

A study on occupational exposure of Sicilian farmers to *Giardia* and *Cryptosporidium*

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Key words

Giardia/*Cryptosporidium* • Calves • Occupational risk

Summary

Introduction. A cross-sectional study was undertaken to determine the prevalence of *Giardia* and *Cryptosporidium* in calves of Palermo area (Sicily) and to evaluate the occupational risk associated with occurrence of zoonotic genotypes.

Methods. A total of 217 faecal samples, from 149 calves (between 2 and 240 days of age) and 68 farmers, were collected in 19 cattle-farms of Palermo area. A questionnaire regarding demographic characteristics and personal hygienic measures was submitted to all farmers. All faecal samples were analyzed by Immunofluorescence assay and Polymerase Chain Reaction (PCR); genotypes were determined by DNA sequencing of Triose Phosphate

Isomerase gene for *Giardia* and Small Subunit Ribosomal RNA gene for *Cryptosporidium*.

Results. None farmer tested was positive for *Giardia* and *Cryptosporidium*, whereas these protozoa were respectively detected in 53 (including 5 with zoonotic *G. duodenalis* genotype A) and 17 (of which 1 with zoonotic *C. ubiquitum*) of the examined calves.

Discussion. The results indicate that the risk of transmitting both protozoa to farmers in Palermo area is negligible although it cannot be considered null because of identification of human genotypes/species in calves.

Introduction

Giardia duodenalis (synonyms are *G. intestinalis* and *G. lamblia*) and *Cryptosporidium* spp. are common parasitic protozoa responsible for enteric illness in humans and animals worldwide [1]. *Cryptosporidium* in particular is a serious pathogen in immunocompromised individuals who may suffer from persistent life-threatening gastroenteritis [2]. The (oo)cysts are excreted in the faeces of infected hosts and transmitted through the faecal-oral route, contaminated water (drinking/recreational), ingestion of contaminated food and direct contact between humans and infected animals [3, 4]. Transmission via direct contact, in particular, is related to workers such as farmers and veterinarians and it suggests that compliance with hygienic standards (i.e. frequent hand-washing or constant use of gloves) to prevent transmission of both protozoa to persons handling animals. The infected hosts shed a lot of (oo)cysts in their faeces, for example one calf (≤ 30 days old) can produce up to 6×10^8 *Cryptosporidium* oocysts per gram of faeces, thereby increasing environmental contamination [5]. The (oo)cysts are robust and very resistant to disinfectants and can last for long periods (months) in the environment [6]. Sources of contamination of water and food may be different, but an important role is played by farm animals that act as reservoirs of infection. *Cryptosporidium* and *Giardia* are common in domestic livestock (cattle, sheep and pigs), where young animals can have a high prevalence of infection, shedding large numbers of (oo)cysts.

In recent years, the zoonotic potential of both protozoa is becoming clearer with the use of molecular techniques to genotype isolates. Molecular characterization of *G. duodenalis* has led to subdivision of the species into seven distinct assemblages (A, B, C, D, E, F, G): humans are infected with assemblages A and B which can also infect wildlife, companion animals and livestock; C and D, isolated from dogs; E, isolated from livestock; F, isolated from felines; and G, isolated from rats. Molecular analyses have also shown that the genus *Cryptosporidium* comprises 19 valid species and nearly twice as many genotypes [6]; *C. parvum* and *C. hominis* are the main species infecting humans [7]. Cattle are infected with at least five *Cryptosporidium* species (*C. parvum*, *C. andersoni*, *C. bovis*, *C. ryanae* and *C. suis*) and are considered the main reservoir of zoonotic *C. parvum* [8]. The distribution of *Cryptosporidium* species in dairy cattle is age-related: *C. parvum* is reported to primarily infect pre-weaned calves (5 days to 5 months) [9], *C. bovis* is found to predominate in 3 month to 2 year-old dairy cattle [8], *C. andersoni* usually infects mature cattle, resisting for years, if not for life. In Canada a prevalence in dairy calves (0-24 weeks of age) has been reported between 45.7 % and 73 % for *G. duodenalis*, and between 40.6 % and 88.7 % for *Cryptosporidium* spp., suggesting a potential risk of zoonotic transmission between cattle and farm workers [10]. In India, *G. duodenalis* assemblage A subgenotype 1 (A1), which is considered the most common zoonotic agent, was identified in both calves and workers, confirming that there is a

risk of occupational transmission of *G. duodenalis* infections between cattle and humans on dairy farms [11]. The strongest epidemiological evidence for zoonotic transmission of cryptosporidiosis is from investigations associating cattle with outbreaks in veterinary students handling infected young calves, animal researchers in contact with infected young calves, and children attending agricultural camps and fairs [12].

