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From Surveillance to Solutions: Integrating Epidemiological Analysis with Web Application Development for Combatting Zoonotic Parasites in Gennargentu - Mandrolisai, Sardinia

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Preface

This thesis is divided in two part: the first part as a 'thesis by publication' format, which includes one review and three published articles in international peer-review journal, and a experimental part which contains a final chapter with the description of the webapp.

The thesis, entitled "From Surveillance to Solutions: Integrating Epidemiological Analysis with Web Application Development for Combatting Zoonotic Parasites in Gennargentu - Mandrolisai, Sardinia", is divided into four chapters:

Chapter 1. covers a Review Article published in "The Journal of Infection in Developing Countries". The aim of this review is to provide a comprehensive overview of zoonotic parasites in a Parasitology Laboratory and their associated risks.

Chapter 2 includes Section 2.1 and Section 2.2, containing two research article, one published in "Parasitology Research" journal and one in "Food and Waterborne Parasitology" journal. The chapter focuses on the epidemiology of *Toxoplasma gondii* in two intermediate hosts, sheep and wild boar, to understand the potential risk of infection in humans and its environmental contamination

Chapter 3 consists of an article published in the international journal "Parasites & Vectors" which focuses on the edutainment approach for raising awareness about *Echinococcus granulosus* and Echinococcosis among primary school children, aiming to prevent the spread of the disease.

Chapter 4 describes the interactive website for zoonosis prevention and the establishment of telemedicine support for farmers, veterinarians, and citizens.

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Abstract

This PhD thesis aims to provide an overview of the main zoonotic parasites. The objective is to offer an epidemiological insight into zoonotic parasitosis to develop a web application for raising awareness and preventing the risk of infection among citizens and farmers in Sardinia region. This endeavour aims to establish a foundation for the development and implementation of a telemedicine tool. The thesis is composed of following chapters:

Chapter 1 contains a review article that comprehend a extensive summary of main zoonotic parasite that could be handled at parasitological laboratories and summarising the standard biosecurity protocols for the infectious agents.

Chapter 2 is divided into two sections: Section 2.1 focuses on the epidemiological and molecular aspects of Apicomplexa parasites (*Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis* spp.) in sheep intended for human consumption. In Section 2.2, the epidemiological aspects of *T. gondii* in Sardinian wild boar are described. Both surveys conducted reveal the widespread presence of *T. gondii* in both species. The prevalence varies depending on age and sex, and differences in prevalence are observed between the two species, leading to distinct epidemiological dynamics between wildlife and farmed animals.

Chapter 3 examines the impact of Cystic Echinococcosis (CE) edutainment on children's awareness and understanding of the disease. An education project conducted among primary school children in Sardinia resulted in increased awareness of CE following the intervention. The findings suggest that edutainment can effectively enhance awareness and reduce the transmission of CE in endemic communities.

Chapter 4 delineates the web application, highlighting its various sections: individual description pages for each parasite, interlinked with other pages detailing the project team's objectives, diagnostic services, and consulting offerings.

Introduction

Livestock farming stands as a cornerstone of Sardinia, encouraging local economies, preserving traditions, and nurturing unique productive specialties that are frequently undervalued. The historical region of Gennargentu Mandrolisai connects the Gennargentu massif to the river valley of the Tirso and the Oristano Gulf; it is extended for 600 km and it is one of the historical regions without access to the sea. (https://www.gennargentumandrolisai.it/vivere/cultura/93) The complexity of the geography significantly shapes the evolution of this sector, leading to underutilized territorial resources (Colavitti et al., 2021). For this reason, this area, which includes 11 municipalities (Aritzo, Atzara, Austis, Belvì, Desulo, Gadoni, Meana Sardo, Ortueri, Sorgono, Teti, Tonara), is part of the SNAI (Strategia Nazionale per le Aree Interne - *National Strategy for Inland Areas*). This strategy embodies an innovative national policy for development and territorial cohesion, with the goal of counteracting the marginalization and demographic decline prevalent in the inland regions of Italy (https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/). These inland municipalities have largely experienced processes of marginalization, marked not only by significant depopulation phenomena starting from 1980—characterized by declining and aging demographics—but also by a notable deterioration in both employment opportunities and the utilization of territorial capital. Furthermore, they have witnessed a decline in the "quality of citizenship," signifying a gradual loss of public and private services. Consequently, they are categorized as "internal areas" within the realm of cohesion indicators due to their spatial peripherality and functional marginality (Battino & Lempreu, 2017)

The landscape of Gennargentu predominantly exhibits a pastoral character. The prevalent animals in the area are primarily sheep, goats, cattle, and pigs. While horses and donkeys are also present, their numbers are comparatively small. Transhumance, once a common practice, has become rather rare and reflects the significant transformation that has occurred throughout the region in recent decades. Livestock are now primarily managed exclusively in free range, marking a departure from traditional transhumance practices (Camarda et al., 2014). In Sardinia, over 45% of the entire Italian sheep stock is raised, representing almost 4% of the European population (ISTAT 2022; Eurostat 2022, Berlinguer et al., 2021). This phenomenon is also reflected in the inland area of Gennargentu Mandrolisai, where

approximately 52173 sheep are bred among the 11 municipalities (Gennargentu Mandrolisai una vita di qualità nel cuore della Sardegna – Strategia d'Area – Strategie Aree Interne). In the Nuoro province, encompassing the Gennargentu Mandrolisai area, about 37% of industries are in the agro-livestock sector, confirming the prevalence of agro-pastoral activities in the region (Rapporto d'area versione 27 febbraio 2006 – Regione Sardegna).

The ongoing economic crisis has resulted in historic lows in investments, particularly in healthcare interventions, thereby posing significant threats to animal production. This situation has led to considerable decreases in production and subsequently driven up management expenses and consumption rates. In this context, zoonoses are of particular interest, referring to diseases that not only affect animals but can also be transmitted from animals to humans, posing significant health problems and relevant social costs (Seimenis, 2012).

Several parasitic zoonotic diseases currently exist in Sardinia, including Cystic Echinococcosis (CE), which poses significant health and economic risks and has long been a major challenge for the National Health Service (Varcasia et al., 2020). The Nuoro province, home to the Gennargentu Mandrolisai area, is considered endemic for various parasitic and zoonotic diseases. Notably, Cystic Echinococcosis and Toxoplasmosis are of particular concern due to their impact on humans and various animal species. Cystic Echinococcosis has been classified by the World Health Organization (WHO) among the seven neglected zoonotic diseases (NTDs) worldwide and is endemic in the Mediterranean region, including Sardinia. (WHO 2020, Varcasia et al., 2020, Joanny et al, 2021). Extensive sheep farming, the presence of numerous stray or shepherd dogs, unsupervised domestic slaughter, and improper carcass disposal, often coupled with a low level of health education, are the most significant factors underlying the persistence of CE in Sardinia. In fact, the prevalence of CE in Sardinian sheep is of 65.3% (Varcasia et al., 2020). In addition, from 2001 to 2010, the island recorded an *Echonococcus* infestation rate in humans of 6.5 patients per 100,000 inhabitants per year, compared to a national average of 2.4 cases per 100,000 inhabitants, with the highest peaks in the Nuoro province where the average annual rate was 12.2 patients/100,000 inhabitants (Brundu et al., 2014). The direct cost of one case of Echinococcosis to the National Health Service (SSN) in Sardinia was estimated at € 5,970.92, resulting in an average annual expenditure of € 746,316.66. The total Disability-adjusted life years (DALYs) were found to be 505.40, representing an increasing threat to Public Health (Mastrandrea et al., 2012).

On the other hand, *Toxoplasma gondii* is considered the fourth most important parasite in the world, owing to its remarkable efficiency (Boireau et al., 2014). Recent studies have highlighted the widespread presence of this parasite in Sardinia. An 85.7% seroprevalence was observed in family-use slaughtered pigs (Pipia et al., 2018), *T. gondii* DNA was detected in 77.5% of sheep heart matrix samples (Dessì et al., 2021), and 37.2% of wild boar heart samples tested positive for *T. gondii* DNA (Sini et al., 2024). These high prevalence of *T. gondii* found revealing a potential risk for meat consumers, especially pregnant woman and immunodeficient individuals (Weiss and Dubey 2009).

For these reasons, zoonotic parasitic diseases have been selected for investigation in Gennargentu Mandrolisai and, more broadly, in the Sardinia region. The goal is to update epidemiological data concerning two significant zoonotic parasites of major concern and evaluate the potential risk of them among human population. With these data, the objective of this thesis is to provide a useful tool, such as an interactive website, to promote public health, education, and environmental protection with a One Health perspective. The underlying idea of this project is to disseminate knowledge about parasitic risks among farmers and citizens, while facilitating real-time consultation between researchers and web app users. The website's objective is to benefit public authorities, promote local economy and traditions, and ultimately revitalize the Gennargentu-Mandrolisai inland area. Specifically, this work will ensure a deeper understanding of the following topics:

- Health: improve awareness of parasitosis, particularly zoonoses, and the risk factors associated with traditional farming.
- Internet and smart connections: strengthen connectivity between the research world and farms located in remote areas. Also, improve computerization and the use of new technologies in the agropastoral sector, within the framework of Farming 4.0.
- Prevention and education: increase awareness of potential risks of parasitosis and zoonoses, and promote the adoption of correct prevention measures.
- Schools: utilize educational institutions to develop human resources and educate future generations about parasitic diseases and preventive measures

In detail, **Chapter 1** comprises a review aimed at providing a comprehensive overview of the main parasitic zoonoses that are typically addressed in parasitological laboratories. It summarizes the standard biosecurity protocols for dealing with infectious agents. Given the

prevalence of these parasites in the Sardinian territory, the chapter offers a comprehensive overview of the main characteristics of parasitic zoonoses, including *Echinococcus* and *Toxoplasma.* **Chapter 2** reports epidemiological surveys detailing the current status and trends of *Toxoplasma gondii* in farmed animals such as sheep, as well as in wildlife animals like wild boar. **Chapter 3** evaluates the effect of an edutainment program on primary schoolchildren's knowledge regarding the infection route of CE and behaviours that can prevent its transmission. The chapter examines the impact of participation in the program on children's understanding and adherence to preventive measures Finally, **Chapter 4** presents the results of the investigation conducted in the territory and applies them to the development of a website. The website serves as an informative and interactive tool for citizen, farmers and researchers.

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Chapter 1

Literature review

Adapted from

Laboratory associated zoonotic parasitic infections: a review of main agents and biosecurity measures

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Abstract

Laboratory workers are exposed to the risk of acquiring infections due to manipulation of infectious materials. The biological hazard for researchers is seven times higher when compared with hospital and public health laboratory workers. Despite the implementation of standardized practices to control infections, there have been multiple cases of Laboratory Associated Infections (LAIs) which usually go unreported. There has been a lack of comprehensive epidemiological data regarding the situation of LAIs for parasitic zoonosis and besides, the available sources are not completely updated. Since most accounts of laboratory infections are organism specific, this study has focused on common pathogenic/zoonotic species which are handled at parasitological laboratories in addition to summarising the standard biosecurity protocols for the infectious agents. The main characteristics of *Cryptosporidium* spp., *Entamoeba* spp, *Giardia duodenalis, Toxoplasma gondii, Leishmania* spp., *Echinococcus* spp., *Schistosoma* spp., *Toxocara canis, Ancylostoma caninum, Strongyloides stercoralis* are considered in this review in order to assess the potential risk of developing occupational infections in the workplace along with stating prevention and prophylactic measures for each species. It was concluded that the LAIs from these agents can be prevented by the use of personal protective measures and through good laboratory practices. However, further studies are necessary to better understand the environmental resistance of cysts, oocysts and eggs, with a view to select the most suitable disinfection methods. Furthermore, it is fundamental to constantly update epidemiological data of infection acquired by laboratory workers, to develop accurate risk indicators .

Keywords: parasite; infection; resistance; prophylaxis; risk; biohazard.
Running title: Laboratory biosecurity of parasitic zoonoses

Introduction

Workers in clinical or research laboratories are exposed to multiple risks of infection and infestation, mainly through accidental exposure, which are not always recognised. In addition, the laboratory workers often underestimate the risk and do not notify the exposure, making it difficult to collect reliable epidemiological data [1]. It was estimated that the biological risk for researchers is seven times higher compared to hospital and public-care workers [2]. In the early 1950s the annual incidence of infection or infestation, calculated as attack rate, was 4.1 per 1000 among researchers [3]. However, epidemiological data on Laboratory Associated Infections (LAIs) are not exhaustive, in particular with regards to parasitological risk, the data are very fragmentary and outdated [2]. Therefore, the preventive measures to be taken remain uncertain and are not always implemented according to the appropriate work practices due to the low perception of risk by laboratory-workers [4]. Handling specimens that may contain viable parasites potentially infectious requires a multi-faceted approach based on developing the standard practices and focusing on the infectious agents to prevent their transmission.

Amid the lack of reliable estimation on the magnitude of LAIs, the control measures are mostly proposed on the basis of experience with one pathogen, on old data which is mostly scattered and on the concept of hazard analysis and the knowledge derived from transmission of a pathogen outside the research laboratories [5, 6]. Risk management based on available information may not suffice in the current laboratory environments, however, it is the only code of information that laboratories must implement until the systematic surveillance of LAIs. Safe laboratory operations are founded on the risk assessment of the pathogens as this process identifies the hazardous characteristics of the infectious agent and the likelihood/chances of exposure that can potentially cause an LAI and consequences of acquiring such an infection [7].

The aim of this study was to collect all the data available in the literature and analyse the risk of the main zoonoses in the veterinary parasite diagnostic laboratory, in order to outline the potential exposure of the employees and draw up prevention measures. The study was divided into a special monographic part concerning zoonotic parasites among protozoa (*Cryptosporidium* spp., *Entamoeba* spp., *Giardia duodenalis, Leishmania* spp*., Toxoplasma gondi)* and helminths *(Echinococcus* spp., *Toxocara canis, Ancylostoma caninum,*

Schistosoma spp., *Strongyloides stercoralis*), and then a specific part on LAIs and preventive measures to be taken in the laboratory.

Cryptosporidium spp.

General information

Cryptosporidium spp. is one of protozoan parasites which infect fish, amphibians, reptiles, birds and mammals [8]. The name of disease is Cryptosporidiosis, is a frequent cause of diarrhea in humans, especially in susceptible subjects like children and immunocompromised individuals [9, 10]. It is primarily transmitted through water (Waterborne disease) but other mechanisms of transmission are ingestion with contaminated food, faecal contact and direct contact with infected people [8, 11]. The infection has been reported in more than 90 countries and on five continents [12] and *Cryptosporidium* was included in the WHO List of Neglected Diseases in 2004.

Cryptosporidium spp. is a coccidian parasite belonging to the Phylum Apicomplexa [13], Family Cryptosporidiidae [14]. This parasite has a monoxenous cycle and alternates an asexual phase with a sexual phase and finally a sporogonic phase with the formation of sporulate oocysts. Till date, 30 species and 60 genotypes have been classified, but only two species, *Cryptosporidium parvum* and *C. hominis,* frequently infect humans. In fact, *C. canis*, *C. felis*, *C. meleagridis*, *C. muris*, *C. cuniculus*, *C. ubiquitum* and *C. viatorum* are potentially zoonotic, but rarely infect humans, while *C. parvum* is responsible for most of the infections in humans, with a significant impact both on human health and the health of domestic farm animals, as has not host specificity [12, 15]. Infection begins with the ingestion of sporulated oocysts (infectious dose is between 9 and 1024 oocysts) [16]. Oocysts may be thin-walled, or thick-walled. Thick-walled oocysts are responsible for the spread of infection, thanks to the high environmental resistance, determined by the presence of the double wall which is composed of hydrophobic proteins [17], while the thin-walled ones determine self and chronic infection [18, 19].

[20][9, 21][22][20][9, 21][23][23, 24][10](Meechan et al., 2020). [4]Occupationally-acquired infections have occurred quite commonly in personnel working with this agent, especially if infected calves were the source of the oocysts, but other infected animals pose potential risks as well. Circumstantial evidence suggests that airborne transmission of

oocysts via droplets of this small organism (i.e., 4–6 μm in diameter) might occur [7]. Sixteen cases of cryptosporidiosis are reported among LAIs [4].

Resistance in the environment and prophylaxis

Oocysts released into the environment can be infectious even for several months due to their protective external structure [25]. Oocysts suspended in deionized water at temperature of 0°C, 5° C, 10°C, and 20°C remain infectious after 6 months, while those kept at a temperature between 25°C and 30°C are active for 3 months and remain infectious only for 1 week at 35°C [26]. Oocysts can survive at -4°C and 4°C in the soil for more than 12 weeks, but at temperatures higher than 25°C, degradation is more rapid (80% decrease after 7 weeks). The degradation of oocysts in bovine feces occurs after 4 weeks (80% decrease), probably due to drying and the presence of microorganisms, either in the feces and in the soil [27].

