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BIOLOGICAL CHARACTERISATION OF *CRYPTOSPORIDIUM PARVUM* ISOLATES OF WILDLIFE RODENTS IN POLAND

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Abstract: The study was undertaken to characterise the *C. parvum* isolates originating from naturally infected woodland and field rodents: *Clethrionomys glareolus* (CG), *Apodemus flavicollis* (AF) and *Microtus arvalis* (MA). We found that the measurements of oocyst dimensions and oocyst morphology did not allow distinction between the parasite isolates from the 3 rodent species. The mean dimensions were: for CG 4.67 × 4.21, for AF 4.65 × 4.14 and for MA 4.66 × 4.16. These 3 groups of isolates have produced significantly different pictures of infection in C57BL/6 mice. The overall mean oocysts output was: in CG-mice 41,739, in AF-mice 18,000, in MA-mice 10,384 oocysts/1 g of faeces. From these data we suggest that rodent isolates of *C. parvum* could represent new subgroups in so-called "mouse" *C. parvum* strain. The successful cross-transmission from wild hosts to laboratory rodents and the close similarity of COWP sequence between our isolates and "mouse" genotype and between "mouse" and zoonotic genotype of *C. parvum* (genotype C) inform us that all these isolates should be treated as potentially hazardous for human health.

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Key words: *Cryptosporidium parvum*, infectivity, oocysts measurements, rodents, Poland.

INTRODUCTION

Cryptosporidium parvum is a protozoan coccidian parasite, which attacks the alimentary and respiratory mucosae of a variety of vertebrates, including man [18]. The infective stages are oocysts, which are released, in high quantities into the environment in the faeces of infected animals [9]. Oocysts are extremely resistant to different environmental conditions and can retain infectivity for 6 months under suitable conditions, e.g. in appropriately moist soil or in surface water [7].

Tyzzer in 1912 [28] first described the parasite from the small intestine of the domestic mouse (*Mus musculus*). *Cryptosporidium* spp. causes diarrhoeal illness in humans, which can be particularly severe in children, elderly persons and in immunocompromised individuals [3, 6, 17]. *C. parvum* infections are the direct cause of death for 15% of HIV infected persons [10].

Controversy still exists about the specificity and virulence of *C. parvum*. Firstly, parasites from the "*C. parvum*" group have been recorded to date in over 100 different mammalian species, and many different cross-transmission studies have indicated the lack of strict host specificity of this parasite [18, 26]. Secondly, some of these cross-transmissions and recent molecular studies have demonstrated the existence of highly specific, host-adapted strains in this group infective only for the primary and immunosuppressed hosts [16, 24]. The epidemiological consequences of these findings have immense importance. To date, infections caused by four different *Cryptosporidium* species and three different *C. parvum* genotypes have been reported in immunocompromised humans world-

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wide [5, 12, 19, 20, 21, 29] and there is every likelihood that more will be reported in the future. Thus, there is an urgent need to sustain the detailed biological and molecular characterisation of the different *C. parvum* isolates derived from reservoir animals.

There is also controversy about the taxonomy of *Cryptosporidium* and the public health significance of parasite isolates from various animals. The classification of parasite species is based on three main biological features: (1) dimensions of oocyst - average length, width and shape; (2) microhabitat localisation in gastrointestinal tract and (3) host specificity - range of naturally infected hosts and the results of cross-transmission studies.

Although the relevance of these criteria for the systematics of *Cryptosporidium* is still problematic and not always clear cut, the availability of molecular genetic techniques should facilitate discrimination of host–adapted *C. parvum* genotypes [4, 23, 25, 27]. However, currently, many of the newly-described genotypes are inadequately characterised with respect to their biological properties. Thus, there is still a need to refine our understanding of the crucial biological properties of isolates that may be useful for defining new parasite species or specific genotypes, e.g., those recommended by Xiao *et al.* [29], such as prepatent and patent periods and intensity of oocyst shedding during infection, infectivity for different species of hosts, or virulence in different hosts.