In a previous study carried out in Sicily [13], we demonstrated that the Oreto river which crosses the city of Palermo was contaminated by *Giardia* and *Cryptosporidium* and that *Giardia* was present in a high concentration in the water of the river. Our subsequent paper confirmed higher prevalence of *Giardia* than *Cryptosporidium* and showed that the Oreto river was contaminated by assemblages A and B of *Giardia*, with a predominance of zoonotic genotype A [14].

The aim of this study is to obtain data about prevalence of these two parasites in calves and farmers who come from cattle farms in the Palermo area, and to determine the occupational risk associated with occurrence of species and genotypes at zoonotic risk.

Methods

COLLECTION OF SPECIMENS

A list of commercial cattle farms was obtained from Veterinary District-Azienda Sanitaria Provinciale 6 (ASP 6) in Sicily. Between February 2009 and July 2010, 19 (19/62) farms from the eastern area of the city of Palermo were chosen due to the recent birth of calves, after which the farmers, who had been contacted by veterinarians of the ASP 6, confirmed their participation in the study. If any farm chose not to participate, it was excluded from the study and replaced by another farm. Selected farms included from 5 to 95 cows: four herds had from 5 to 10 animals, seven from 11 to 40, five from 41 to 70, three from 71 to 95; 11 farms were in the wild, the remaining 8 were cattle shed farms.

A total of 149 calves faecal samples were collected directly from the rectum of each animal using a disposable latex glove; 69 samples were collected during winter months and 80 during summer months. The calves, with or without diarrhoea, aged between 2 and 240 days of age (52 ranged from 2 to 60 days, 74 from 61 to 150 days, 23 from 151 to 240 days); 17 calves (7-150 days age) showed evidence of watery diarrhoea.

Human faecal samples were obtained from the faeces of 68 farmers (66 males and 2 females; the age ranged from 18 to 63 years) and examined for the presence of *Giardia* and *Cryptosporidium*. Simultaneously with the collection of samples, workers received a medical-social questionnaire comprising 15 questions.

All faecal samples were placed in a sterile vessel and transported to the laboratory in a proper recipient at 4° C for analysis within 24 hours of collection.

CONCENTRATION OF (OO)CYSTS FROM FAECES

The animal faecal samples were subjected to purification by a sucrose flotation technique as previously described [15]. Approximately 3 g of faeces were suspended in 10 mL of phosphate buffered saline (PBS). The suspension was filtered through a surgical gauze sponge and the filtrate was stratified into 5 ml of 1 M sucrose (Sigma-Aldrich) solution. The suspension was centrifuged at 800 x g for 5 min without brake. Following centrifugation, the interface and the upper layer of liquid was transferred to a clean tube and recentrifuged at 800 x g for 5 min. The supernatant was decanted and the pellet resuspended in PBS to a volume of 1 ml to examine by immunofluorescence assay.

All human faecal samples were examined by formol-ether concentration. Briefly, 2-3 g of faeces were suspended in 10mL of saline solution and filtered through a surgical gauze. The filtrate was then centrifuged at 1500 rpm for 2 min. The supernatant was decanted and the pellet was suspended in 7 ml of formalin at 7% and 3 ml of ethyl acetate. The sample was mixed for 3 min and centrifuged at 1500 rpm for 2 min. The supernatant was discharged and the final pellet was examined by immunofluorescence assay (IFA).

IMMUNOFLUORESCENCE ASSAY (IFA)

All resulting pellet were processed by IFA (Merifluor *Cryptosporidium/Giardia* assay; Meridian Biosciences) for the simultaneous detection of *Cryptosporidium* and *Giardia*. The slides were observed with an epifluorescence microscope at 400 x magnification for the detection of FITC-mAb labeled oocysts/cysts. Presence of stained oocysts/cysts was identified according to morphology.

MOLECULAR ANALYSIS

The IFA positive *Giardia* and *Cryptosporidium* samples were subjected to molecular analysis. DNA was extracted as described by da Silva et al. [16]. The PCR of *Giardia* and *Cryptosporidium* were performed by amplification of the triose phosphate isomerase (TPI) and small subunit ribosomal RNA (ssuRNA) genes, respectively. Nested-PCR was used to amplify fragments of both genes [17]. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized after ethidium bromide staining.

