharagozlou et al. 2006, Baroudi et al. 2013). These results show high variability among breeds worldwide, but different methodological approaches of surveys, differences in hygiene and host age likely play important role. In contrast to previous studies that frequently reported clinical cryptosporidiosis in turkeys and chickens (Goodwin 1988, Goodwin et al. 1988, Baroudi et al. 2013, Nakamura and Meireles 2015), infection with *C. meleagridis* was not associated with diarrhoea and mortality in the present study.

The importance of cryptosporidiosis in commercial poultry production has not yet been determined because few studies have examined the relationship between natural infection by *Cryptosporidium* spp. and production losses (Nakamura and Meireles 2015). The common occurrence of *C. meleagridis*, mainly in domestic birds, may have an impact on public health. *Cryptosporidium meleagridis* is the third most common human-pathogenic species in both developing and developed countries (Cama et al. 2008). All isolates of *C. meleagridis* detected in the present study belonged to family IIIg, which has been previously detected in birds and humans (Abal-Fabeiro et al. 2013, Baroudi et al. 2013, Stensvold et al. 2014). Two subtypes of *C. meleagridis*, IIIgA23G2R1 and IIIgA22G3R1, have not been reported previously from domestic chickens, turkeys and wild goose. The presence of subtype IIIgA22G3R1 in wild and farmed birds in this study suggests circulation

Fig. 2. Maximum likelihood tree (model General Time Reversible G + I) based on partial sequences of gp60 gene of species and genotypes of *Cryptosporidium* Tyzzer, 1910, including *Cryptosporidium meleagridis* Slavin, 1955 detected in the present study. Newly obtained sequences are bolded. Numbers at the nodes represent the bootstrap values (maximum likelihood/maximum parsimony; 1,000 replicates) gaining more than 50% support. Branch length scale bar indicates number of substitution per site.

of parasites between wild and domestic animals. Due to the lack of gp60 subtyping on human cryptosporidiosis in North Africa, it is not known if the *C. meleagridis* subtypes reported in the present study are associated with human disease, although infections by this species has been previously reported in Tunisia (Essid et al. 2008, Rahmouni et al. 2014).

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