Oocysts can survive even in saltwater for a long time, some oocysts still remain active after 40 days in 35% salinity and at a temperature of 18°C [28]. Humidity is a crucial factor for the survival of oocysts: only 3% of oocysts remained viable for two hours at room temperature and all oocysts died after this time [26, 29]. Exposure to UV radiation can affect the viability of oocysts; [29], report that this condition can lead to a 90% reduction of oocysts after 3 hours.

The ability to survive both wastewater treatments and drinking water treatments has been extensively studied for *Cryptosporidium* spp., as a factor of considerable epidemiological importance [25]. The classic processes of wastewater treatments such as chlorination are ineffective and on the other hand, traditional treatments of drinking water, such as coagulation, flocculation and filtration, do not sufficiently reduce the presence of oocysts. The most effective treatments are of new generation: microfiltration, reverse osmosis, ultrafiltration, use of UV combined with advanced oxidation and UV disinfection with peroxide [30]. Environmental oocysts are also resistant to various chemical compounds such as sodium hypochlorite, denatured alcohol, 70% and absolute ethanol [31]. Oocysts are inactive after exposure of only 2 hours to hydrogen peroxide and after 12 hours to 6% sodium hypochlorite [31]. The commercial disinfectants are not effective against *Cryptosporidium*

spp. 3% hydrogen peroxide devitalizes *Cryptosporidium* oocysts after sufficient exposure. For this purpose, it is recommended to disinfect the surfaces to remove organic material followed by application of 3% undiluted hydrogen peroxide for 30 minutes which can then be removed with absorbent paper and letting the surface dry for 10/30 min [7].

Entamoeba spp.

General information

Entamoeba spp. are protozoan parasites that form pseudopods and belong to the phylum Amoebozoa, Class Archamoebae, and Family Entamoebidae. The genus *Entamoeba* (Casagrandi & Barbagallo, 1895) includes different species that infect humans and different animals as reptiles, birds and amphibians and others [32, 33]. About seven species (*E. histolytica, Entamoeba coli, Entamoeba hartmanni, Entamoeba polecki, Entamoeba dispar, Entamoeba moshkovskii, and Entamoeba bangladeshi*) are able to inhabit the human intestine and one (*Entamoeba gingivalis*) that can be found in the oral cavity.

Although *E. polecki* and *E. moshkovskii* have occasionally been implicated as a cause gastrointestinal symptom, most *Entamoeba* species are generally commensal organisms of the gut, with the exception of *E. histolytica*, which is responsible of different health problems ranging from mild diarrhoea to invasive and extraintestinal infections. *E. histolytica* infects up to 50 million people worldwide, with nearly 100,000 deaths per year [34], primarily in developing countries such as Africa, India, Central and South America [35]. The pathogenic *E. histolytica* and the non-pathogenic species *E. dispar* and *E. moshkovskii* form morphologically indistinguishable cysts with four nuclei averaging 10-16 μm in size [36]. Only molecular techniques allow distinguishing commensal species from species with confirmed pathogenicity [37]. The morphological identity of the three species makes it difficult to differentiate pathogenic and non-pathogenic *Entamoeba* by microscopic examination, adding to the disease's complexity. Previous statistics reported that 90% of *E. histolytica* cases were asymptomatic, and only 10% had some symptoms, resulting in an overestimation of the prevalence of human intestinal amoebiasis[38].

The cycle of infection begins when the cyst form of the organism is ingested in fecally contaminated food or water. The acid-resistant cyst passes unharmed through the stomach until it reaches the small intestine where it excysts to form eight trophozoites, the motile and

invasive form of the species. Trophozoites migrate to the large intestine, where they may either colonize the bowel lumen as commensal flora or invade into the colonic epithelium, causing inflammation and destruction of the bowel wall. What influences this decision to invade is as yet unknown, but potential factors include genetic differences between amoeba strains and host variations, such as intestinal flora, nutritional state, and immunocompetence. After invasion, amoebae can enter the portal circulation and be transported to various target organs such as the liver, brain, and lungs. Extraintestinal amoebiasis is most commonly found in the liver [39].

Although ingestion of contaminated food or water is the most common transmission route, person-to-person transmission can also occur in settings with high crowding and poor personal hygiene, such as mental hospitals and day care centers. In animal models, only 10-100 cysts are needed to cause amoebic dysentery, a contagious dose comparable to the notoriously contagious *Shigella* spp., which can be transmitted by as few as 10-100 organisms [40]. There is no notified Acquired Laboratory Infection of *Entamoeba*, although the possibility is real.

Resistance in the environment and prophylaxis

The cysts can survive days to weeks in the external environment. Since *Entamoeba* cysts are infectious when excreted, laboratory personnel should take routine precautions when working with stool specimens and fecally contaminated material, such as washing their hands thoroughly after handling specimens. Even well-preserved specimens should be handled with caution because parasites in poorly preserved specimens may still be viable. Commercially available iodine-containing disinfectants, as well as high concentrations of chlorine (1 cup of full-strength commercial bleach [5% chlorine] per gallon of water [1:16, vol/vol]), are effective against *Entamoeba* spp. when used as directed [1].

Giardia duodenalis

General information

Giardia duodenalis, also known as *Giardia lamblia* or *Giardia intestinalis*, is the most common cause of parasitic diarrhea in the world [41]. Giardiasis (the name of disease) has been included in the "Neglected Disease Initiative" project of the World Health Organization (WHO) since 2004, due to its prevalence and incidence in the poorest areas of the world

[42]. For this reason, is discussed as the most important parasitic cause of travelers' diarrhea, together with *Cryptosporidium* spp.. Transmission occurs via fecal-oral pathway, most frequently through the ingestion of contaminated food and water, making it also classified as "Foodborne disease" and "Waterborne disease" [43]. Furthermore, person-to-person and animal-person transmission is possible, although it is less prevalent [44].

Giardia duodenalis is considered as a species complex, that includes 8 genetic group or assemblages, identified with the letters from A to H. The assemblages A and B are subdivided into subgroups, and they are the only ones recognized as causing infection both in mammals and in humans, showing a zoonotic potential that cannot be underestimated [45].

Giardia duodenalis is a flagellated protozoan classified as diplomonads [46]. It has two stages: trophozoite, an active and vegetative form, and cyst which is the infectious form, relatively inert and resistant into the environment [47]. After ingestion of cysts, exposure to pancreatic enzymes and acidic pH of the stomach stimulates excystation with the release of two trophozoites, thus initiating the infectious process [47–49]. Trophozoites reproduces by binary fission in the crypts of the duodenal mucosa and in the proximal part of the jejunum; in the ileum, encystation takes place [50]. Its strong resistance to all external agents is due to the structure of the cystic wall which is composed of chitin [51] and the presence of three membranes: the outer membrane is linked to the filamentous elements of the cystic wall, while the two inner membranes enclose the peritrophic space, where the innermost one is the plasma membrane of the trophozoite [52]. Unlike other parasites such as nematodes, *G. duodenalis* do not require maturation periods or activation after their excretion, but are immediately able to infect a new host [53].

The parasite is monoxenous, has a simple and direct life cycle. Infection occurs after ingestion of the cystic form. The infectious dose for humans is between 10 and 100 cysts [50, 54, 55]. The infection can have a completely asymptomatic course to mild or severe course, even asymptomatic subjects eliminate cysts through feces. Age, immune status, co-infection with other protozoon parasites and microbiota of the host are factors that can influence the disease course. Children and immunocompromised individuals are considered to be at risk in which diarrhea can become chronic [11]. Laboratorians could be infected through the wrong manipulation of stool specimens, the workers in cleaning of disposable materials may be infected by accident. Nevertheless, among the LAIs, only two cases of giardiasis are reported [4].

Resistance in the environment and prophylaxis

Cysts of *G. duodenalis* have a marked resistance in the environment, sometimes to water purification treatments too, which allows the spread of infection through contaminated foods [56]. The resistance of cysts in the environment depends on climatic factors, such as temperature and humidity: they resist about 77 days at 8°C, from 5 to 24 days at 21°C and about 4 days at 37°C in distilled water [27, 57] and remain mostly stables at 4°C for 11 weeks in water, 7 weeks in soil and 1 week in bovine feces [27]. Analyses conducted at -4 and 25°C in soil and faecal samples, show that cysts are non-infectious after 1 week; in water they are inactivated in less than 1 week at -4°C and in 2 weeks at 25°C [27] (Table 1). *Giardia* cysts are also sensitive to dryness and heat (>40°C) [58]; they show a survival of 1 hour and a half in marine waters (salinity of 35ppm) exposed to solar radiation, highlighting a significant role of salinity in their inactivation and also certain susceptibility to UV rays [29]. The UV could represent an effective medium of water disinfection [59]. The ability to survive potabilization treatments is one of the critical factors related to this protozoan. It has been widely reported that cysts can remain in the environment after a disinfection treatment, due to their physical and chemical structure. The classic disinfection processes – coagulation, flocculation, sedimentation, and filtration – are ineffective in eliminating *Giardia* spp. cysts [30]. Modern drinking water treatment systems, such as ozone treatment, reverse osmosis, UV disinfection combined with oxidation, microfiltration and ultrafiltration are more effective compared to the classical processes [29]. In fact, *Giardia* cysts have a certain resistance to numerous chemical compounds: disinfectants such as chlorine and chloramines have proved to be ineffective against the protozoan, while they are sensitive to sodium hypochlorite [60].

The prevention of giardiasis is based on interventions of infection control and effective water purification. Hand hygiene assumes significant importance to reduce the chance of transmission between people, therefore, hand washing, proper disposal of waste (for example, diapers) and treatment of the symptomatic people (especially children) can effectively prevent the spread in nurseries. Hand washing with soap and water is preferable than the use of sanitizing hand gel without rinsing, because of its effectiveness for the trophozoite form, but not for the cyst [61]. The disinfection of tools and laboratory surfaces can be done through 5% chlorine concentration solution which is effective against *G. duodenalis* [4].

Toxoplasma gondii

General information

Toxoplasma gondii is one of the most widespread parasites in the world, both in warm-blooded animals and humans [62]. Toxoplasmosis is one of the most important foodborne parasitic zoonoses, occupying the fourth place in the global ranking of food-borne parasites drawn up by the Food and Agriculture Organization of the United Nations (FAO) and the WHO [70, 71]. *Toxoplasma* is a protozoan belong to the phylum Apicomplexa, family Sarcocystidae and have only one genus *Toxoplasma* [63], and one species, namely *T. gondii* [64]. Three main genetic lineages - type I, II and III - isolated in North America, Europe and Africa, together with high percentage of atypical genotypes have been so far identified. Type II is the most common followed by type III and atypical genotypes in farm animals while the type I is rarely found in these animals [65]. Type II strains have a high capacity to produce cysts in animal models and are frequently associated with infections in agricultural animals, instead type III strains appear to be more common in animals, although in general they are not associated with disease. Type I strains, although rare in animals, have shown increased prevalence in some cases of congenital infection and in AIDS patients, suggesting that they are more likely to cause disease in humans [66]. In addition, there are new strains that show unexpected virulence features in humans [67]. All warm-blooded animals can be the intermediate hosts, including farm and wild animals, as well as human, while the definitive hosts are represented by the felids, especially the cat [68]. In the intermediate hosts, infection occurs through the ingestion of tissue cysts or sporulated oocysts and two stages of asexual development of the parasite take place [68]. In the definitive host felids, both the asexual and sexual reproduction phases take place. The cat, through the feces, releases up to 100 million oocysts which do not sporulate until about 15 days in the external environment [69], eventually undergoing sporulation and becoming infectious within 1-5 days [70] The parasite is transmitted mainly through the ingestion of tissue cysts in raw or undercooked meat, or through oocysts expelled from the cat with the feces that had time to sporulate and consequently become infectious. Other possible transmission routes are water, blood transfusions or organ transplants, and vertical transmission from mother to child [4]. Laboratorians can become infected through ingestion of sporulated oocysts from feline fecal specimens or through skin or mucosal contact with either tachyzoites or bradyzoites in

humans or animal tissue or culture. All *T. gondii* isolates should be considered pathogenic for humans even if they are avirulent for mice [71]. Forty-seven laboratory acquired cases of *T. gondii* infection have been reported [4].

Resistance in the environment and prophylaxis

The infectious sporulated oocysts have a high environmental resistance: they can survive in the soil for one year and a half [72], in water at temperature of 4°C for four and a half years and at 20-25°C for six months[73]. In marine waters with a salinity of 15 %, sporulated oocysts survive for at least 24 months [74]. The resistance depends on the oocyst's stage of sporulation: for example, exposure to 37°C for 24 hours is lethal for unsporulated oocysts, while sporulated oocysts survive at least 32 days at temperature of 35°C and 9 days at 40°C [73]. At a temperature of 4°C, unsporulated oocysts are inactivated after 6-10 weeks [75].

Tissue cysts, on the other hand, are inactivated by cooking or freezing, respectively at 66°C and -12°C in less than one second [76, 77]. Under conditions of salinity equivalent to 3.3% NaCl, tissue cysts survive for at least 21 days at 10°C, 14 days at 15°C and 3 days at 20°C. At NaCl concentrations of 6%, tissue cysts do not survive when exposed for 7-14 days [78].

Cases of toxoplasmosis from the consumption of drinking water have been documented, which highlights the ineffectiveness of the chlorination of water for human consumption. In fact, under laboratory conditions, 4 hour treatments at different concentrations of chloramines, free chlorine or chlorine dioxide, are ineffective against *T. gondii*, as well as treatment with ozone. Sporulated oocysts maintain their viability even after exposure to 100 mg/L of free chlorine for 30 min and for 2, 4, 8, 16 and for 24 hours, as well as to 6 mg/L of ozone for 1, 2, 4, 8 and 12 minutes and to 9.4 mg/L of ozone for 20 minutes [79]. UV treatments, on the other hand, have considerable effect leading to inactivation of 99.9% of sporulated oocysts [80]. The data obtained are attributable to the particular structure of sporulated oocyst which protects the sporozoites from chemical damage of acids, solvents and other oxidizing elements. The disinfection efficacy was demonstrated at exposure at concentrations of 10% and 5% ammonium hydroxide for 10 and 30 minutes respectively [81]. However, ammonium hydroxide is a potent toxicant and presents concrete health risks [82]. The laboratory instruments and glassware contaminated with *T. gondii* oocysts must be sterilized through the use of heat [4] .

Leishmania spp.

General information

Leishmania (Kinetoplastida, Trypanosomatidae) is a genus of protozoan parasites that are transmitted by the bite of blood sucking female phlebotomine sandfly (Diptera, Psychodidae). Leishmaniasis is one of the most significant of the neglected tropical diseases, with 350 million people in 88 countries worldwide living at risk of developing one of the many forms of the disease [83]. The *Leishmania* infection occurs when sandflies ingest amastigote forms of the parasite while feeding on a reservoir host, and then, during another blood feeding, the sandfly regurgitates metacyclic promastigotes into the host [83, 84].

About 20 species of *Leishmania* infect mammals and many of them caused human leishmaniasis [85], with different clinical outcome based on the species. Human Cutaneous Leishmaniasis (CL) is caused by most *Leishmania* species in the subgenus *Leishmania*, such us *Leishmania major* from Africa and Asia, and *Leishmania mexicana* from Central and South America, and by many species in the subgenus *Viannia*, which are restricted to Latin America (for example *Leishmania brasiliensis*). Mucosal leishmaniasis (ML) is caused by *Leishmania tropica*, *Leishmania major*, *Leishmania infantum*, *Leishmania donovani* in the Old World (Africa, Europe, Asia), while several species of *Vianna* subgenus can cause ML in the New World (The Americas) [86]. Any parasite causing cutaneous or mucosal leishmaniasis can visceralize, but only two species of the subgenus *Leishmania* routinely do so, and these are the causative agents of most human Visceral Leishmaniasis (VL) worldwide, that are *Leishmania donovani* e *Leishmania infantum* [87]. *Leishmania infantum* is the zoonotic form, with dogs as main reservoir, occurs in the Mediterranean basin, China, the Middle East, and South America. *Leishmania donovani* is the antroponotic form, with human-to-human transmission without animal reservoir. This form is prevalent in East Africa, Bangladesh, India, and Nepal [87].