The secondary PCR products were purified using Microcon PCR centrifugal filter devices (Millipore Corp., Bedford, MA) and sequenced on an ABI 3100 automated sequencer (Perkin Elmer). Sequence accuracy was confirmed by sequencing an independent PCR product on each strand. Multiple alignments were performed using the computer software package Clustal X [18].

Published *Giardia* TPI and *Cryptosporidium* ssuRNA nucleotide sequences were aligned with the homologous sequences determined in the present work.

QUESTIONNAIRE

During this study 74 farmers were willing to participate and were enrolled into the survey; six subjects refused to answer the questionnaire and were therefore excluded from the study. All enrolled participants (n=68) declared not to be in a immunosuppressed status.

A series of multiple choice questions (n = 15) were asked regarding the attitude and practices towards preventive measures taken to avoid occupational infections as well as socio-demographic characteristics (sex, age, length of service, distance between farm and home, household composition) and medical information (diarrhoea events) were also collected. Subjects were asked the circumstances of hand washing (i.e. during handling animals, at the beginning or end of the working day) and the use of gloves, the custom of biting one's nails and of changing clothing before going home. Questions about changing clothes and shoes were used to understand infectious risk levels for themselves and for their families as the clothing, and in particular the shoes, could be carriers of *Giardia* and *Cryptosporidium* (oo)cysts and therefore play a role in their transmission to other environments. Moreover, the participants were asked to answer two questions related to a number of personal cases of diarrhoea during the last year and to those that occurred in their families during the last 6 months.

Statistical analysis

All collected data were entered into an electronic database and analyzed using Epi Info software, version 7. Calves age was categorized into three groups (2 to 60 days; 61 to 150 days and 151 to 240 days). Absolute and relative frequencies were calculated for qualitative variables, while quantitative non-normally distributed variables were summarized as median and/or range. Normal distribution was verified by the Shapiro-Wilk test for normality.

Results

Assemblages/Species of *Giardia* and *Cryptosporidium*

Giardia and *Cryptosporidium* were found in 78.9% and in 52.6% of the cattle farms, respectively. The overall proportion of animal samples containing detectable *G. duodenalis* cysts and *Cryptosporidium* oocysts was 35.6% and 11.4%, respectively. Mixed infections of *Giardia* and *Cryptosporidium* were found in 14 dairy calves out of 149; of these, 10 were detected in animals from 2 to 60 days of age, 2 in those between 61-150 days of age and 2 between 180-240 days of age. Moreover, we observed no seasonality among the *Giardia* and *Cryptosporidium* infections, instead as previously reported in Norway [19] where the prevalence of both parasites was higher in samples taken during winter than in samples taken during summer.

There was a large variation in prevalence of *Giardia* and *Cryptosporidium* infected animals among age groups. Only for *Giardia* there was a trend for decreasing intensity of infection with increasing age of calves. In fact, prevalence of *Giardia* varied from 65.4% to 20.2% and 17.4%, while for *Cryptosporidium* it varied from 21.1% to 2.7% and 17.4% (Tab. I). Faecal consistency at sampling was watery in 14 younger calves (under 60 days) and in 3 animals of age-group 61-150 days; none of the 23 calves older than 150 days showed diarrhoea. The watery consistency of the stools was associated with increased detection of both parasites. *Giardia* was detected in 16 while *Cryptosporidium* in 6 of 17 diarrhoeic faecal samples (Tab. I).

All 68 human faecal samples were negative for detection of *G. duodenalis* and *Cryptosporidium* spp. including farmers (n = 15) who had contact with infected animals and did not wash their hands at work.

Of the animal samples tested positive for *Giardia* and *Cryptosporidium* by IFA, the PCR was successful in 34 (25 ranged from 2 to 60 days, 8 from 61 to 150 days, 1 from 151 to 240 days) and 12 (10 ranged from 2 to 60

Tab. I. Age-related prevalence of *Giardia* and *Cryptosporidium* in 149 calves between 2 and 240 days of age, coming from 19 farms, as determined by IFA.