The aim of the present study was to characterise the *C. parvum* isolates originating from naturally infected woodland and field rodents which are known to be the important reservoir hosts in Poland [2].

MATERIALS AND METHODS

Measurements of oocysts

Material. During field studies, faecal smears were made from pellets found in the traps used for catching rodents. Bank voles and yellow-necked mice were trapped in a forest area and common voles trapped in grassy fallow fields adjoining the forest site [2]. Slides were stained by a modified Ziehl-Neelsen procedure [11].

Measurements. Oocysts were scanned and measured under 1,000 × magnification in an Olympus BX50F4 microscope provided with the software package Analysis Pro 2.11. For each oocyst, 2 measurements were taken: the length along the longest axis, and the width, perpendicular to the length.

Cross-transmission study

Animals. 46 pathogen-free C57BL/6 mice of both sexes, 12 weeks old, were obtained from Department of Genetics and Laboratory Animal Breeding, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw, Poland. All mice received Dexamethasone in the drinking water at a concentration equivalent to 0.25 mg/g body weight per day beginning from 11th day before inoculation until the end of the experiment.

Parasites. During field work on *C. parvum* infections in wildlife rodents in NE Poland [2] individual isolates of parasites were collected from 3 species of rodents: 12 from bank voles (*Clethrionomys glareolus*); 9 from yellow-necked mice (*Apodemus flavicollis*) and 12 from common voles (*Microtus arvalis*). Oocysts of *C. parvum* genotype C originated from a naturally-infected calf and were passaged through C57BL/6 mice and used as control of infections for comparison with the rodent isolates.

Preparation of inocula. *C. parvum* oocysts were concentrated using the modified Sheather's sugar flotation technique, as previously described [2, 8]. The immunofluorescent kit (IFA) MerIFluor *Cryptosporidium/Giardia* (Meridian Diagnostics, Cincinnati, Ohio, USA) was used for detection of parasites and for counting out infective doses.

Oocysts of each isolate were processed separately and then stored in phosphate–buffered saline pH = 7.2 at a temperature +4ºC. The number of oocysts in infective doses varied depending on the intensity of oocyst shedding from hosts carrying the original infection. The doses are shown in Table 2.

Administration of inocula to mice. Mice were divided into the following 5 groups:

(1) 9 mice were infected with 2.4×10^4 oocyst of *C. parvum* genotype C (CPC-mice);

(2) 12 mice were infected with different doses of *C. parvum* oocysts originating from naturally-infected bank voles (CG-mice);

(3) 9 mice were infected with different doses of *C. parvum* oocysts originating from naturally-infected yellow-necked mice (AF-mice);

(4) 12 mice were infected with different doses of *C. parvum* oocysts originating from naturally-infected common voles (MA-mice);

(5) 3 mice were left uninfected to act as the control group.

The animals were inoculated orally with the oocysts suspended in 0.2 ml of water with a 23-gauge straight feeding needle, and were housed in individual cages with wire bottoms to enable the faecal samples to be collected and to prevent autoinfection. Food and drinking water were available *ad libitum*.

Monitoring of infection. Faecal samples from experimental animals were collected at 7-day intervals post-infection (dpi), starting on day 7 until day 60 pi. Samples were concentrated using Sheather's sugar flotation as described above. IFA was used for estimation of the intensity of oocysts shedding per 1g of faeces.

Statistical analysis. Comparison of the course of infection (expressed in oocysts shedding) between 3 wild *C. parvum* isolates was performed by 3-way analysis of variance (3-way ANOVA) with normal errors after

normalization of the data by LOG10 (x+1) transformation. The day of post-infection (8 levels), No. of separated isolates (n = 33), and isolate host origin (3 levels) were entered as factors.

Comparison of the dimensions of oocysts from the 3 wild *C. parvum* isolates was conducted by 2-way analysis of variance (2-way ANOVA) with normal errors. The number of separated isolates (n = 25) and the host origin of each isolate (3 levels) were entered as factors. All calculations were carried out using the software package Statgraphics Version 7.