The main three phenotypic categories of *Leishmania* disease are cutaneous, mucosal, and visceral leishmaniasis, but CL and VL are the most severe clinical forms of the disease [86, 88]. In the CL, the first sign of an infection is typically a small erythema that develops at the site where an infected sandfly has bitten the host. The erythema develops into a papule, then a nodule that progressively ulcerates over a period of 2 weeks to 6 months to become the lesion that is feature of CL. Resolution of disease results in a lifelong cutaneous scar, which, depending on its size and location, may cause substantial trauma in affected individuals [89].

The most lethal form of leishmaniasis, VL (also known as kala-azar) can cause systemic infection affecting the liver, spleen, hematogenous and lymphatic system [88]. The disease is progressive and a symptomatic infection left untreated is generally fatal, with a mortality rate of 75-95%. Death usually occurs within 2 years, although spontaneous cures may occur [87]. Asymptomatic infection represents approximately 20–60% of *Leishmania* spp. infection in endemic areas [88]. Currently, no vaccination against leishmaniasis is available for humans. The primary prevention is based on the management of animal reservoir host and control of the sandfly population [90]. However, deforestation, agricultural practices and urbanization have led vectors to feed on human beings rather than synanthropic reservoirs [89]. Furthermore, with climate change, the incidence and geographical distribution of leishmaniasis is expected to increase [86]. Secondary and tertiary prevention are dependent on the optimum management of cases and may be assisted by the use of clinical guidelines [90].

Fourteen cases of LAIs caused by *Leishmania* spp. have been reported, due to negligence (e.g. during mouth pipetting, re-capping a needle), accidental percutaneous exposure and needlestick injury [4].

Resistance in the environment and prophylaxis

Studies conducted in vitro shown that viscerotropic *L. tropica* survived as intracellular parasites in monocytes for 25 days in the red blood cell fraction kept at 4°C, five days in the platelet fraction kept at 24°C, 35 days in the red blood cell fraction frozen with glycerol and for 30 days in unprocessed whole blood left at 4°C. Identical experiments with *L. donovani* resulted in comparable survival data. Intracellular parasites survived longer than did stationary phase extracellular promastigotes or free amastigotes [91]. For this reason, blood specimens should be handled with care, as well as needles and sharp objects. Data on the resistance to different temperatures of *Leishmania* in blood are given in Tables 4.

Echinococcus spp.

General information

Echinococcus spp. are a Taeniidae cestode parasites, the genus *Echinococcus* represents a group of nine species, but only two are important for public health: *Echinococcus*

granulosus, which causes cystic echinococcosis (CE) and *Echinococcus multilocularis* which is responsible for alveolar echinococcosis (AE). *Echinococcus vogeli* and *Echinococcus oligarthra*, neotropical species, cause polycystic echinococcosis in tropical areas (Central and South America) while two other species have recently been identified: *Echinococcus shiquicus* in small mammals of Tibet and *Echinococcus felidis* in African lions. The zoonotic potential of the latter two species is still unknown [92]. The CE is a zoonosis caused by cestodes belonging to the species complex *Echinococcus granulosus sensu lato* (*s.l.*) [93]. This metacestodosis has a cosmopolitan distribution and represents a significant public health problem in different regions of the world [94]. Despite the efforts made in the field of prevention and control, WHO includes echinococcosis among the 17 neglected tropical diseases (NTDs) [95, 96]. The presence of extensive sheep farms, the consistent presence of stray or shepherd dogs, unsupervised home slaughter and improper disposal of carcasses are the predominant factors for the persistence of CE in endemic region [97]. The biological cycle of *E. granulosus* is indirect and requires two hosts, a definitive and an intermediate host. The adult form of *E. granulosus* resides in the small intestine of the definitive host, represented mostly by the dog [92], while the intermediate host are manly sheep, but also buffalo, horses, cattle, pigs, camels, and cervids. The ingestion of eggs by the intermediate host leads the release of oncospheres, which migrate through the circulatory system into various organs, especially in the liver and lungs, developing hydatids. The definitive host becomes infected by ingesting the visceral organs of the intermediate host infested with hydatids [98]. Humans, occasional/accidental intermediate hosts, become infected by ingesting eggs that can contaminate water and plants, and can develop hydatids in different organs. Transmission of *E. multilocularis* occurs in a sylvatic cycle, which is sometimes linked via infected small mammals to domestic dogs and cats. In the sylvatic cycle, foxes play a key role as definitive hosts and small mammals, mainly rodents, are the intermediate hosts. However, dogs and cats can also serve as competent definitive host [98, 99]. *Echinococcus multilocularis* (the small fox tapeworm) is widely distributed within but restricted to the Northern Hemisphere [98].

Several studies indicate in humans a higher frequency of CE in the liver rather than in the lungs (average rate 2.5:1), even less frequent cysts are detected in the spleen, kidneys, heart, bones and central nervous system [92, 100]. Concerning AE, data from patients with single-organ involvement indicate that initially establish almost exclusively in the liver (approximately 99% of the cases) and are rarely found in extra-hepatic sites [99]. The

infection in the initial phase is always asymptomatic, and for some patients it might remain asymptomatic for years (the incubation period vary between less than 5 and up to 15 years for AE) or for entire life or manifests into different clinical form depending on the number, size and stage of development of metacestode, of the organ involved, the localization of hydatid cysts (if they cause pressure in adjacent tissues and organs) and the host defense mechanisms [99]. Clinical symptoms usually appear after several months or years. Liver cysts can cause pain in the abdominal region, hepatomegaly, cholestasis, biliary cirrhosis and ascites and in the rare case of metacestode ruptures, anaphylaxis might occur. In the case of pulmonary cysts, the most frequent signs and symptoms are chronic cough, dyspnea, hemoptysis, pleurisy and lung abscesses. Larval growth in the bones is atypical, and when it occurs, invasion of the medullary cavities and spongiosa is common and causes extensive erosion of the bone. The development of the cyst at the cerebral level is atypical as well and may result in certain neurological disorders [92, 99].

No Laboratory-associated infections with any cestode parasite have been reported [7].

Resistance in the environment and prophylaxis

The eggs of *Echinococcus* spp. released into the environment are already infective and easily spread through insects and birds. In the environment they remain viable for a variable period, depending on temperature and humidity conditions. Eggs of *E. multilocularis* remain infective for approximately 1 year in a suitable, moist environment at lower temperatures, but they are sensitive to desiccation and high temperatures Their high resistance to low temperatures is a precondition for their survival in Arctic regions [101]. Both *E. granulosus* and *E. multilocularis* eggs can survive at 50°C for 24 h but are killed at 70°C within 96 h and at 80° C to 83°C within 48 h. Deep-freezing at -70°C for at least 4 days or at -80°C for at least 2 days is recommended for inactivating *E. multilocularis* eggs in carcasses or intestines of final hosts or in fecal material before examination in the laboratory [99, 102]. At 4°C, eggs can remain infectious for over 300 days, but the viability is significantly reduced in case of rise in temperature. Eggs are devitalized within 2-14 days at temperature range of 37-39°C [103]. The eggs are sensitive to heat, the inactivation begins at temperatures of 50°C and completes at 60°C; 10 minutes at 72°C is the most effective method for the destruction of eggs [104, 105]. The eggs of *Echinococcus* spp. are sensitive to drying. At a relative humidity of 25% eggs of *E. granulosus* were killed within 4 days and at 0% within 1 day. Eggs of *E. multilocularis* lost infectivity to rodents after exposure at

+25°C and a relative humidity (RH) of 27% for 2 days, at +43°C and 15% RH for 2 h, and at +45°C and 85%-95% RH for 3 h [101]. Data on the resistance to different temperatures and relative humidity of *Echinococcus* are given in Table 5. The exposure to 0.7% sodium hypochlorite for 7 minutes at 25–27 °C is effective for egg inactivation [105], but sodium hypochlorite solution (NaOCl) at a minimum concentration of 3.75% in water disrupts the embryophores of *Echinococcus* spp. eggs and damages the majority of the oncospheres within a few minutes. Finally, the eggs of *E. granulosus* retained viability in ethanol (50%, 70%, 95%) after 5 min to 60 min exposure [101]. The disinfection of workbenches and equipment can be carried out with commercial bleach, which contains 50 g/l of free chlorine and must be diluted 1:10 to obtain 5.0 g/l, which is effective *E. granulosus* [4].

***Schistosoma* spp.**

General information

Schistosomiasis is a water-borne infectious disease caused by blood flukes of the genus *Schistosoma* that affects humans and domestic and wild animals in many tropical and subtropical regions. Human schistosomiasis, included by the WHO in the list of "Neglected Tropical Diseases", is mainly caused by *Schistosoma haematobium, Schistosoma mansoni* and *Schistosoma japonicum*, whereas *Schistosoma guineensis, Schistosoma intercalatum* and *Schistosoma mekongi* have lower global prevalence [106]. After malaria, schistosomiasis is the most common parasitic disease in humans. It is currently endemic in over 70 tropical and subtropical countries, where it is thought to affect more than 240 million people. Additionally, it poses a threat to an additional 780 million people who live in areas where they are susceptible to infection [107].

Despite, to our knowledge, there is no information on the current prevalence of animal schistosomiasis, in the past it affected more than 165 million animals and was widely distributed throughout Africa, the Mediterranean Basin, and Southwestern Asia [108]. At least ten different *Schistosoma* species have the potential to cause the illness, with *Schistosoma bovis* standing out due to its pathogenicity for domestic ruminants [109].

The zoonotic potential of *S. bovis* and its effects on human health have been recently revealed. Indeed, the occurrence of *S. bovis* x *S. haematobium* hybrids in human urine and stool samples have been described in people from several villages along the Senegal River Basin in Northern Senegal [110] as well as in a recent outbreak of urogenital human schistosomiasis that occurred in Corsica [111].

Other *Schistosoma* species that parasitize birds and mammals can also cause cercarial dermatitis in humans, but this is clinically distinct from schistosomiasis [112].

In general, *Schistosoma* spp. eggs are eliminated through faeces or urine, depending on the species. When the eggs hatch, they release miracidia, which swim and penetrate specific snail intermediate hosts. The stages in the snail include two generations of sporocysts and the production of cercariae. When the infective cercariae are released from the snail, they swim, penetrate the skin of the vertebrate host (humans or animals), and shed their forked tails, transforming into schistosomulae. They migrate through the venous circulation to the lungs, then to the heart, and finally to the liver, where they mature and exit through the portal vein system. Male and female adult worms copulate and live in the mesenteric venules, which vary by species. Females lay eggs in the portal and perivesical systems' small venules. The eggs are gradually moved toward the lumen of the intestine or the bladder and ureters, where they are eliminated with faeces or urine [113].

Such cercariae, which swim freely, could infect laboratories working with aquaria for snail intermediate hosts; dissecting or crushing infected schistosome-infected snails could also expose workers to droplets containing cercariae. Therefore, workers performing such tasks ought to use gloves. Furthermore, people at risk of schistosomiasis should wear a long-sleeved gown or coat and shoes rather than sandals to reduce the amount of exposed skin. So far, at least nine laboratory-acquired cases of schistosomiasis have been reported in workers who came into contact with infected snails while not wearing protective clothing [1, 114].

Resistance in the environment and prophylaxis

Environmental factors, particularly those affecting the intermediate host snail, play a significant role in transmission. Variations in the weather conditions, such as alterations in temperature, rainfall/precipitation, flood, drought, and pH among others, have been recognized to have a significant impact on the lifespan and fecundity rate of both snails and the penetration of cercariae into the skin of the definitive host [115].

Concerning the prevention measures, travellers should be aware of the possibility of infection when engaging in activities that involve direct contact with water in endemic areas [116]. People who are inadvertently exposed to potentially contaminated water (such as by falling into a river) should vigorously dry off with a towel to try to remove any parasites before they penetrate the skin.

However, avoiding water contact may be extremely challenging, if not impossible, for residents of rural areas where schistosomiasis is endemic. Schistosomiasis is a disease associated with poverty, and although it can be prevented, it is frequently not present [117]. Preventive measures should include the access to clean water and sanitary facilities. The risk from occupational exposure, such as fishing in rivers and lakes, will also be little affected by these measures, even though these pursuits are frequently the sole or primary source of income for poor families [118].

Furthermore, schistosomiasis can be prevented by using molluscicides in fresh water, but it can be challenging, expensive, and environmentally hazardous. In endemic areas, widespread praziquantel treatment and education campaigns are used to control the disease. Despite intensive development efforts, currently no schistosomiasis vaccines are still available [119].

Toxocara spp.

General information

Toxocara is an important ascaridoid genus containing species of human and animal health significance [120]. The genus contains 21 species, but *Toxocara canis* and *Toxocara cati* are mainly responsible for human toxocariosis. The final hosts are the dog and the cat respectively, while the other animals, upon which dogs and cats usually prey, like rodents, are the paratenic hosts [121, 122]. Humans represents an accidental host [123]. These parasites present a complex biological cycle with different possible infestation routes based on the age and immunological status of the definitive host consumption of raw or undercooked meat containing encysted larvae of *Toxocara* spp., coming either from paratenic hosts or directly from the environment, which migrate within the body and are encysted in several muscles and organs [122, 124].

Toxocariosis in humans occurs predominantly with an asymptomatic or subclinical symptoms, but symptomatic infestations can generate visceral *larva migrans* (VLM) syndromes, ocular *larva migrans* (OLM) or neurotoxocariosis [122]. VLM is typical in children aged 1 to 7 years of age, but can also be found in adults. Symptomatic infestations present with fever, gastrointestinal symptoms, hepatomegaly, abdominal pain, loss of appetite and weight loss [122, 125]. OLM occurs mainly in children aged 5 and 10 years, and the syndrome typically manifests itself with unilateral vision accompanied by occasional

strabismus [122, 126]. Instead, neurotoxocariosis presents a symptomatology affecting the central nervous system (CNS), but the frequency and location of *Toxocara* spp. in the CNS in humans remains unknown [122, 127, 128]. The different species of *Toxocara* are widespread throughout the world with higher concentrations in areas with a high population of domestic dogs and cats. However, toxocariosis is predominant in tropical and subtropical regions and in developing countries[124]There is no reported case for *Toxocara canis*, but despite this, is crucial to pay attention because ascarid eggs are sticky, then contaminated laboratory surfaces and equipment must be thoroughly continuous cleaning to prevent worker exposure.

Resistance in the environment and prophylaxis

Toxocara spp. eggs remain infectious under different environmental conditions. In fact, the larva is well protected by the wall that surrounds it, composed of chitin and fibrous layers [129] which is resistant to different chemical agents such as formalin and different inorganic acids [122]. Under suitable environmental conditions (15-35°C temperature, 85% relative humidity) the larva develops inside the egg after 2-5 weeks and is potentially infectious for paratenic and definitive hosts [130]. The eggs are inactivated at -15°C and are sensitive to direct sunlight, although they can survive in favorable climatic conditions for 6 years [129]. Sodium hypochlorite at 7% is the most effective and economical disinfectant against *Toxocara* spp. eggs [129–131]. It is crucial to follow some important prevention rules, including the control of the hygiene of environments designed to animals, for example using sodium hypochlorite and exposing to direct sunlight, applying good health and hygiene practices. Data on the resistance to different temperatures and relative humidity of *Toxocara* are given in Table 6.

Ancylostoma* spp. and *Uncinaria stenocephala

General information

Ancylostoma caninum, Ancylostoma braziliense, Ancylostoma ceylanicum and *Uncinaria stenocephala* are the parasites completing their lifecycles with dogs as the definitive hosts, and in the case of *A. braziliense*, *A. ceylanicum* and *U. stenocephala* the cats as well [121]. Defined as "hookworms" for their morphology, they belong to the Order Strongylida, Family Ancylostomatidae [132].

Their biological cycle in the hosts is of direct nature. The host acquires the infection through the free-living L3 larvae, which can be taken either orally, through the predation of paratenic hosts or percutaneous penetration [7]. The infestation of puppies by *A. caninum* through transplacental and transmammary routes has also been documented [121, 133, 134]. *Uncinaria stenocephala*, also called "northern *Ancylostoma*", tolerates harsh climates and is present throughout Europe. *Ancylostoma caninum* is widespread mainly in central and southern Europe, while *A. braziliense* is widespread in tropical and subtropical countries and *A. ceylanicum* is distributed in Asia [135, 136].

Apart from infection in animals, these parasites also infect humans which are the accidental hosts because they do not play any role in the completion of biological cycle. In humans, the infectious larvae (L3), penetrate through the skin causing cutaneous *larva migrans* (CLM) also called "creeping eruption" which cause intense itching with serpiginous linear skin lesions having erythematous-papulo-vesicular appearance, which can grow a few millimeters per day [69]. If left untreated, the parasitic forms resolved spontaneously from one to two months, till larvae die. In rare cases, the larvae migrate into the intestine causing eosinophilic enteritis with abdominal pain, anorexia, diarrhea and nausea [137–139].