Calves age	Total calves	Faecal consistency	<i>Giardia</i> Positive IFA (%)	<i>Cryptosporidium</i> Positive IFA (%)	Mixed infections (<i>Giardia</i> / <i>Cryptosporidium</i>) (%)
2-60 days	52	14 watery	14	5	5
		38 normal	20 (65.4)	6 (21.1)	5 (10.9)
61-150 days	74	3 watery	2	1	2
		71 normal	13 (20.2)	1 (2.7)	0 (2.7)
151-240 days	23	0 watery	0	0	0
		23 normal	4 (17.4)	4 (17.4)	2 (8.1)
Tot 149			(35.6)	(11.4)	(9.4)

Tab. II. Age-related positive samples for *Giardia* and *Cryptosporidium* by PCR and identification of genotypes as determined by sequencing of the TPI gene for *Giardia* and SSUrRNA gene for *Cryptosporidium*.

Calves age	<i>Giardia</i>	<i>G. duodenalis</i> genotypes (n)	<i>Cryptosporidium</i>	<i>Cryptosporidium</i> species (n)
	No. of positive samples by PCR/No. of positive samples by IFA		No. of positive samples by PCR/No. of positive specimens by IFA	
2-60 days	25/34	A (4); E (15) ^a	10/10	<i>C. ryanae</i> (4) <i>C. bovis</i> (5) <i>C. ubiquitum</i> (1)
61-150 days	8/15	A (1); E (6) ^b	1/1	<i>C. bovis</i> (1)
151-240 days	1/4	E (1)	1/1	<i>C. bovis</i> (1)

^a 19/25 PCR products were successfully sequenced^b 7/8 PCR products were successfully sequenced

days, 1 from 61 to 150 days, 1 from 151 to 240 days) samples respectively. *Giardia* genetic sequencing was successful on 27 of the 34 PCR positive samples.

Giardia genotypes showed 100% homology with assemblage E (n = 22) (GenBank accession number AB569406), and assemblage A (n = 5) (GenBank accession number AB569398); the zoonotic genotype *Giardia* assemblage B was not detected.

Of the 12 samples from which DNA amplification and genetic sequencing was obtained for *Cryptosporidium*, 7 samples exhibited 100% homology with *C. bovis* (GenBank accession number HQ179575), 4 samples had 100% homology with *C. ryanae* (GenBank accession number HQ179574), and finally 1 sample showed 100% homology with *C. ubiquitum* (GenBank accession number EU827382) (Tab. II).

QUESTIONNAIRE RESPONSES

68/74 farmers returned the completed questionnaire. The analysis of the demographic and practice characteristics of the study group showed that the majority was male (n = 66); the age of respondents ranged from 18 to 63 years, with a median age of 35 years. 38 respondents had worked for more than 15 years, while 20 had worked for 4 to 15 years and 10 for less than 4 years. The household composition of 49 participants ranged from 3 to 5 members: of 14 participants from 6 to 7 and finally of 5 participants from 1 to 2. The distance between farm and home is more than 1 kilometre for 58 farmers, while 10 people live next to the farm. The behaviours related to use of protective equipment and to personal hygiene and their clinical status were also evaluated as shown in Table III.

Discussion

To our knowledge, this is the first study to report prevalence and molecular characterization data of *G. duodenalis* and *Cryptosporidium* spp. in cattle farms in the Palermo area. Data collected from 19 farms in Palermo area indicate that giardiasis and cryptosporidiosis are

common infections of calves and that the prevalence of *Giardia* infection is higher than that of *Cryptosporidium*. This result is in agreement with the previously reported findings in Spain [20] and in Australia [21], but not with those described in Portugal, where the prevalence of *Cryptosporidium* exceeded that of *Giardia* [22]. Moreover, ours and other data in literature report two main points: a) the decreasing age-related prevalence of *Giardia* in calves, b) the concurrent *G. duodenalis* and *Cryptosporidium* spp. infections [23].

We have found two distinct genotypes of *G. duodenalis*: assemblage A, which is also isolated from humans and other animals and proposed to be zoonotic [23], and assemblage E, which is believed to be specific for livestock [24]. However, recent studies conducted in Uganda and in Egypt revealed the presence of assemblage E in non-human primates (red colobus monkeys) and in humans respectively [25, 26]. Assemblage E has been reported in the area of Gharbia (Egypt) where two thirds of the inhabitants belong to a rural community who live in close contact with their livestock and implement inadequate hygiene practices. However, because of a high prevalence of assemblage E in calves [25] and of the possible cattle-primate transmission link, a strong implementation for future research into the epidemiology, cross-species transmission ecology and clinical consequences of *G. duodenalis* infection in humans are required. In this study it is probable that both the lack of the above mentioned inadequate conditions of hygiene in our area may be cause for the no transmission of zoonotic and non-zoonotic genotypes/species to farmers.