RESULTS

Measurements of oocysts. Measurements were collected for 226 oocysts from 13 individuals of *C. glareolus*; for 68 oocysts from 6 individuals of *A. flavicollis*; for 113 oocysts from 6 individuals of *M. arvalis* and for 78 calf oocysts. The means and ranges for all isolates are presented in Table 1. There were no differences in oocysts morphology and shape coefficients between the three rodents isolates and between them and the calf isolate (Fig. 1, Tab. 1). Although the differences in length and width between isolates from the 3 host species were not significant (species of origin × oocyst length: $F_{2,406}$ = 1.178, p = NS; species of origin × oocyst width: $F_{2,406}$ = 1.680, p = NS) overall the rodent *C. parvum* oocysts were slightly bigger than calf *C. parvum* oocysts (Tab. 1). In contrast to the similarity of oocyst dimensions from the 3 rodents species, differences in length and width between individual animal isolates were significant (isolate number × oocyst length: $F_{24,406}$ = 1.940, p = 0.0070; isolate number × oocyst width: $F_{24,406}$ = 2.224, p = 0.0013).

Cross-transmission study

Infectivity, prepatent and patent periods. All 33 *C. parvum* rodent isolates produced infections in C57BL/6

Table 1. Comparison of *C. parvum* oocysts dimensions from 3 rodent host species.

Species and number of host	Dimensions of oocysts						
	n	Length (µm)		Width (µm)		Shape coefficient length : width	
		Range	Mean	Range	Mean	Range	Mean
Clethrionomys glareolus							
20/116	21	3.96–5.30	4.68	3.50–5.07	4.33	1.00–1.36	1.09
99/33	12	3.41–5.23	4.40	3.10–5.23	4.03	1.00–1.27	1.10
D104	14	3.73–5.12	4.57	3.47–4.89	4.11	1.01–1.32	1.12
D111	27	3.82–5.33	4.65	3.54–4.96	4.21	1.00–1.33	1.11
D118	18	3.67–5.62	4.71	3.60–5.30	4.28	1.01–1.24	1.10
D120	27	4.09–6.11	4.78	3.56–5.34	4.26	1.01–1.27	1.12
D142	22	3.85–5.43	4.58	3.30–5.20	4.17	1.01–1.24	1.10
D156	22	3.92–5.38	4.66	3.60–4.69	4.11	1.01–1.37	1.14
D178	11	3.88–5.03	4.60	3.59–4.69	4.22	1.02–1.21	1.09
D96	11	3.95–5.25	4.82	3.61–5.13	4.25	1.02–1.30	1.14
n220	8	4.03–4.82	4.43	3.52–4.64	3.94	1.01–1.25	1.13
n254	27	3.73–5.68	4.88	3.55–5.01	4.37	1.00–1.27	1.12
n296	6	3.94–4.99	4.43	3.80–4.51	4.20	1.02–1.11	1.05
total	226	3.67-5.68	4.67	3.10-5.34	4.21	1.00-1.37	1.11
Apodemus flavicollis							
20/105	27	3.74–5.74	4.56	3.30–4.84	4.15	1.01–1.26	1.10
20/117	12	4.13–5.79	5.08	4.04–5.77	4.64	1.00–1.26	1.10
20/133	6	4.29–5.57	4.84	3.39–4.84	4.22	1.00–1.27	1.15
20/143	8	3.66–5.68	4.44	3.00–5.33	3.86	1.04–1.63	1.16
D176	11	3.76–5.17	4.56	3.21–4.95	3.88	1.01–1.37	1.18
n338	4	3.76–5.25	4.39	3.64–4.08	3.80	1.03–1.37	1.16
total	68	3.66–5.79	4.65	3.00–5.77	4.14	1.00–1.63	1.13
Microtus arvalis							
20/200	23	3.69–5.46	4.62	3.51–5.27	4.13	1.02–1.38	1.12
20/20	25	4.22–5.79	4.83	3.56–5.34	4.29	1.02–1.29	1.13
20/209	22	3.80–5.70	4.68	3.27–5.28	4.13	1.00–1.35	1.14
20/212	15	3.45–6.09	4.53	3.39–5.00	3.95	1.01–1.34	1.15
20/21	21	3.22–5.22	4.58	3.21–4.60	4.16	1.00–1.27	1.10
20/214	7	4.33–5.13	4.67	3.88–4.78	4.31	1.02–1.18	1.08
total	113	3.22–6.09	4.66	3.21–5.34	4.16	1.00–1.38	1.12