An exception is represented by *A. ceylanicum,* which does not lead to CLM, but develops as adult in humans, and is capable of causing chronic infestation and anemia, even in healthy individuals [140, 141].

CLM is a frequent disease in subtropical countries, including the southern states of the USA, due to fecal contamination of the soil by infested animals [138].[142] Autochthonous cases of *A. caninum* have been reported in Europe and specifically in the UK, Germany, Italy and Serbia [140]. Only one case of LAIs caused by *Ancylostoma* spp. has been reported [4].

Resistance in the environment and prophylaxis

In the environment like the grass, the larvae of *A. caninum* survived best at a temperature range of 0°C to approximately 20°C. To kill larvae is recommended that sunlight be allowed to be present at least 2 hours every day on areas where there might be they are present. Is recommended to follow some prevention measures such as collection of dog and cat feces, regular cleaning of the litter, avoid walking barefoot, even on the beach, and use of sunbeds instead of towels when in direct contact with the sand [140]. The iodine concentrations of 70 ppm have been shown to kill infective larvae of *A. caninum* immersed in an aqueous iodine solution for one to five minutes [7].

Strongyloides stercoralis

General information

Strongyloides stercoralis is a zoonotic parasite, widespread throughout the world, which infests dogs, cats and primates including humans [143, 144]. *Strongyloides stercoralis* is one of the STH and is listed among the NTDs [133].

Strongyloides stercoralis (Rhabditida: Strongyloididae) has a biological cycle that can include a parasitic life phase and a free-living phase, depending on climatic conditions. Rhabditiform larvae of *S. stercoralis* are excreted through the feces of an infected individual and develop into filariform larvae (L3) that can infect a new host through the percutaneous or oral route. Larvae reach the pulmonary capillaries, penetrate the alveoli and pass through the larynx and pharynx finally reaching the small intestine after swallowing to mature into adults. Through the oral route, the larvae of *S. stercoralis* follow the same cycle, penetrating first through the intestinal mucosa and then carrying out the migration described above. Adult females deposit about 50 unfertilized eggs daily, which hatch in the intestinal wall, migrate into the intestinal lumen and are excreted through the feces. Sometimes the larvae penetrate the wall of the colon or the skin of the perianal area, establishing a self-infection that leads to a chronic form of infestation or spread into other organs. In the latter case, the infestation can be fatal [145, 146]. In dogs, transmission to puppies by the galactogen route is also followed [134]. Strongyloidiasis in humans includes a number of nonspecific gastrointestinal symptoms such as diarrhea, abdominal pain and urticaria. However, most infestations, including chronic ones, remain asymptomatic. Asymptomatic infestations can be dangerous in the case of immunosuppressive treatments, especially with corticosteroids, because they can lead to disseminated infestation [146]. LAIs with *Strongyloides stercoralis* have been reported; furthermore 4 cases of *Strongyloides* spp. laboratory infections acquired from infected animals have also been reported [4].

Resistance in the environment and prophylaxis

Positive samples of *S. stercoralis* stored at 4°C for 24, 48, and 72 hourswere reexamined, which still had viable larvae after 72 hours of refrigeration [147]Iodine concentration of 50ppm kill the infective *S. stercoralis* larvae in 5 minutes, in vitro exposure to 70% ethanol has been shown to kill infective larvae within 3-5 minutes [7].

LAIs – Laboratory Associated Infections

Laboratory acquired infections (LAIs) include all infections associated with laboratory work carried out in the clinical laboratories, research, teaching (medical and veterinary) and production facilities [2]. Both symptomatic and asymptomatic (subclinical) infections are comprised among the LAIs. Although these phenomena are not new in laboratories, the information collected, through publications and questionnaires, mainly considers symptomatic infections and associated symptoms, with minimal data on asymptomatic cases. In addition, in some cases it is difficult to establish if the infectious disease is caused by a microorganism present only in the laboratory or even in the community. All these elements lead to assert that it is impossible to establish a real incidence of LAIs [2]. Till date, there is no centralized system for reporting the infections in the laboratory, therefore, epidemiological studies represent an indispensable tool to assess the nature and extent of this phenomenon.

In a review conducted by Herwaldt [4], 47 cases of *T. gondii* were reported from 1940 until the 90s, among which 23 were from the USA and 20 from Europe. In the majority of cases, infection occurred through parenteral exposure (through sharp injuries) followed by cases due to ingestion of oocysts and contact with mucous membranes by laboratorians. Of the 47 cases reported, only 9 were asymptomatic, the other subjects had symptoms such as fever, headache, general malaise and lymphadenopathy, in 4 cases encephalitis had occurred and in two of these cases, myocarditis was reported. There was only one lethal case in 1951 caused by encephalitis and myocarditis [148]. Despite the infrequency of LAIs, the laboratory staff must remain cautious as many exposures remain unrecognized and accidental incidents may happen.

Fourteen cases of *Leishmania* spp. infection have been reported during the decades among 1930 – 2005: five cases of *L. donovani* and one case of *Leishmania chagasi* (considered same species of *L.infantum* in the Old World), three cases of *Leishmanua braziliensis,* two cases of *Leishmania tropica*, one case of *Leishmania mexicana* and finally one case of *Leishmania guyanensis* [4]. All the infections were symptomatic, and only one person infected by *L. donovani* species complex developed manifestations of visceral involvement. In fact, although both *L. donovani* and *L. chagasi* are typically considered etiologic agents of visceral leishmaniasis, both can also cause cutaneous infection. Laboratorians that developed CL had symptoms such as nodule, papular lesion at the site of exposure, someone also lymphadenopathy and lymphangitis. Fortunately, none of these cases was fatal. In

laboratory settings, leishmaniasis could be acquired through inadvertent contact with an infected sand fly, like natural route exposure, but transmission could also occur through contact with cultured parasites or specimens from infected persons or animals. In fact, nine of reported cases were caused by parenteral exposure (e.g. needlestick injury), two cases by biting of infected animals, one case due to nonintact skin, one case by mouth pipetting (mucosal contact) and finally one accident was not recognised [4].

In case of intestinal protozoa, a very few case reports are available among both researchers and healthcare professionals: 2 cases of giardiasis and 16 cases of cryptosporidiosis have been reported to date, probably because these infections can be diagnosed and treated easily and also the disease is typically gastrointestinal rather than systemic [4]. Even in our laboratory, unfortunately, despite all the biosecurity precautions undertaken, a student working on the epidemiology of cryptosporidiosis of calves from 1995-1996, manifested several episodes of transient diarrhea attributable to these protozoa, as demonstrated by the analyzes carried out during the last episode (Scala, personal communication).

Four cases of helminth infestations reported so far form the scientific research laboratories were attributable to *S. stercoralis*, all contracted through skin contact, and only one has been attributed to *Ancylostoma* spp. [4]. Furthermore, nine laboratory-acquired cases of schistosomiasis have been so far reported [1]. The lack of reports reflects the fact that helminth infestations are generally less common than protozoan infections in the laboratory environments. In fact, the use of gloves and laboratory coat, together with appropriate organizational measures (decontamination and disinfection) are able to stem the possibility of infestation.

Although the epidemiological information collected is not exhaustive, it is clear that the adoption of adequate biosecurity measures is necessary to prevent infections and infestations. It is necessary to implement information and training of personnel, good hygiene practices, the correct use of Personal Protective Equipment (PPE) and Collective Protective Equipment and the improvement of the work organization and facilities. In fact, it is evident that the lack of adequate control plans, programmed for parasitology laboratory staff certainly leads to a considerable underestimation of the real cases of "parasitic" LAIs. For this reason, it is advisable to create a surveillance system, providing periodical check on the lab workers, with simple and cheap tests. For example, seroprevalence of IgG against *G. duodenalis*, *Cryptosporidium* spp. and *Toxoplasma gondii*, or Portable US and dot-ELISA for CE surveys that have been demonstrated applicable, with a few limitation [149]. In addition, it is recommended to notify any cases of suspected transmission of infections to

the occupational physician, to set up the appropriate measures of prophylaxis and control of infection.

Biosecurity measures

One of the most frequent consequences when working with biological material is to acquire an infection/infestation. History shows how workers working in the laboratory show a higher risk of infection than the rest of the population [150].

The parasites described earlier are considered Biological Agents (BA) that we can encounter in laboratories for research and diagnostics, creating occupational biological risk, since the lab activities involve constant contact with biological material. Although biological agents do not fit into strict categories, it is possible to assess the relative risk of a microorganism according to the classification in 4 risk groups (RG) drawn up by the WHO [151] and adopted by European Union with the Directive 2000/54/EC (table 7):

- RG1 (none or low individual or community risk): BA that is unlikely to cause disease;
- RG2 (moderate individual or community risk): BA that can cause disease in human subjects, capable to constitute a risk to workers, but with low probability that it spread in the community; effective prophylactic or therapeutic measures are normally available;
- RG3 (high individual risk, low community risk): BA capable of causing serious illness, represent a serious risk to workers, can spread in the community, but effective prophylactic or therapeutic measures are available;
- RG4 (high individual and community risk): BA that can cause serious illness and is a serious risk for workers, present a high risk of propagation in the community, effective prophylactic or therapeutic measures are not normally available.

All parasites considered in this review are included in the RG2 category, except for *E. granulosus, L. donovani* and *L. braziliensis*, included the RG3 category with double asterisk (**), which is capable of causing serious disease with no airborne transmission risks and limited chance of spread and for which there are usually effective preventive measures (EU Directive note 8, annex III) [152]. For this reason, the prophylactic measures are the same adopted for the BA of the RG2 group.

It is important to underline that this classification takes into account only the effects of BA on the immunocompetent worker, and not the possible effects on the worker of risk categories, that is who has a higher probability of contracting a disease (or contracting a disease in a more serious form), following exposure of the pathogen, compared to the majority of the general population. Indeed, as reported in the WHO Biosafety Manual [151], referring only to risk groups is not sufficient for risk assessment. Other factors to consider are the natural routes of infection and other routes due to manipulation in the laboratory, the type of activity that is carried out in the laboratory (homogenization, sonication, centrifugation, aerosolization, etc.), the stability and resistance of the microorganism/pathogen in the environment, the concentration of the agent that is manipulated and the reports of previous laboratory-acquired infections [153]. The prevention of biological risk is based on the adoption of technical, organizational and procedural measures, on the choice and correct use of appropriate PPE, the health education and health surveillance of workers.

Laboratories are distinguish in basic laboratories (Biosafety Level-1 and -2) and containment lab Biosafety Level-3 and -4). The assignment of biosafety level takes into account structural features, available equipment, and activities performed. More information about Biosafety Levels BSL-2 BSL-3 and BSL-4, listed in the Annex VI of the Directive 2000/54/EC, are reported in Table 8.

The preventive measures taken in level 2 containment laboratories (Biosafety Levels-2 and Animal Biosafety Level-2) are recommended to be adopted in the research and diagnostic parasitology laboratories to achieve an acceptable level of safety [7]. It is absolutely recommended to use the universal standard procedures, that include hand washing, the use of gloves, lab coat, protective masks, goggles and visors, and other precautions to prevent accidental exposure. Therefore, the laboratory operator must use disposable gloves at each handling step of potentially infectious samples, before and during the sample preparation, wash the laboratory equipment and wash the hands with water and neutral soap. It is mandatory to use lab coat with stretch sleeves to protect the wrists and wear clothes that can cover the legs. It is also recommended to wear face masks to reduce the possibility of transmission by air, as in the case of *Cryptosporidium* spp. or *G. duodenalis* [7]. Workers belonging to any risk category, such as immunocompromised and, in the case of *T. gondii*, pregnant women, must avoid working in workplace with zoonotic risk [4]. In case of *Leishmania* spp. it is important to avoid accidental needlestick and also blood samples

should be handled with care, even though fewer parasites generally are found in the bloodstream than in infected tissues.

Manipulation of biological agents requires identification of the best practices and integration of multiple strategies to control the possibility of spread of infections besides responding to unforeseen circumstances. In the parasitology laboratories, it is important to correctly perform the decontamination, disinfection and sterilization procedures, due to the ability of oocyst cysts and eggs to resist the external environment, resulting in all respects one of the major critical points for the prevention of any infestations [154]

Briefly, decontamination consists of cleaning of an instrument, device, or area with ordinary soap and water to primarily reduce the risk of infection. It is an essential pre-requisite to disinfection or sterilization processes to ensure the optimal activity of the disinfectants or sterilization processes. In fact, cleaning can be used to remove microorganisms and other associated contaminants (e.g., feces, blood, etc) from a surface by physical means. Disinfection is generally a less-lethal process than sterilization; it eliminates nearly all recognized pathogenic microorganisms, but not necessarily all microbial forms. Disinfection does not ensure a kill level and lacks the margin of safety achieved by sterilization procedures [7]. Factors affecting disinfection are linked to physical and structural characteristics of several parasites, and for this reason, is essential to know the nature of the parasite which one can come into contact with but, above all, which chemical and or physical agent is most appropriate for its inactivation.Furthermore, is important to develop and test new disinfectant that could be effective on several parasites species. In fact, Due to the current pandemic situation caused by SARS-COV-2, the need of effective precautionary methods is increasing, leading to the development of new effective chemical solutions such as hydrogen peroxide combined with silver nanoparticles [155, 156]. Other interventions could be the engineering and installation of self-disinfecting surfaces at the laboratories which could reduce the preliminary chances of contamination [157]. Additionally, plasma disinfection (ionized gas) could also be used to inactivate the pathogenic organisms in the parasitological examination facilities [158].

The moist heat used with autoclaves, with saturated steam under pressure, is the most reliable measure to sterilize the laboratory materials. Different heating cycles for varying time periods ensure sterilization [151] for example, 3 minutes at 134°C, 10 min at 126°C, 15 min at 121°C and/or 25 min at 115°C [7]

It is equally important to develop standard code of practice for handling the infectious material to reduce the pathogen transmission. The disposable material must be correctly

disposed in containers with the indication "hazardous medical waste at infectious risk". Sharp objects (slides, blades and syringes) should be disposed in rigid yellow containers (halibox) while, for the remaining disposable material, the container is made by disposable packaging, with an internal polyethylene bag inserted in a rigid and waterproof external container. In fact, waste of research of veterinary diagnostic activities is considered potentially hazardous at infectious risk. Carcasses and anatomical parts coming from the diagnostic activity of the Experimental Zooprophylactic Institutes, from the Departments of Veterinary Medicine and from the Scientific Research Institutes and Centers are classified in category according to Regulation (EC) No 1774/2002 [159]. These are disposed in approved and certified incineration plant, or they are processed in an approved plant according to specific method.

The main theme behind implementation of the control measures for the infectious agents is the protection of laboratory workers and general public through proper management (handling and disposal) of biological (infectious) wastes. A biological safety program, developed to minimize the risks associated with the handling and disposal of pathogenic organisms, is based on the transmission mode, pathogenicity of the organism and the susceptibility of the host. In the specific case of parasitic biological agents, it is important to evaluate for each parasite a method of decontamination, disinfection and possibly appropriate sterilization. In fact, from the document drawn up by the CDC, Biosafety in Microbiological and Biomedical laboratories, emerges that zoonotic parasites have a grade 2 stability, for which an inactivation through commercial disinfectants, detergents, temperature extremes (pasteurization), or steam is required [7]. The above mentioned regulations and advices should be applied especially in developing countries, where there is a lack of normative and surveillance. It is recommended to develop an epidemiological system, based on medical surveillance and periodical systematic analysis, to track all the potential cases, creating a database. In this way, the biological risk reaches an acceptable level. Hence, risks from the biological hazards can be reduced through the usage of containment devices and protective barriers, but especially following appropriate procedures to handle zoonotic pathogens. Above all, the foundation of biosafety measures rests on training of the laboratorians to make them understand the need for these safety [6].

Conclusion

The present review had the purpose of raise awareness among the operators of the diagnostic and research facilities who work in the field of parasitology, for educating them about safety by acquiring a greater consciousness of what kind of biological risks they could be exposed to during the laboratory activities. A reduction in infection and infestation risks posed by the handling of parasites can be achieved through the systematic and timely application of a unified strategy. This includes the management of occupational exposures to the various biological agents, the training of laboratory staff and implementation of standard cleaning and disinfection measures. A safety plan identifying potential biological hazards should be at the core of effective management strategy to minimize the accidental exposures. It is evident that for a greater understanding and analysis of the topic examined in the review, it is necessary to update data regularly on both epidemiology and environmental resistance and chemical agents of cysts / oocysts and eggs, which are reflected in the work environment, such as the laboratory (of parasitology), together with the development of new disinfectants, easier and safer to use.