In particular, in the present study there are three main hygienic measures implemented by the participants: 1) no biting of nails, 2) change of shoes before returning home, 3) hand-washing prior to eating, drinking, or smoking at work and after contact with animals. Nails, in particular long nails, are a receptacle of microorganisms and the absence of the practice of nail-biting (82.4% of respondents) is a healthy measure that can prevent infections in individuals handling infected animals. Changing shoes at the end of the day (36.7% of respondents) is an important hygienic measure because

Tab. III. Compliance with hygienic measures and reports of diarrhoea in 68 farmers who responded to the survey.

Personal hygienic measures	Frequency	N	%
Use of gloves at work	always	12	17.6
	sometimes	18	26.5
	never	38	55.9
Washing hands before drinking/eating/ smoking at work	always	37	54.4
	sometimes	16	23.5
	never	15	22.1
Washing hands after animal contacts	always	40	58.8
	sometimes	3	4.4
	never	25	36.8
Washing hands at the end of working day	always	13	19.1
	sometimes	14	20.6
	never	41	60.3
Washing hands return home	always	25	36.8
	sometimes	10	14.7
	never	33	48.5
Practice of nail biting	always	10	14.7
	sometimes	2	2.9
	never	56	82.4
Change of work clothes at the end of the day	always	25	36.7
	sometimes	31	45.6
	never	12	17.7
Change of work shoes at the end of the day	always	43	63.2
	sometimes	13	19.1
	never	12	17.7
Diarrhoea during last year	no episode	49	72.0
	1 episode	8	11.8
	2 episodes	7	10.3
	> 2 episodes	4	5.9
Diarrhoea in family during last 6 months	no episode	62	91.2
	1 episode	6	8.8
	2 episodes	0	0
	> 2 episodes	0	0

the soles could be carriers of *Giardia* and *Cryptosporidium* (oo)cysts (present in soil) which then spread at home, with consequent risks of infection among family members, especially children who often play on the ground. Hands represent the principal route of transmission (i.e. person-to-person or animal-to-person) of many pathogens that can survive for a long time on the skin, in particular *Giardia* and *Cryptosporidium* that produce robust (oo)cysts, therefore a high compliance with hand-washing is of crucial importance to prevent the transmission of microorganisms that can be transmitted orally when hands are brought near the mouth to eat, drink or smoke. However, it is important to emphasize that other infectious occupational risks (i.e. mycosis) [27] associated with animal contact highlight a need for the use of personal protective equipment, and the infrequent use of gloves in this report is a critical point that identifies farmers of the Palermo area as a target group for future educational campaigns of public health, as educational interventions may improve knowledge and practice related to prevention.

Furthermore, we have not found either the zoonotic *Giardia* assemblage B (which has only occasionally been reported in calves), nor the zoonotic *C. parvum* found up to 100% of calves at 1 and 2 weeks of age [9]. On the contrary, *C. bovis* (considered cattle-specific) was

the most common species identified (n=7), particularly in age-group 2-60 days (n = 5), as already reported in China, India, Georgia and western North Dakota [28-30]. Recently, *C. bovis* has been identified in farm workers in rural areas both in India [11] and in Australia [31], providing evidence of zoonotic transmission and the possible association of infected calves and human infection with *Cryptosporidium*; interestingly, in both studies no clinical symptom was reported from infected individuals.

Conclusion

The absence of *C. parvum*, a major pathogen in humans, the occurrence only once of *C. ubiquitum*, potentially zoonotic, the relative low prevalence of *C. bovis* as well as the small number (n = 5) of zoonotic *G. duodenalis* assemblage A, suggest that calves may not be significant in the epidemiology of human giardiasis and cryptosporidiosis in the Palermo area.

For *Giardia* and *Cryptosporidium* there is a need for molecular epidemiological studies to be undertaken in well-defined foci of transmission in order to fully determine the frequency and importance of zoonotic transmission, in particular because cryptosporidiosis affects both immunocompetent and immunocompromised individuals

and in these last subjects is responsible of severe life-threatening illness [2]. Although the risk of transmitting *Giardia* and *Cryptosporidium* to occupationally exposed subjects in farms in Palermo area is negligible due to the low prevalence of protozoa and biosecurity measures implemented, the obtained data suggest that calves

can act as a source of giardiasis and cryptosporidiosis for farm workers and responsible of environmental contamination. We believe that more studies involving extensive sampling of both calves and farmers are necessary in Palermo area and in other geographic areas of Sicily.

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