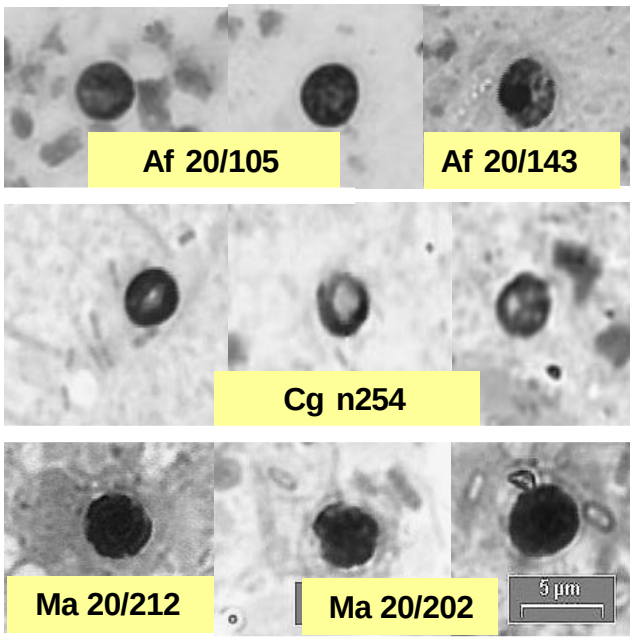

Figure 1. Morphology of *Cryptosporidium parvum* oocysts derived from three rodent species. Af - oocysts from *Apodemus flavicollis*, Cg - oocysts from *Clethrionomys glareolus*, Ma - oocysts from *Microtus arvalis*.

mice (Tab. 2), as did also the calf isolate. For all CPC- and AF-mice, for 11 of the 12 MA-mice and for 10 of the 12 CG-mice the prepatent period lasted less then 7 dpi. The remaining 3 mice (1 MA-mouse and 2 CG-mice) started oocyst shedding between 7–14 dpi. The patent period during which mice shed oocysts lasted more than 8 weeks for all CG-mice and the majority of MA- and AF-mice, until the end of the experiment 60 dpi. Only one of the 12 MA-mice (MA-10 mouse) stopped shedding oocysts between the 5th and 6th week of the experiment and only one of the 9 AF-mice (AF-12 mouse) ended oocyst shedding between the 6th and 7th week of experiment.

Comparison of courses of infection. There were significant differences in the course of infection between the 3 groups of rodent *C. parvum* isolates (species of origin × oocysts shedding: $F_{2,263} = 9.695$, $p = 0.0010$) (Fig. 2). The highest overall mean oocysts output was found in CG-mice (41,739 oocyst/1 g of faeces) with a maximum 14 dpi of over 65,000 oocysts/1 g of faeces. Sixty dpi, the mean output for CG-mice, was much higher than from the other two groups, almost exceeding 40,000 oocysts/1 g of faeces (Fig. 2). The highest individual oocyst output was also found in CG-mice - over 324,000 oocysts/1 g of faeces for CG-5 mouse 35 dpi, and over 280,000 oocysts/1 g of faeces for CG-4 and CG-3 mice 14 and 41 dpi, respectively (Tab. 2). By the end of the experiment, CG-mice were producing between 416 and 57,000 oocysts/1 g of faeces (mean = 39,000 oocysts/1 g of faeces) (Fig. 2).