List of abbreviations

AE: Alveolar Echinococcosis
BA: Biological Agent
BSL: Biosafety Level
CDC: Centers for Disease Control and Prevention
CE: Cystic Echinococcosis
CL: Cutaneous Leishmaniosis
CLM: Cutaneous Larva Migrans
CNS: Central Nervous System
FAO: Food and Agriculture Organization
LAIs: Laboratory Associated Infections
NTDs: Neglected Tropical Diseases
OLM: Ocular Larva Migrans
PPE: Personal Protective Equipment
RG: Risk Group
STH: Soil Transmitted Helminths
VL: Visceral Leishmaniosis

VLM: Visceral Larva Migrans
WHO: World Health Organization

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Tables

Table 1. Maximum duration of resistance of *Giardia* spp. cyst in water, soil and bovine feces [27]

Medium	Temperature	Resistance of cyst (per week)
Water	-4°C	<1
	4°C	11
	25°C	2
Soil	-4°C	<1
	4°C	7
	25°C	1
Bovine feces	-4°C	<1
	4°C	1
	25°C	1

Table 2. Maximum period of viability of *Cryptosporidium* spp. oocyst [8, 27, 28]

Materials	Temperature	Resistance of cyst (weeks)
Water	0°C / 20°C	24
	25°C / 30°C	12
	35°C	1
Soil	-4°C / 4°C	>12

	25°C	7
Bovine feces	25°C	7
Water with NaCl 35%	18°C	>7

Table 3. Resistance of *Toxoplasma gondii* oocyst

Medium	Temperature	Resistance of cyst (sporulate)
Water	4°C	54 months
	20 - 25°C	6 months
Water with NaCl 15 ‰		24 months
	35°C	32 days
	40°C	9 days
	-4	

Table 4. Resistance of *Leishmania* spp.

Medium	Temperature	Time of viability
Red blood cell fraction	4°C	25 days
	24°C	5 days
Unprocessed blood	4°C	30 days

Red blood cell fraction with glycerol	Frozen (-4°C)	35 days

Table 5. Resistance of *Echinococcus* spp.

Temperature	Relative humidity (RH) (only for *E. multilocularis*)	Time of viability
-80°C	-	2 days
-70°C	-	4 days
4 °C	-	300 days
37°C – 39°C	-	2-14 days
50°C	-	24 hours
72°C	-	10 min
25°C	27%	2 days
43°C	15%	2 hours
45°C	85% - 95%	3 hours

Table 6. Resistance of *Toxocara* spp.

Temperature	Relative humidity	Resistance of larvae
15°C – 35°C	85%	2-5 weeks (time to develop inside the egg) – 6 years

-15°C	-	Inactivate

Table 7. Classification of Biological Agents from Directive 2000/54/EC [152]

Group	Effects on humans	Risk to workers	Prophylactic and therapeutic measures	Example of BA
Risk Group 1	Low chances of causing disease	Very low	-	-
Risk Group 2	Can cause diseases	Low risk; little chance of spreading in the community	Normally available	*Giardia duodenalis*; *Entomoeba hystolytica* *Cryptosporidium* spp; *Toxoplasma gondii*; *Toxocara* spp.; *Strongiloides* spp.; *Ancylostoma* spp.
Risk Group 3	Capable of causing serious illness	Serious risk; manage to spread in the community	Normally available	*Escherichia coli* (es. O157:H7 or O103); E. *granulosus*; *E. multiocularis*; *E vogeli Leishmania*

				brasiliensis; *Leishmania donovani*
Risk Group 4	Serious illnesses	Serious risk; can spread very easily in the community	Normally not available	Congo–Crimea hemorrhagic fever; Lassa Virus; Ebola Virus

Table 8. Containment measures against Laboratory Biological Agents extracted from Annex VI of Directive 2000/54/EC [152]

Containment measures	Containment levels		
	BSL-2	BSL-3	BSL-4
The workplace is to be separated from any other activities in the same building	No	Recommended	Yes
The workplace is to be sealable to permit fumigation	No	Recommended	Yes
Infected material including any animal is to be handled in a safety cabinet or isolation or other suitable containment	Where appropriate	Yes, where infection is by airborne route	Yes

Input air and extract air to the workplace are to be filtered using (HEPA) or likewise	No	Yes, on extract air	Yes, on input and extract air
The workplace is to be maintained at an air pressure negative to atmosphere	No	Recommended	Yes
Surfaces impervious to water and easy to clean	Yes, for bench and floor t	Yes, for bench, floor and other surfaces determined by risk assessmen	Yes, for bench, walls, floor and ceiling
Surfaces resistant to acids, alkalis, solvents, disinfectants	Recommended	Yes	Yes
Access is to be restricted to nominated workers only	Recommended	Yes	Yes, via airlock
Efficient vector control, for example rodents and insects	Recommended	Yes	Yes
Specified disinfection procedures	Yes	Yes	Yes
Safe storage of a biological agent	Yes	Yes	Yes, secure storage
Personnel should shower before leaving the contained area	No	Recommended	Recommended
Validated inactivation process for the safe disposal of animal carcases	Recommended	Yes, on or off site	Yes, on site

A laboratory is to contain its own equipment	No	Recommended	Yes
An observation window, or, alternative, is to be present, so that occupants can be seen	Recommended	Recommended	Yes

Chapter 2

Section 2.1

Research Article

Adapted from

A survey on Apicomplexa protozoa in sheep slaughtered for human consumption

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Abstract

Infections with the Apicomplexa *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis* spp. are common causes of reproductive disorders in sheep. However, few epidemiological studies regarding co-infections with these three protozoa are reported in sheep in Italy. For this reason, this study aims to evaluate possible co-infections with *T. gondii*, *N. caninum*, and *Sarcocystis* spp. in sheep slaughtered for human consumption. From April to July 2019, individual blood, brain, heart, and diaphragm samples were collected from 138 sheep after slaughtering. The presence of IgG anti-*Toxoplasma* in serum samples was evaluated through ELISA. DNA of the three protozoa was investigated using specifc PCRs. Co-infection with *T. gondii*, *N. caninum* and *Sarcocystis* spp. was found in 66.7% of the examined sheep. Antibodies against T. gondii were found in the 36.2% of serum samples. The presence of *T. gondii* DNA was detected in the 67.4%, 77.5%, and 21.7% of the brain, heart, and diaphragm samples, respectively. *Neospora caninum* DNA was found in 72.5% of the examined brain samples. *Sarcocystis* spp. DNA was detected in 92% and 52.2% of the heart and diaphragm samples, respectively. Sequence analysis of the *Sarcocystis* spp. revealed the sole presence of *Sarcocystis tenella*. The present study demonstrates that sheep have a high risk of infection with the three Apicomplexa investigated, suggesting the need to adopt adequate measures to prevent the spread of these parasitic infections considering their clinical and economic impact on ovine production. Furthermore, the possible role sheep play in the zoonotic transmission of toxoplasmosis to humans was highlighted.

Keywords: *Toxoplasma gondii,·Neospora caninum, Sarcocystis tenella*, Sheep, Foodborne parasites, Italy

Introduction

The phylum Apicomplexa includes parasites of veterinary and medical signifcance as well as economic interest (Ortega-Mora et al. 2007; Gajadhar et al. 2015). Three important protozoa within this phylum, *Toxoplasma gondii, Neospora caninum,* and *Sarcocystis* spp., negatively impact the reproductive efficiency of farmed ruminants including sheep (Buxton 1998; Ortega-Mora et al. 2007; Lindsay and Dubey 2020). While *N. caninum* is mainly known to cause reproductive failure in cattle, *T. gondii* is recognized to be one of the principal causes of abortion in sheep (González Warleta et al. 2014; Hecker et al. 2019). Sarcocystosis in small ruminants is associated with foetal infection and abortions to a lesser extent (Buxton 1998; Ortega-Mora et al. 2007). *Toxoplasma gondii* and *N. caninum* exhibit a similar two-stage asexual life cycle in the intermediate host and a host-specific sexual cycle in the definite host (Ortega-Mora et al. 2007; Lindsay and Dubey 2020). The first, *T. gondii*, is by felids (definitive hosts), and its infective stage is capable of infecting virtually all warm-blooded animals, including humans (Innes et al. 2009; Gajadhar et al. 2015). In sheep, clinical toxoplasmosis appears when the infection occurs during early to mid-gestation. In such cases, spreading tachyzoites can cause transplacental infection (exogenous trans-placental transmission) leading to parasitism of placental and foetal tissues followed by foetal death and resorption, abortion, stillbirth, or weakly born lambs often together with a mummified foetus (Buxton 1998; Taylor 2000; Innes et al. 2009; Lindsay and Dubey 2020). Foe *N. caninum*, canids act as definitive hosts and ruminants (including sheep) as intermediate hosts (Ortega-Mora et al. 2007; Dubey et al. 2017). Like the previous, tachyzoites disseminate to numerous organs, possibly including trans-placentally to the foetus, causing tissue damage (Ortega-Mora et al. 2007; Dubey et al. 2017). Additionally, as in cattle, it is likely that reactivation of dormant tissue cysts during

gestation allowing for tachyzoites to spread to the foetal tissues (endogenous trans-placental transmission) represents a major infection route for this parasite in sheep (González Warleta et al. 2014, 2018; Filho et al. 2017). Contrary to *T.gondii*, ovine neosporosis was previously not considered to impact the reproductive success of flocks significantly (Buxton 1998; Taylor 2000; Hässig et al. 2003). However, recent reports and studies are pointing towards *N. caninum* to be the cause of reproductive dysgenesis in sheep more often than previously thought (West et al. 2006; Masala et al. 2007; Howe et al. 2012; Moreno et al. 2012; González-Warleta et al. 2014; 2018; Hecker et al. 2019). Besides, experimentally induced

neosporosis during the first and second thirds of gestation was shown to be 100% fatal for the foetus, leading to foetal resorption or abortion (Arranz-Solís et al. 2015; Dubey et al. 2017). The third protozoa of interest, *Sarcocystis* spp., have an obligatory prey-predator life cycle in which prey ingest sporocysts presents in food or water contaminated by the faeces of predators (Taylor 2000; Lindsay and Dubey 2020). Asexual reproduction occurs in the intermediate host and includes multiple generations of merogony with the formation of bradyzoite *Sarcocysts* in the intermediate host's muscle cells (Buxton 1998; Gajadhar et al. 2015; Lindsay and Dubey 2020). Overall, sheep function as intermediate hosts for six species of *Sarcocystis*: *Sarcocystis tenella, Sarcocystis arieticanis, Sarcocystis gigantea, Sarcocystis medusiformis, Sarcocystis microps,* and *Sarcocystis mihoensis* (Hu et al. 2017; Gjerde et al. 2020). Pathogenic species consist of those with canid definite hosts (*S. tenella, S. arieticanis*) (Buxton 1998; Ortega-Mora et al. 2007; Dubey et al. 2015a) where primary infection during gestation can lead to foetal death, abortion, or premature lambs (Taylor 2000; Ortega Mora et al. 2007; Dubey et al. 2015a). Antibodies against *T. gondii* have been detected in small ruminants worldwide, and based on previously published reviews, it is clear this parasite to be highly prevalent in sheep (Dubey 2009; Stelzer et al. 2019). Similarly, the presence of *N. caninum* in sheep has been documented in most parts of the world including Europe, the Middle East, Asia, Australia, New Zealand, and South America (Dubey et al. 2017). *Sarcocystis* spp. are some of the most common parasites in livestock (Hecker et al. 2018), and *S. tenella, S. arieticanis,* and *S. gigantea* seem to have a global distribution (Dubey et al. 2015a; Gjerde et al. 2020). The presence of *S. medusiformis* has only been recorded in New Zealand, Australia, Iran, Jordan, Spain, and Sardinia, Italy (Dubey et al. 2015a; Gjerde et al. 2020), and S. *microps* and *S. mihoensis* are rarely reported (Hu et al. 2017; Gjerde et al. 2020). In Italy, depending on the region and technique, a *T. gondii* seroprevalence between 28 and 83% has been reported (Fusco et al. 2007; Masala et al. 2007; Natale et al. 2007; Vesco et al. 2007; Zedda et al. 2010; Cenci-Goga et al. 2013; Gazzonis et al. 2015; Bacci et al. 2016). For *N. caninum*, a seroprevalence of 19–46% can be found in the current scientific literature (Tamponi et al. 2015; Gazzonis et al. 2016). Fewer studies on Sarcocystosis in Italian sheep have been published. However, current data suggests pathogenic species to be highly common as the presence of these parasites was detected in 78-100% of examined slaughterhouse samples (Pipia et al. 2016; Bacci et al. 2016; Pagano et al. 2020). Even though current data show *T. gondii, N. caninum* and *Sarcocystis* spp. to be widespread in sheep in Italy, epidemiological studies concerning the

co-infection of these three protozoa commonly recognized to cause significant economic losses are scarce. Furthermore, the few studies where multiple ovine Apicomplexa are included solely report two of the three parasites are discussed above (Masala et al. 2007; Bacci et al. 2016; Gazzonis et al. 2016). For this reason, this study aims to evaluate possible co-infections with *T. gondii, N. caninum,* and *Sarcocystis* spp. in sheep slaughtered for human consumption in Sardinia, where approximately half (over 3 million of sheep) of the entire Italian sheep population is reared (ISTAT 2020), through the use of biomolecular and serological methods.

Material and methods

Sample collection

From April to July 2019, individual blood and tissue samples (brain, heart and diaphragm) were collected, at the time of slaughtering, from 138 Sarda sheep, females, aged between 3 and 7 years and semi-extensively reared. The amount of tissue samples was 50 g for the brain and heart, while 5 g was collected from the diaphragm. Samples were collected in abattoirs from 8 different municipalities in Sardinia. Each animal was assigned a unique ID number, and samples were marked accordingly. Samples were transported to the Parasitology Laboratory of the Veterinary Teaching Hospital of the University of Sassari immediately after collection. Upon arrival, blood samples were centrifuged at 2000 rpm for 10 min, and the obtained sera were stored at – 20 °C. Each tissue sample (brain, heart, and diaphragm) was homogenized into small pieces (approximately 1 mm × 1 mm) using an Ultra Turrax® homogenizer (IKA, Staufen, Germany). All devices used were washed several times with sodium hypochlorite solution (2.5%) followed by distillate water to avoid DNA cross-contamination between the samples, as previously described (Santos et al. 2010). After homogenization, an aliquot of 50 mg was stored at – 20 °C for biomolecular examination.

Biomolecular analysis

DNA was extracted from 50 mg of homogenized tissue (brain, heart, and diaphragm) using a commercial kit (G-spinTM total DNA extraction kit, Korea), according to the manufacturer instructions. Three different polymerase chain reaction (PCR) protocols were applied to

detect the DNA of *T. gondii, Sarcocystis* spp*.,* and *N. caninum*, respectively. Each PCR reaction was carried out in a final volume of 25 µl containing 10X PCR buffer, 1.5 mm MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dNTP), and 0.2 µM of Thermus aquaticus DNA polymerase (Thermo Fisher Scientifc, Massachusetts USA). For all T. *gondii* samples (brain, heart, and diaphragm), a nested PCR (nPCR) was performed in order to amplify a 302 bp fragment of the internal transcribed spacer 1 (ITS1) region as previously described (Halová et al. 2013). In detail, the external primers NN1 (5′-CCT TTGAATCCCAAGCAAAACATGAG-3′) and NN2 (5′-CGAGCCAAGACATCCATTGCTGA-3′) and the internal primers ITSfw (5′-GATTTGCATTCAAGAAGCGTGATA GTAT-3′) and ITSrev (5′-AGTTTAGGAAGCAATCTG AAAGCACATC-3′ were used for the first and second PCR reaction, respectively. The thermal cycler conditions were 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 65 °C for 45 s, and 72 °C for 1 min, followed by 5 min at 72 °C for the first PCR reaction and 95 °C for 5 min, 50 cycles of 94°C for 40 s, and 60°C and 72 °C for 1 min followed by 7 min at 72°C for the second PCR reaction. *Neospora caninum* DNA was detected in brain samples through an PCR amplifying the 224 bp NC5 target region as reported by Yao et al. (2009). Briefly, the external primers were Np6+ (5′-CTCGCAGTCAACCTACGTCTTCT-3’) and Np21+ (5′-CCCAGTGCGTCCAATCCTGTAAC-3′), while the internal primer were Np9 (5′-GTTGCTCTGCTGACGTGTCGTTG-3′) and Np10 (5′ CTCAACACAGAACACTGAACTCTCG 3′); the thermal cycler conditions were the same for the first and second PCR reactions: 94°C for 5 min, 35 cycles of 94 °C per 30 s, 63 °C for 20 s, and 72 °C for 30 s followed by 10 min at 72 °C. Finally, *Sarcocystis* spp. DNA extracted from heart and diaphragm samples was detected through conventional PCR targeting a fragment of the rRNA 18S gene (609bp) according to Hamidinejat et al. (2014). Specifically, the primers used were Sar-F1 (5′ GCACTTGATGAATTCTGG CA 3’) and Sar-F2 5′ CACCACCCATAGAATCAAG 3′), and the thermal cycler conditions consisted in 94 °C for 5 min, 30 cycles of 94 °C for 2 min, 57°C for 30 s, and 72 °C for 2 min, followed by 72°C for 5 min. All PCR reactions were run in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystem, Foster City, CA, USA). The PCR amplification products were resolved using electrophoresis in 2% agarose gels and visualized by UVIdoc HD2 (UVITEC, Cambridge, UK). PCR-positive samples were purified using Nucleospin Gel and PCR clean up (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to an external sequencing

service (Eurofns Genomics, Ebersberg, Germany) in order to confirm the specificity of the PCR amplification. The sequences obtained were compared with those found in the National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

Serological survey

Sera were tested for the presence of IgG anti-*Toxoplasma* using a commercial enzyme linked immuno-adsorbent (ELISA) kit (PrioCHECK® Toxoplasma Ab SR, Prionics, Schlieren-Zurich, Switzerland). The kit included ELISA plates coated with cell culture-derived *T. gondii*-tachyzoite antigens, a peroxidase-labelled anti-small ruminant secondary antibody, tetramethyl benzidine (TMB) as a chromogenic substrate, control sera, and buffer solutions. Serum samples were tested at a 1:100 dilution with sample diluent buffer. Optical density (OD) was measured at a wavelength of 450 nm (reference filter 620 nm), and the results were interpreted by calculating, for each sample, a percentage of positivity (PP) relative to the OD of the positive control (PP sample = OD450 nm sample/OD450 nm positive control × 100). A PP value exceeding 20 was considered as positive and below 20 as negative, as suggested by the manufacturer.

Statistical analysis

The data generated for *T. gondii, N. caninum*, and *Sarcocystis* spp. were recorded on a spreadsheet (Microsoft Excel®, Microsoft Corp., Redmond, WA) and subsequently analysed by Chi-square test ($\chi 2$) (Epi-info 6.04, CDC, Atlanta, GA, USA). Results were considered statistically significant for $P < 0.05$.

Results

All sheep included within this research (100%, 138/138) were found to be positive for at least one of the three targeted protozoa, at least one of matrices and diagnostic techniques used. Co-infection with *T. gondii, N. caninum*, and *Sarcocystis* spp. was detected in 66.7% (92/138) of the examined sheep. Details are shown in Fig. 1.

Molecular analysis

The presence of *T. gondii* DNA was detected in the 67.4% (93/138), 77.5% (107/138), and 21.7% (30/138) of the brain, heart, and diaphragm samples, respectively ($\chi 2 = 98.75$; $P < 0.001$). No statistically significant differences were observed between samples from the heart and brain ($\chi 2 = 3.56$; $P = 0.059$), while *T. gondii* prevalence was significantly higher in the heart ($\chi 2 = 85.21$; $P < 0.001$) and the brain ($\chi 2 = 58.21$; $P < 0.001$) compared to the diaphragm. *Neospora caninum* DNA was found in 72.5% (100/138) of the examined brain samples. The sequence analysis of the NC5 gene identified the isolates as *N. caninum* with a homology of 98.86–100% (accession number LN714488), confirming the PCR amplification results. Finally, ovine heart and diaphragm tissues did not show any macroscopic cysts even though PCR revealed *Sarcocystis* spp. DNA in 92% (127/138) and 52.2% (72/138) of the heart and diaphragm samples, respectively ($\chi 2 = 54.49$; $P < 0.001$). Sequencing of the 18s rRNA gene from the *Sarcocystis* spp. isolates showed a homology of 100% with *S. tenella* sequences deposited in GenBank (accession number KP263759).

Serological survey

Antibodies against *T. gondii* were found in the 36.2% (50/138) of serum samples. In 44 of these 50 seropositive animals, DNA could be detected in the brain, heart, or diaphragm. Thus, 31.9% (44/138) of the examined sheep were positive for T. gondii by both methods used, PCR and ELISA.

Discussion

The present survey highlights the presence of co-infection with *T. gondii*, *N. caninum*, and *Sarcocystis* spp. in sheep slaughtered for human consumption in Sardinia. Additionally, high prevalence rates of these protozoa were recorded in different matrices (serum, brain, heart, and diaphragm tissue samples).

Results herein reported show the widespread presence of *T. gondii* in sheep farms in Sardinia, as recently reported in pigs and cattle on the same island (Pipia et al. 2018; Gazzonis et al. 2020). The seroprevalence of *T. gondii* recorded in the present survey (36.2%) is considerably lower than that reported in Sardinia more than 10 years ago (51.3%)

(Natale et al. 2007). Similar results were reported in Portugal (33.6%) and Iran (33.62%) (Lopes et al. 2013; Izadyar et al. 2019).

A higher percentage of positive samples was found using PCR (performed in different matrices) than ELISA (on serum samples). This agrees with other studies where a higher *T. gondii* prevalence was found through PCR compared to serological methods (Rasti et al. 2017; Yousefvand et al. 2021). Such differences most likely result from molecular methods having a higher precision, sensitivity, and specificity than serological methods (Martínez-Flores et al. 2017; Abd El-Razik et al. 2018). Furthermore, through PCR analysis, active *T. gondii* infections can be identified as both living and dead parasites can be detected (Robert-Gangneux and Dardè 2012; Liu et al. 2015). Instead, ELISA test used in the present survey can solely identify chronic infection through the detection of IgG anti-*Toxoplasma* produced by the infected host (Robert-Gangneux and Dardè 2012; Liu et al. 2015).

PCR results showed the prevalence of *T. gondii* to be significantly higher in the heart (77.5%) and brain (67.4%) compared to diaphragm samples (21.7%). Given these results, and in accordance with previous research, authors suggest heart samples to be the best choice for the isolation of *T. gondii* (Dubey et al. 2015b). In addition to skeletal and cardiac muscles, the central nervous system has been proven to be a preference site for tissue cysts (Tenter 2009). However, the distribution of cysts and the parasite burden in different tissues may depend on the *T. gondii* strain, the infective stage (oocysts, tachyzoites and bradyzoites), and the time of infection, increasing in heart and skeletal muscles over time (Dadimoghaddam et al. 2014; Juránková et al. 2014; Swierzy et al. 2014; Yousefvand et al. 2021).

An underestimation of the *T. gondii* prevalence in the diaphragm samples within this research may have occurred because for diaphragm, only 5 g was homogenized, whereas for brain and heart tissue, 50 g of each was homogenized, resulting in a potentially higher chance of including tissue cyst for those latter tissues. Indeed, the distribution of *T. gondii* parasites within tissues is heterogenous, and thus, the parasites could have been present in the unexamined parts of the diaphragm (Robert-Gangneux and Dardè 2012). It is worthy to note the high prevalence of *T. gondii* found in sheep slaughtered for human consumption in this research, revealing a potential risk for consumers, especially pregnant woman and immunodeficient individuals (Weiss and Dubey 2009). Indeed, the consumption of raw or undercooked meat containing *T. gondii* tissue cysts is among the main routes of transmission of this parasite to humans, together with oocyst contaminated water and contact with cat faeces carrying *T. gondii* oocysts (Dubey 2021). Thus, this research allowed to confirm sheep meat as a possible source of toxoplasmosis for humans (Belluco et al. 2016; OIE

World Organization for Animal Health 2021). This is especially true considering the popularity of sheep products on the island. Nevertheless, in Sardinia, several projects have been undertaken to improve the global value of sheep meat-based food products, e.g. the production of sheep sausages, ham, and air-dried whole shoulder (Mangia et al. 2006). Although the curing processes applied in their production make these products microbiologically safe for human consumption, these processes have not been validated for the inactivation of *T. gondii* cysts present in meat and require further attention (Herrero et al. 2017; Hill et al. 2018; Fredericks et al. 2020). Finally, given the high presence of *T. gondii* found in the heart and brain of sheep examined within this research, authors recommend at risk people to avoid the consumption of products derived from these sources or better to consume them after having been cooked thoroughly.

A potential risk for the livestock industry is also highlighted here since toxoplasmosis is recognized to be responsible of great economic losses due to foetal death in addition to costs linked to veterinary services (e.g. diagnosis, costs for anti-inflammatory substances to reduce the fever in acute toxoplasmosis, treatment of fertility problems after abortion) (Stelzer et al. 2019; Nayeri et al 2021).

Similarly, the significance of the high prevalence of *N. caninum* (72.5%) reported here should not be underestimated. Despite that the clinical, epidemiological, and economic importance of *N. caninum* infections in sheep is still unclear, the potential role of this protozoa in ovine reproductive problems has been highlighted by several authors (Howe et al., 2012; González-Warleta et al. 2014; Al-Shaeli et al. 2020). Additionally, *N. caninum* has previously been isolated from ovine abortion samples in Sardinia, emphasizing its causal role in sheep abortions (Masala et al. 2007).

Besides this, the presence of numerous stray and shepherd dogs in Sardinia (and their close contact with sheep) could contribute to the spread of this parasitosis to more sensitive animal species such as cattle in which *N. caninum* is considered the highly prevalent cause of abortion (Al-Shaeli et al. 2020; Varcasia et al. 2020). Luckily there is no evidence of the zoonotic potential of this protozoan even though high frequency of *N. caninum* antibodies has been found in humans, especially immunocompromised patients (Lobato et al. 2006; Duarte et al. 2020).

Most data regarding ovine neosporosis have been obtained through serological essays while those related to the detection of *N. caninum* DNA in naturally infected adult sheep are few (Castañeda-Hernández et al. 2014; Arbabi et al. 2016; Amdouni et al. 2018). Amplification of the NC5 gene is one of the most suitable techniques for the detection of *N. caninum* due

to its sensitivity and specificity, allowing for the discrimination between the related apicomplexan parasites examined in the present survey and for the identification of active infections, contrary to serological tests that only indicate parasite exposure (Castañeda-Hernández et al. 2014; Arbabi et al. 2016). Results obtained here confirm the presence of this protozoa in sheep in Sardinia as previously reported in a sero-epidemiological survey where a prevalence of 44.4% and 46.4% was recorded by ELISA in blood and milk, respectively (Tamponi et al. 2015). However, this research reported the prevalence of *Neospora* DNA only in brain samples, chosen as it is the predilection site of this parasite (Dubey 2009). Further studies are needed to assess the presence of *Neospora* DNA in the other tissue samples and anti-*Neospora* antibodies in the serum.

Occurrence of *Sarcocystis* spp. was evaluated in ovine heart and diaphragm samples by PCR and revealed a prevalence of 92% and 52.2%, respectively. The absence of visible macroscopic cysts suggests the presence of microscopic species, and in fact, sequence analysis identified *S. tenella* as the only species involved. This finding is in accordance with a previous survey on sheep sarcocystosis carried out in Sardinia that reported *S. tenella* with a high prevalence (95.5%) (Pipia et al. 2016). These microscopic cysts producing species are considered the most pathogenic in sheep, responsible for fever, loss of appetite, and anaemia (Dubey 1988; Bacci et al. 2016). Furthermore, *S. tenella* can cause abortion or premature birth of offspring in pregnant sheep (Bacci et al. 2016). Other, less pathogenic species, *S. gigantea* and *S. medusiformis*, were not observed in heart and diaphragm tissues of all the examined animals. These macroscopic *Sarcocystis* transmitted by felids can be found in various tissues and organs depending on the species: *S. gigantea* is mainly detected in the oesophagus, larynx, and tongue, while *S. medusiformis* cysts are found in the diaphragm, abdominal muscles, and the carcass (Dong et al. 2018). A previous study carried out in Sardinia showed the oesophagus and abdominal muscles of sheep to be the most affected by these macroscopic species of *Sarcocystis*, while no macroscopic cysts were found in the diaphragm and heart, in agreement with our results (Pipia et al. 2016). Furthermore, *S. tenella* and *S. arieticanis* were reported as more prevalent in comparison to *S. gigantea* and *S. medusiformis* in China, Brazil, and Iraq (Dong et al. 2018; Minuzzi et al. 2019; Abdullah 2021).

The detection of canid transmitted sarcocystosis in the examined sheep underlines the significant role of dogs in the spread of *S. tenella* among others (such as *N. caninum*) on the island. In Sardinia, sheep flocks have a great chance of coming into contact with dog faeces considering the extensive farming practices applied, the high presence of shepherd dogs, and

the defecation behaviour of dogs in general (which increase the environmental spread of various parasites, including *S. tenella*, and the risk of transmitting infection to sheep during grazing) (Smith et al. 2014; Varcasia et al. 2020). Likewise, the extensive sheep farming applied in Sardinia contributes to the contact risk of sheep with cat faeces and the consequent transmission of parasites such as *S. gigantea* and *S. medusiformis*. However, the cat's defecation behaviour, consisting of burying its faeces, leads to a lower spreading potential of oocysts and sporocysts, decreasing the risk of infection for sheep, possibly explaining the absence of *Sarcocystis* species transmitted by cats in the sheep examined (Tamponi et al. 2020; El-Morsey et al. 2021). On the other hand, for *T. gondii*, where cats also function as definitive hosts, a high prevalence was found, highlighting the possibility that vertical transmission could play an important role in the transmission of this protozoa in sheep (Minuzzi et al. 2019).

Despite that the species responsible for sarcocystosis in sheep (and *Sarcocystis* spp. in general) are host specific (Dubey et al. 2015a), their implication in the development of toxic effects has been studied experimentally in other animal species. In particular, protein extracted from *S. gigantea* was be found to be toxic in mice and rats (Al-Hyali et al. 2009, 2010) and an antigen obtained from *S. tenella* caused toxic manifestations in rabbits (Mandour 1969). In any case, ovine *Sarcocystis* are believed to be non-zoonotic (Dubey et al. 2015a).

In conclusion, the present study demonstrates that sheep have a high risk of infection with the three Apicomplexa investigated (*T. gondii*, *N. caninum*, and *Sarcocystis* spp.) and co-infections are frequent. Our results suggest the brain and heart to be suitable matrices for the molecular detection of the investigated protozoa (*T. gondii*: brain and heart, *N. caninum*: brain, *S. tenella*: heart). Overall, any ovine meat for human consumption should be cooked or prepared adequately in order to inactivate infective parasite stages. Finally, adequate control programs and sanitary measures (e.g. promotion of appropriate disposal of ovine placentas and carcasses, avoiding unsupervised home slaughtering, limiting the access of dogs and cats to livestock) should be adopted in order to prevent the spread of these parasitic infections considering their clinical and economic impact on ovine production and the possible role sheep play in the zoonotic transmission of toxoplasmosis to humans.