The intensity of infection measured as an overall mean oocysts output was about 50% lower in the group of AF-

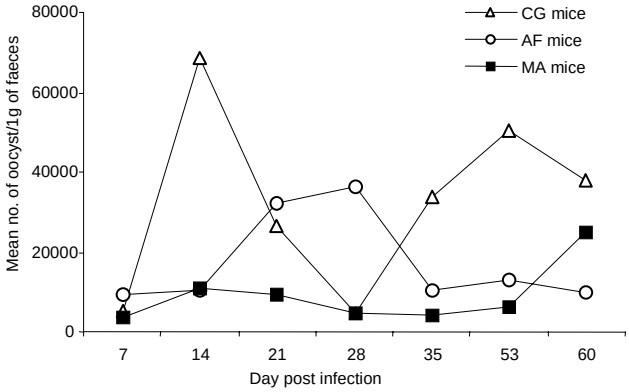

Figure 2. Comparison of the course of *Cryptosporidium parvum* infections between 3 rodent isolates. The faecal oocysts concentration was determined by extraction of oocysts from faeces, purification by sugar solution gradient centrifugation and immunodetection using the MeriFluor *Cryptosporidium/Giardia* test kit for direct immunofluorescence.

mice, just exceeding 18,000 oocysts/1 g of faeces. Maximum oocysts output per day was observed in this group two weeks later, 28 dpi, and exceeded 38,000 oocysts/1 g of faeces (Fig. 2). The highest individual oocysts output for AF-mice exceeded 105,000 oocysts/1 g of faeces for mouse AF-7 27 dpi (Tab. 2). By the end of the experiment, 8 AF-mice produced between 750 and 30,000 oocysts/1 g of faeces with the mean output only 25% of that in CG-mice (10,000 oocysts/1 g of faeces) (Fig. 2).

The lowest overall mean oocysts output was found in MA-mice (10,384 oocysts/1 g of faeces). The intensity of infection measured as an overall mean oocysts output was the lowest in this group throughout the experiment, excepting 60 dpi, when the maximum daily oocysts output was found to be almost 30,000 oocysts/1 g of faeces (Fig. 2). The highest daily individual oocysts output from MA-mice was very similar to that found in AF-mice and exceeded over 95,000 oocysts/1g of faeces for MA-11 and MA-12 mice, 60 dpi (Tab. 2). By the end of the experiment, MA-mice produced between 159 and 96,000 oocysts/1 g of faeces.

DISCUSSION

The present field and experimental study was the first attempt to characterise rodent *C. parvum* isolates in Poland by their biological properties. We found that the measurements of oocyst dimensions and oocysts morphology did not allow distinction between the parasite isolates from the three rodent species.

The dimensions of oocysts, average length, width and shape were identical for *C. parvum* originating from *C. glareolus*, *A. flavicollis* and *M. arvalis*. Moreover, differences in oocysts dimensions between individual hosts, irrespective of species, were more marked than those between the 3 rodent isolates. However, only 1 species from the "*C. parvum*" group parasite can be distinguish on the basis of oocysts dimensions, namely *C. felis* [24]. All the other strains and species (e.g. "dog"

Table 2. Doses and oocyst shedding intensities after infection of *C. parvum* isolates originating from *C. glareolus*, *A. flavicollis*, *M. arvalis*.

Number of primary host	Number of experimental mice	Infective dose (No. of oocysts per inoculum)	Infectivity result	Oocyst output (no. of oocysts/1g faeces)	
				mean	result
Clethrionomys glareolus					
n220	CG-1	200	+	32462	6562–106111
n 254	CG-2	4000	+	89457	200–290454
n 296	CG-3	1460	+	61032	0–283000
99/33	CG-4	220	+	71202	0–324225
D96	CG-5	1365	+	744	0–3500
D104	CG-6	900	+	11146	0–50482
D111	CG-7	1014	+	18194	698–74863
D118	CG-8	576	+	23765	266–72496
D120	CG-9	324	+	37283	51–135227
D142	CG-10	5220	+	14076	214–53750
D156	CG-11	1950	+	26977	385–91071
D178	CG-12	1845	+	25731	3524–113196
mean		1590		41639	0–290454
Apodemus flavicollis					
D95	AF-1	99	+	9783	2872–20800
D164	AF-2	36	+	22274	2300–84643
D176	AF-3	108	+	3100	1912–24070
D189	AF-4	36	+	43417	5100–105116
20/105	AF-5	33	+	11662	1802–35854
20/116	AF-6	39	+	21040	750–59000
20/117	AF-7	33	+	12711	5714–30156
20/133	AF-8	39	+	21049	3333–89531
20/143	AF-9	33	+	5696	256–15593
mean		51		18114	256–105116
Microtus arvalis					
D5	MA-1	210	+	7605	0–20221
D17	MA-2	35	+	585	0–1786
H1	MA-3	504	+	12355	2721–26474
H2	MA-4	72	+	36894	641- 17105
20/120	MA-5	1485	+	5244	43–15385
20/129	MA-6	280	+	74	0–493
20/200	MA-7	288	+	18677	769–96154
20/202	MA-8	264	+	31626	455–95250
20/209	MA-9	132	+	4406	188–11500
20/212	MA-10	104	+	6011	510–17066
20/213	MA-11	99	+	10493	102–26118
20/214	MA-12	138	+	11252	272–28520
mean		301		10384	0–96154