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Figures

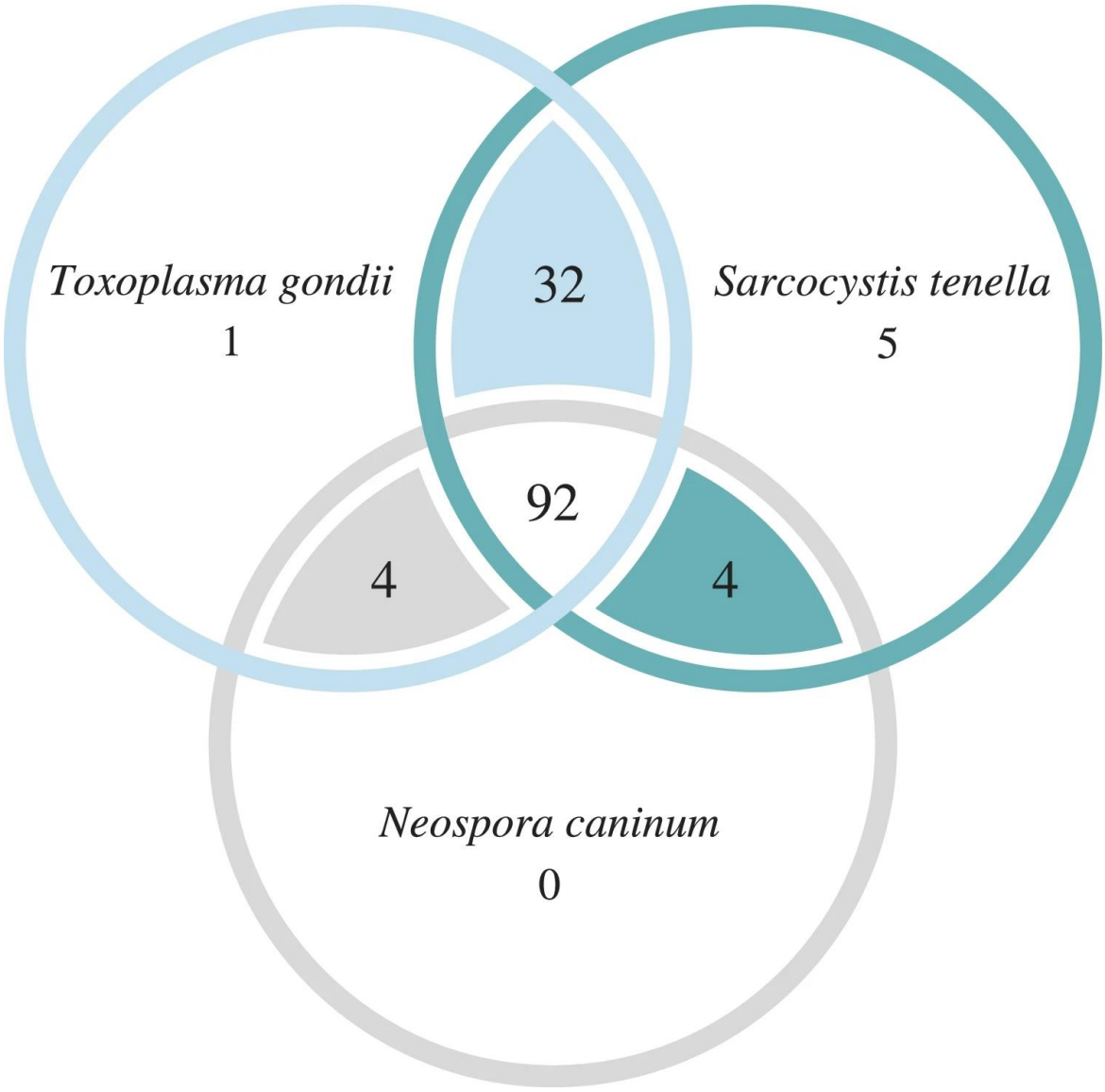

Fig. 1 Venn diagram showing the prevalence of *Toxoplasma gondii, Neospora caninum* and *Sarcocystis tenella* found in the examined animals and their intersection

Section 2.2

Research Article

Adapted from

Seroepidemiological and biomolecular survey on *Toxoplasma gondii* in Sardinian wild boar (*Sus scrofa*)

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Abstract

Toxoplasma gondii is a zoonotic parasite able of infecting all warm-blooded animals. Toxoplasmosis is one of the major foodborne diseases globally. The consumption of wild boar (*Sus scrofa*) meat from recreational hunting has been linked to outbreaks of human toxoplasmosis. The island of Sardinia (Italy) contains a large wild boar population, thus providing an opportunity to assess the distribution of *Toxoplasma* in this species and the associated risks of transmission to humans. A total of 562 wild boars were screened: heart and meat juice samples were tested for *T. gondii* DNA *via* nested-PCR and IgG anti-*Toxoplasma* by commercial ELISA. Anti-*Toxoplasma* IgG were detected in 24.6% (138/562) of animals, while 37.2% (209/562) of the heart samples were PCR positive. The prevalence of *T. gondii* antibodies and DNA highlights the potential role of wild boar as an important reservoir for this parasite. The study suggests that wild boar could play a significant role in spreading the parasite to humans. As wild boar numbers are increasing throughout their range, their potential role in transmitting toxoplasmosis should be communicated to stakeholders, and the impact of different population control methods on disease transmission should be thoroughly assessed to mitigate potential threats effectively.

Keywords: *Toxoplasma gondii,* Foodborne disease, Zoonosis, Human infection, host-parasite distribution, meat consumption.

Introduction

Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan parasite *Toxoplasma gondii* (Phylum Apicomplexa). While *T. gondii* is known to infect all warm-blooded animals, only felids act as definitive hosts and shed oocysts in their faeces. Wild boar (*Sus scrofa*) and other intermediate hosts can become infected *via* several routes: i) by ingesting sporulated oocysts excreted by felids that contaminate the external environment; ii) by consuming raw or undercooked meat of infected intermediate hosts containing tissue cysts or iii) congenitally *via* transplacental transmission. Due to its remarkable efficiency, *T. gondii* is considered the fourth most important parasite in the world (Boireau et al., 2014). One-third of the human population is estimated to be chronically infected by *T. gondii* (Almeria & Dubey, 2021). Although most infections are asymptomatic, life-threatening or

fatal complications in humans can occur, particularly in immunocompromised individuals. In addition, primary infections acquired during pregnancy may result in a range of adverse outcomes, including foetal ocular infection, cranial and neurological deformities, stillbirth, and miscarriage (McCall et al., 2022). The consumption of raw and undercooked meat from farm animals is an essential source of human infection; poor hygiene practices while handling contaminated feedstuffs may further contribute to human transmission (Santoro et al. 2019). The ever increasing numbers of wild boar worldwide, and particularly in Europe (Jori et al., 2021; Massei et al., 2015), as well as the growing popularity of wild boar meat in different parts of the world (Rostami et al., 2017), underpins the increased risk of toxoplasmosis transmission through consumption of meat derived from recreational hunting. Indeed, over the last few years, epidemiological investigations have been conducted worldwide to assess the role of wild boar in the transmission of *T. gondii* (Olsen et al., 2019; Rostami et al., 2017). In Europe, *T. gondii* seroprevalence in wild boar is highly variable both within and between countries, ranging from 10 to 50% (A. L. Gazzonis et al., 2018). Moreover, in several European countries, limited data are available on prevalence of toxoplasmosis in wild boar populations. In Mediterranean islands, a single epidemiological survey on wild boar has been carried out in Corsica (Richomme et al., 2010). In Sardinia, prevalence for *T. gondii* in pigs and sheep is 54.5% (Pipia et al., 2018a), and 77.5 % (Dessì et al., 2022), respectively. Nevertheless, thus far, no information on wild boar is available. Therefore, the present study aimed to investigate the presence of *T. gondii* in wild boar in Sardinia using biomolecular and serological techniques, and to discuss the findings in relation to potential risks for human infection.

Materials and methods

The sample size for this survey (n = 377) was calculated a priori using the Raosoft Digital Sample Size Calculator (RaoSoft, Inc., Seattle, WA; http://www.raosoft.com/samplesize.html), with 5% margin of error, and a 95% confidence interval (CI). A population size of 18,750 wild boar was determined based on the data provided by the Carta delle Vocazioni Faunistiche della Sardegna (Apollonio et al., 2011). A prevalence rate of 50% was considered. Wild boar examined in the present study (n = 562) originated from the hunting seasons of 2021-2022 throughout Sardinia (40°N 9°E) (23 812, 6 km²). Animal data were recorded according to age range, gender and municipality of

origin, and the carcass examination was performed by veterinaries during routine post mortem surveillance procedures for the control of African Swine Fever and trichinellosis. The age of each animal was estimated by patterns of tooth eruption and replacement according to Massei and Toso (1993). Adults were defined as animals of ≥ 12 months-old while juveniles were defined as <12 months old. For five (n = 5) samples, age and gender were not recorded but these samples were included in the study; however, they were not included in the risk factor analysis (see below). Organs and viscera from abdominal and thoracic cavities were inspected and removed from each carcass and the hearts sent for parasitological examination to the Parasitology Laboratory of the Department of Veterinary Medicine, University of Sassari, Italy. Upon arrival, the hearts were portioned into 50 g aliquots and stored at – 20 °C in a plastic bag to collect the heart meat juice for subsequent serological analysis. The heart meat juice was collected after thawing each bag containing samples at room temperature and then transferred into 1.5 ml Eppendorf microtubes (Nöckler et al., 2005). Heart tissue samples were homogenized using an Ultra Terrex® homogenizer (IKA, Stiffen, Germany). To avoid DNA cross contamination between samples, all devices were washed several times with sodium hypochlorite solution (2.5%) followed by distilled water (Santos et al., 2010). After homogenization, aliquots of 25 mg of each sample were stored at – 20 °C for subsequent biomolecular examination. DNA was extracted from 25 mg of homogenized heart tissues using a commercial kit (G-spins total DNA extraction kit, Korea), according to the manufacturer's instructions. *Toxoplasma gondii* DNA was detected through a Nested PCR, as described by Pipia et al. (2018a). Briefly, 302 base pairs (bp) – fragment of the Internal Transcribed Spacers-1 (ITS1) region of 18S-5,8S rRNA was amplified, according to Halová et al. (2013). Each PCR reaction was conducted in a final volume of 25µl containing PCR buffer (10X), MgCl2 (1.0 mm), deoxynucleotide triphosphate (dNTPs) (0.5 mm of each), primer FOR (0.5 µm), primer REV (0.5 µm), 0,75 U Taq polymerase (Biotechrabbit GmbH, Berlin Germany). Positive and negative controls were included in each PCR reaction. As reference material, *T. gondii* genomic DNA was obtained from *T. gondii* positive swine heart sample from a previous study conducted in the same laboratory by Pipia et al (2018). After extraction, DNA concentration was determined using a NanoDrop© Lite spectrophotometer. Additionally, internal extraction was conducted. PCR amplification products were resolved in 2% agarose gel and visualized by UVIdoc HD2 (UVITEC, Cambridge, UK). Fragment length estimation was carried out using the Thermo Scientific GeneRuler molecular weight standard (100 bp DNA ladder). PCR-positive samples were purified using nucleospin gel and PCR clean up

(Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sequenced by a commercial service (Eurofins Genomics, Ebersberg, Germany) in order to confirm the specificity of the PCR amplifications. The sequences obtained were compared with those available in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

The heart meat juice from each sample was tested for the presence of anti-*Toxoplasma* IgG using a commercial enzyme linked immuno-adsorbent (ELISA) kit (PrioCHECK® *Toxoplasma* Ab SR, Prionics, Schlieren-Zurich, Switzerland), as previously described (Dessì et al. 2021). All animal data, along with the results of PCR and ELISA, were recorded on a spreadsheet (Microsoft Excel®, Microsoft Corp., Redmond, WA). The prevalence at the individual level was computed with an associated confidence interval of 95% (95% CI). Data were subsequently analysed using *Chi*-square test (χ^2) (Epi-info 6.04, CDC, Atlanta, GA, USA), and results were considered statistically significant if $P < 0.05$.

Results

The present study revealed an overall prevalence of 37.1% for *T. gondii* (209/562; CI95%: 33.2-41.2) detected by PCR. Female boar yielded a prevalence of 39.7% (106/267; CI95%: 33.8-45.6), while males of 34.8% (101/290; CI95%: 29.3-40.3), albeit no statistically significant difference in infection prevalence was recorded between genders ($\chi2=1.41$; $P=0.23$). A prevalence of 39.4% (158/401; CI95%: 34.6-44.2) and 31.4% (49/156; CI95%: 24.1-38.7) was detected in adults and juveniles, respectively, although this difference was also not statistically significant ($\chi2=3.07$; P=0.079). Of the5 samples for which gender and age of wild boar had not been recorded 2 tested positive by PCR. Nested PCR results were confirmed by sequencing of representative samples, which displayed 99% homology to *T. gondii* sequences available in GenBank (accession number JX456457.1) (Table 1).

An overall prevalence of 24.6% (138/562; CI95%: 21.0-28.1) for anti-*Toxoplasma* IgG was detected in samples subjected to ELISA; in particular, a significantly higher prevalence (29.0%; 84/290; CI95%: 23.7-34.2) was detected in males than in females (i.e. 19.1%; 51/267; CI95%: 14.4-23.8) ($\chi2=7.366$; P=0.006). Likewise, the adult cohort (i.e. 27.9%; 112/401; CI95%: 25.3-34.1) displayed significantly higher seroprevalence than juveniles (i.e. 14.7%; 23/156; CI95%: 9.2-20.3) ($\chi2=10.635$; P=0.001) (Table 1). Three out of the 5 unidentified samples were positive for IgG antibody *T. gondii.*

Table 1. Number of positive cases, prevalence of *Toxoplasma* antibodies and PCR results divided per age (adult ≥ 12 months, juveniles < 12 months) and gender

Animal category	Examined wild boar	Number of Positive Samples		Prevalence		CI (95%)	
		ELISA	PCR	ELISA	PCR	ELISA	PCR
Male	290	84	101	29.0%[a]	34.8%[c]	23.7-34.2	33.8-45.6
Female	267	51	106	19.1%[a]	39.7%[c]	14.4-23.8	29.3-40.3
Adult	401	112	158	27.9%[b]	39.4%[d]	25.3-34.1	34.6-44.2
Juveniles	156	23	49	14.7%[b]	31.4%[d]	9.2-20.3	24.1-38.7

CI 95% confidence interval; [a]$\chi2$=7.366; P=0.006); [b]$\chi2$=10.635; P=0.001; [c]$\chi2$ = 1.41; P value=0.23; [d]$\chi2$=3.07; P value=0.079

Discussion

The present survey represents the first comprehensive epidemiological study of *T. gondii* in wild boar in a large Mediterranean island. The sample size in this study (n=562) exceeded the minimum number of 377 samples initially required, thus strengthening the robustness of the experimental design. The 37.1% prevalence of *T. gondii* recorded in the wild boar population indicates a widespread occurrence of the parasite throughout the island. Nevertheless, the *T. gondii* prevalence recorded by PCR is lower than that detected in other studies conducted in Southern Italy by Santoro et al. (2019) (44%) and Sgroi et al. (2020) (39.6%), but higher than that reported in Northern Italy (16%) (Ferroglio et al., 2014). Seroprevalence rates of *T. gondii* in wild boar vary considerably throughout the globe, i.e. 22.7% in Japan (Saito et al., 2021), 36% in Korea (Jeong et al., 2014), 27.7% in the United States (Sandfoss et al., 2011), 12.5% in Argentina (Winter et al., 2019) and 15.6% Brazil (Brandão et al., 2019). In Europe, high seroprevalence rate have been reported in Slovenia

(62%) (Bandelj et al., 2021), Poland (48%) (Puchalska et al., 2021), Sweden (50%) (Wallander et al., 2015), the Czech Republic (40%) (Račka et al., 2015), Romania (56.6%) (Grema et al., 2015), and Corsica (55%) (Richomme et al., 2010). Conversely, relatively low seroprevalence rates have been recorded in Slovakia (8.1%) (Antolová et al., 2007) and Switzerland (6.7%) (Berger-Schoch et al., 2011). A PCR study conducted in the Czech Republic showed a low prevalence (8.8%) while a higher seroprevalence rate (15.4%) was recorded (Slany et al., 2016), likely attributed to the use of diaphragmatic instead of cardiac tissue. The overall seroprevalence (24.5%) recorded in our study is similar to that reported in Germany (24.40%) (Bier et al., 2020), and in the Netherlands (24.4%) (Opsteegh et al., 2011), but higher than those recorded in Spain (14%) (Lizana et al., 2021) and Central Italy (14%) (Ranucci et al., 2013). These variations emphasize the challenges with comparing seroprevalences among different surveys, which are conducted under different circumstances in geographically distinct areas, and through the application of different diagnostic techniques and epidemiological approaches (Veronesi et al., 2011). It is essential to note that this study used meat juice as a serological matrix, which differs from blood serum. ELISAs applied to this material are characterised by lower sensitivity than blood serum. However, specific antibody levels in meat juice vary depending on the muscle(s) sample, with heart meat juice characterised by significantly higher levels of antibodies compared with other muscles (Wallander et al., 2015). ELISA can only identify chronic infections *via* the detection of anti-*Toxoplasma* IgG, while PCR analysis detects nucleic acids from *T. gondii* (Dessì et al., 2022). Importantly, previous studies have shown that the detection of *T. gondii* antibodies in wild boar is positively correlated with the presence of bradyzoites in the animal muscles (Bártová et al., 2006; Richomme et al., 2010).