C. parvum strain, *C. wrairi*, *C. meleagridis*) have morpho-logically identical oocysts [29].

Although our study was conducted using a susceptible laboratory mouse strain [22] and all animals were immunosuppressed before and during the experiment, the successful cross-transmission from wild hosts to laboratory rodents suggests that host specificity is not strict in this case, and that the rodent *C. parvum* isolates are infective to more than the host species of origin. It is therefore possible that *C. parvum* isolates from rodents may be infective to other hosts including man, and hence rodents as their reservoir host should be considered a potential hazard for the health of human communities in the vicinity.

Many transmission experiments have revealed that *C. parvum* infections with oocysts of domestic mouse (*M. musculus*) origin were readily transmissible to livestock animals i.e. calves and goats and also rabbits [13, 18]. Successful *C. parvum* cross-transmission have also been reported for other species of rodents. Matsui *et al.* [14] have transmitted *C. parvum* from naturally-infected Siberian chipmunk (*Tamias sibiricus*) to laboratory

mice; however, they failed to infect laboratory rats, guinea-pigs and rabbits with this isolate.

In our primary molecular study [1] we have shown by sequence analysis of the COWP gene fragment from the 3 *C. parvum* isolates originating from *C. glareolus*, *A. flavicollis* and *M. arvalis* that these animals carried a *C. parvum* genotype almost identical with "mouse" genotype previously described from *Mus musculus* [15]. Thus, small rodents should be considered as an important reservoir of *C. parvum* genotypes closely related to the zoonotic genotype 2 and potentially hazardous to humans.

However, the differences in the sequences of the COWP gene were too small to distinguish between 3 rodent *C. parvum* isolates [1]. In the present study, despite differences between individual isolates and the different infective doses used, we found significant differences in the course of infection among the 3 experimental groups. The course of infection with *C. parvum* in C57BL/6 mice was fairly typical in the case of CG-mice, with a characteristic acute phase during the first 2 weeks, and a long, chronic phase in the following weeks during which oocysts output fell, suggesting progressive self-cure. In AF-mice initial oocysts output was low, and the acute phase was observed later and was less intense. The poorest oocysts output initially was when the oocysts were derived from the isolates from *M. arvalis*, and the general pattern was for a low intensity output reflecting a chronic infection without a clear acute phase.

In the presented study we have demonstrated biological characterisation of 3 *C. parvum* isolates originated from 3 species of common rural rodents in Poland. We have found that oocysts dimensions and oocysts morphology are not valuable tools for distinguishing between isolates, however these 3 groups of isolates have produced significantly different pictures of infection in C57BL/6 mice. Taking together these data and primary molecular characterisation of isolates [1] we suppose that rodent isolates of *C. parvum* show some signs of specification and could represent new subgroups in so called "mouse" *C. parvum* strain. However, the successful cross-transmission from wild hosts to laboratory rodents and the close similarity of COWP sequence between our isolates and "mouse" genotype and between "mouse" and zoonotic genotype of *C. parvum* (genotype C) inform us that all these isolates should be treated as potentially hazardous for human health.

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