In our survey, animal gender and age were identified as risk factors for *T. gondii* infection, with significantly higher seroprevalences recorded in males (29.0%) compared to females (19.1%) and in adults (27.9%) compared with juveniles (14.7%). The higher seroprevalence in male wild boar is not consistent with data from other studies, that reported no gender-related difference in seroprevalence (Opsteegh et al., 2011; Ranucci et al., 2013; Richomme et al., 2010). This may be explained by the larger home ranges of males compared to females (Cavazza et al., 2023; Laguna et al., 2022).The higher prevalence observed in adult wild boar is also not consistent with data from other studies and it may be related to longer exposure to the parasite compared with juveniles (Antolová et al., 2007; Opsteegh et al., 2011; Ranucci et al., 2013). However, whether anti-*T. gondii* immunity in wild boar is a lifelong condition remains to be determined. Opsteegh et al. (2011) showed that animals up

to 10 months of age display a step-increase in seroprevalence, with titres of anti-*Toxoplasma* antibodies stabilising thereafter. This suggest that susceptibility to infection may remain throughout an animal's life. Based on our PCR data, gender and age likely to represent a risk factors, with a higher PCR prevalence in females (39.7%) than males (34.8%) and in young animals (39.4%) compared to adults (31.2%), although differences between categories are minimal. The stress associated with pregnancy and breastfeeding in females may lead to an increased susceptibility to *T. gondii* infection, a hypothesis that requires testing. Similarly, the higher prevalence recorded in young wild boar may be linked to instances of transplacental transmission and/or the developing immune system (Dubey et al., 1990; Pipia et al., 2018b). However, this data contrast with widespread knowledge that *T. gondii* prevalence rates are higher among older animals compared to piglets. This discrepancy is usually attributed to the higher likelihood of older wild boars coming into contact with infective oocysts or infected intermediate hosts (Herrero et al., 2016).

Our data support the hypothesis that infected wild boar may represent a key-sentinel of environmental contamination with *T. gondii*, due to their capacity for adaptation to different habitats, wide geographical distribution and high reproductive rates (Sgroi et al., 2020). Recreational hunting can impact wild boar social and spatial behaviour (Keuling & Massei, 2021). For instance, a study in Catalonia (Spain) found six of 40 wild boar, which had been ear-tagged and later culled by hunters, at a mean linear distance of 45.8 km (min. 30, max. 89.8) from their origin (Casas-Díaz et al., 2013). Wild boar could play an important role in maintaining the *T. gondii* sylvatic cycle due to their scavenging and predatory behaviour, and frequent interactions with a wide range of different hosts (including sheep, cattle, birds, rodents and foxes).

Hunter malpractices, such as inconsiderate disposal of animal offal in the environment following evisceration, could exacerbate the perpetuation of *Toxoplasma* life cycle (Sgroi et al., 2019). Wildlife animals, like wild boar, predators (including cats) or pigs, can scavenge offal and viscera, indirectly amplifying transmission risks within the food chain and, consequently, the risk of human exposure (Ranucci et al., 2013). The detection of *T. gondii* in wild boar meat highlights a potential risk to humans via the growing market of wild boar sausages and cured meat (Richomme et al., 2010). These latter products have previously been shown a risk for infection with pathogenic microorganisms, including *T. gondii* (Fredericks et al., 2019; Hill & Dubey, 2018). Sausages and fresh meat processed through smoking, salting, drying, and injection of solutions containing sodium chloride, potassium lactate and sodium lactate are intended for direct consumption. However, some of these

techniques may inactivate bradyzoites pathogen transmission remains possible (Hill et al., 2006; Hill & Dubey, 2018; Kijlstra & Jongert, 2008). In particular, the "Salsiccia Sarda" (Sardinian sausage), a short-aged, cured salami, made with meat and fat from pork, is a traditional Sardinian product included in the national list of traditional agri-food products (XXIII Revision of the list of traditional agri-food products produced, Italian Republic, 22.05.2023). Curing for >12 months is recommended to ensure the inactivation of *T. gondii* in many salami (Fredericks et al., 2019). Nevertheless, for the Sardinian sausage, this time is reduced to a maximum of 20-25 days, potentially allowing any *T. gondii* cysts to maintain their infectious properties throughout the curing process.

The ever-rising number of wild boar in many countries, Sardinia included, has led hunters to consume wild boar game meat by also producing fresh sausages, thus increasing the risk of transmission to humans. Consumption of raw or undercooked wild boar meat may lead to infection, as evidenced by several reported cases of acute toxoplasmosis among hunters and their families who consumed meat from infected wild boar (Choi et al., 1997). It is essential to point out that simple and inexpensive methods, such as heating, cooking, and freezing, are effective in inactivating *Toxoplasma* cysts. Several studies have demonstrated that heating water to 60°C for 1 minute can efficiently inactivate tachyzoites. Additionally, cooking meat at 67°C is effective in killing the parasite. Freezing meat for at least three days at -20°C reduces the *T. gondii* load in contaminated meat. Cross-contamination between raw and processed food products must nevertheless be prevented (Mirza Alizadeh et al., 2018). Consequently, hunters can also become infected during evisceration practices and at various stages of venison processing (Tenter et al., 2001).

The prevalence of *T. gondii* in pigs, particularly those intended for family consumption, is higher than that in wild boar, as shown by Pipia et al. (2018) in Sardinian organic pig farms (85.7% and 54.8% seroprevalence and PCR, respectively). This could be attributed to several factors, including high environmental contamination, occurrence of wild and /or stray cats around the farms, the practice of raising domestic pigs alongside other animals (sheep, goats, or cattle) and the persistence of a sylvatic cycle, e.g. though wild boar. Given the widespread consumption and easy access to pig meat, this aspect merits further consideration.

To address this concern, the Biological Hazard Panel of the EFSA has recently proposed the implementation of *Toxoplasma* monitoring programmes (EFSA, 2007). These programs aim to produce *Toxoplasma*-free meat via selection of seronegative animals and the introduction of validated tests and certification, leading to the issuance of '*Toxoplasma*-free meat' label for *Toxoplasma*-free farms (Kijlstra & Jongert, 2008). Furthermore, the development and

standardization of methods for testing of pathogen survival in dry-cured meat products may assist with mitigating the risk of *Toxoplasma* transmission to consumers (Herrero et al., 2016). At present, Italian law does not enforce health checks on meat obtained from hunted wild boar. Biological samples from hunted wild boar are typically tested for the presence of *African Swine Fever* (ASF) and *Trichinella* spp. (according to the European Regulation 2015/1375), which outlines rules for *Trichinella* control in meat and no specific checks are required for other pathogens. However, this game meat is frequently offered in typical country restaurants ("agritourism") to locals and tourists alike, who often consume raw homemade products (e.g., sausages or "guanciale"). Due to this potential risk to public health, these measures could be adopted to reduce the potential role of wild boar in the zoonotic transmission of *T. gondii*, alongside an educational effort to raise public health awareness.

Conclusions

This study underscores the high prevalence of *T. gondii* in Sardinia's wild boar population that poses a substantial risk of transmission to humans. To address this concern, it is recommended for regulatory agencies to implement comprehensive control programmes. These may include public education, implementation of specific regulatory legislations to adopt methods of wild boar density reduction (e.g. through controlled hunting or fertility control programmes) and monitoring of the whole food chain. Subsequently, the adoption of specific sanitary measures and training courses focusing on appropriate application of safe hygiene practices among hunters, such as contamination prevention, cooking and freezing, are pivotal to improve and ultimately guarantee food safety.

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Chapter 3

Research Article

Adapted from

Fight the parasite' – raising awareness of cystic echinococcosis in primary schoolchildren in endemic countries

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Abstract

Background: Cystic echinococcosis (CE) is a widespread zoonosis causing significant economic concern and morbidity in humans. Scarce education on sources of infection and containment measures is considered a key factor responsible for persistent transmission within communities. Recently, edutainment approaches have captured the attention of health education (HE) professionals, due to the benefits of integrating cognitive and emotional learning processes. **Methods:** To this end, a study was carried out in Sardinia, Italy, between 2020 and 2022, amid the SARS-Covid-19 pandemic. The project, designed to involve primary schoolchildren (*via* remote or face-to-face learning depending on the evolving Covid-19 containment measures) consisted of four distinct phases: (1) creation of material for schoolchildren and teachers focused on cystic echinococcosis; (2) pre-intervention evaluation of CE knowledge (i.e. True False Don't Know (TFD) pre-intervention questionnaire based on CE-related knowledge and practices); (3) Edutainment activity (e.g. interactive lessons enhanced by the comic booklet and the "Fight the parasite" cartoon video, hands on educational activities and drawing activities on CE); (4) post-intervention evaluation of CE knowledge (*via* TFD post-intervention questionnaire on CE-related knowledge and practices) and on-site edutainment tour in primary schools taking part to the project. **Results:** The percentage of correct answers increased from 65% in the pre-intervention questionnaire to 87.9% in the post-intervention administration ($\chi 2 = 648.12$; $df = 1$, $P < 0.0001$), whilst the percentage of uncertain answers (i.e., 'I don't know') decreased from 23% in the pre-intervention questionnaire to 5% in the post-intervention administration ($\chi 2 = 603.44$; $df = 1$, $P < 0.0001$), thus indicating significantly enhanced understanding of CE among participating schoolchildren. **Conclusions:** The results of the present survey indicate that the use of digital educational tools, the use of video animations as a model for science communication, as well as other participatory teaching methods enabled children to retain key knowledge of routes of CE transmission and ways to prevent it.

Keywords: Cystic echinococcosis, *Echinococcus granulosus*, health communication, public engagement, edutainment

Background

Cystic echinococcosis (CE) is a widespread zoonosis caused by the larval stage of the tapeworm *Echinococcus granulosus sensu lato* (*s.l.*). The disease is listed among the most severe parasitic diseases in humans and is prioritized by the World Health Organization (WHO) as one of the 17 neglected tropical diseases [1]. The infection causes significant morbidity in humans, with over 1 million cases reported worldwide [2]. According to recent CE burden estimates, about 300,000 disability adjusted life years (DALY's) and approximately $200 million USD are spent annually for the treatment of CE in humans [3]. Dogs and wild canids frequently act as definitive hosts harboring the adult stages of this parasite [4]. Eggs are shed in the feces of the definitive host and are thus excreted in the environment. Subsequently, eggs are accidentally ingested by suitable intermediate hosts, that include several species of herbivorous and omnivorous mammals. Humans may also serve as accidental hosts following close contact with infected canids or consumption of fruits and vegetables contaminated by the parasite eggs [5, 6].

Extensive livestock farming, the presence of numerous stray or shepherd dogs, unsupervised home slaughtering, and improper disposal of carcasses are among the most important factors underlying the persistence of CE in endemic areas [7].

However, human behavior (e.g. man-dog relationships and home slaughtering practices) remains a key element determining continued transmission in these areas; consequently, health information and health education (HE) have long been deemed necessary to achieve successful CE control [8, 9] The integration of HE in school-based programs is recommended by the WHO [9]. Indeed, it has been reported that negative health behaviors starting from childhood may persist through adulthood; thus, modifying children behaviour via health education has the potential to significantly lower risky practices later in life [10]. HE is therefore an effective strategy and a key tool to achieve short- to long-term control of CE [11]; the positive effects of health education have already been validated in principle [12].

Within the broad concept of health education, 'edutainment' has garnered the attention of HE professionals over the last several decades; edutainment is an educational strategy based on 'warm cognition', a branch of study that recognizes the interaction between cognitive and emotional processes in learning [13]. Edutainment integrates Entertainment-Education and pedagogy [14] and takes advantage of educational videos, cartoons, and comic books to communicate complex scientific topics to children [15].

Even though a number of HE programs have been established as part of CE control programmes, very few edutainment initiatives have been carried out so far. Thus, in this study, we sought to design and complete an edutainment programme for primary schoolchildren in an area of Italy (Sardinia) endemic for CE. This health educational project, entitled "Fight the parasite", seeks to develop effective teaching materials and health education techniques adaptable to CE endemic regions worldwide [14]. The project also aims to determine the level of schoolchildren knowledge of routes of infection and behaviours that may halt transmission of CE prior to and following participation in the edutainment programme.

Methods

The project was carried out from 2020 to 2022, amid the SARS-Covid-19 pandemic. Originally, the project was designed to be delivered to primary schoolchildren either *via* remote or face-to-face learning, depending on national and regional Covid-19 mitigation guidelines at the time of the study. The project involved the following steps: (1) Creation of material for schoolchildren and teachers including the comic multilingual educational booklet, the teacher's guidebook for CE, the educational "Fight the parasite" cartoon video and the pre and post intervention questionnaires; (2) Pre-intervention evaluation of CE knowledge (*via* TFD pre-intervention questionnaire based on CE-related knowledge and practices); (3) Edutainment activities (e.g. interactive lessons enhanced by the comic booklet and the "Fight the parasite" cartoon video, hands on educational activities and drawing session on CE); (4) Post-intervention evaluation of CE knowledge (*via* TFD post-intervention questionnaire based on CE-related knowledge and practices) and on-site edutainment tour in primary schools taking part to the study. The engaging resources were built on the characteristics of a successful resume described by the CDC, Characteristics of an Effective Health Education Curriculum [16].

Creation of material for schoolchildren and teachers

Comic multilingual educational booklet with activities and games

From July 2020 to September 2020, comic educational booklets were created in Italian, English and Spanish, featuring educational games and activities. First, key risk factors of CE infection and preventive behaviours of schoolchildren were evaluated in order to develop a health education package that met the needs of those it is targeting [17]. Subsequently, cultural, behavioural, individual, environmental and educational factors that may influence the efforts to control CE infections were considered to transform the assessed risk factors of CE into effective educational messages, as described in Green's Precede-Proceed model [18]. The interactive booklet was inspired by The Worm Hunters Project [19]. The booklets were created using the graphic design software Canva (https://www.canva.com/) [20] and downloaded as PDF files for distribution to schools. The booklet (Additional file 1: Text S1) has been annexed to the present paper (see Supplementary material section) with the purpose to be replicated in any primary school of the world.

Teacher's guidebook for CE

The CE teacher guide (Additional file 2: Text S2) was developed as a digital information tool for education and training in both Italian, English and Spanish. The steps followed in producing the teacher's guidebook included: assessing the situation (i.e. CE state-of-the-art study and literature search), defining the learning content of the guidebook, choosing content and references, writing text transforming the language of the information found in the literature making it accessible to all levels of society [21], designing and drawing illustrations using an Apple Pencil (Apple Inc., Cupertino, CA) and IPad 6 (Apple Inc., Cupertino, CA). The booklet was laid out using the Canva graphic design tool [20], editing and proofreading [22]. The teacher guide (Additional file 2: Text S2) included information on the epidemiology and life cycle of the parasite in animals and humans, risk factors for infection and suggestions to halt transmission.

Development and production of the educational "Fight the parasite" cartoon video

The "Fight the parasite" cartoon video (Additional file 3: Movie files S1) was developed between December 2021 and August 2022; this video (2:22 minutes in length) consists of color educational animation featuring an entertaining narrative [23] about CE transmission and prevention [15]. The video was created according to the conscious use of visuals in educational planning [24, 25] and developed to favor meaningful learning [26], engagement, health education, awareness and decision-making processes [27]. Prior to video

development, educational research was performed on health and hygiene practices and behaviors to prevent infection by *E. granulosus*, and primary risk factors for parasite transmission, key messages were selected (i.e., main CE prevention strategies, transmission routes) and the story script was drafted and reviewed. The "Fight the parasite" video was developed to achieve the following objectives (according to HE methodology) [9]: (1) cognition: analysis of problems and solutions to describe and provide knowledge of CE and the parasite lifecycle in animals and humans; (2) planning: design of appropriate solutions in order to achieve parasite eradication; (3) operation: action and adoption of appropriate behavior to guide end users in any socioeconomic context toward appropriate lifestyles. Over the course of several brainstorming sessions, the text storyline was drafted as a written document including live dialogue and voiceover. In the video, researchers and schoolchildren were depicted as the heroes of a special mission aimed at eradicating *E. granulosus*. The storytelling did not refer to contextual persons and was suitable for the children language as well as their surrounding context, i.e., the cultural and disciplinary basin of reference. The comic strips were synchronized with the script and according to the content and key messages of the project. The subjects were shown as anthropomorphized animals [28] and illustrated using the Apple Pencil, an intuitive tool for iPad apps and Procreate, a raster graphics editor app for digital painting for iPadOS (Savage Interactive Pty Ltd). The "Fight the parasite clips were filmed with the visual-effects and post-production technique of chroma key compositing. Graphic resources, professional voiceover dialogue, English subtitles, and sound were subsequently added and merged using the professional video editing program Apple Final Cut software, V. 10.6.3 (Apple Inc., Cupertino, CA).

Pre-intervention evaluation of CE knowledge

From March to June 2022, all of research participants were interviewed using True False Don't Know (TFD) questionnaires based on CE-related knowledge and practices (Additional file 4: Text S3). The classes involved, after agreement with teachers, did not receive any information regarding CE before our intervention, to avoid bias. Each participating schoolchild was interviewed in the classroom prior to the beginning of the participatory classroom meeting. Each questionnaire included five questions pertaining to knowledge and practices [29] on CE (see Additional file 4: Text S3).

Edutainment activity

Participant schoolchildren were subsequently engaged in one-hour lecture on CE [30]. The activity was led by the members of the research team and focused on CE infection routes, transmission and prevention. The interactive lessons [29] included classroom discussion and hands on educational activities [15], including use of a light microscope for observation of alcohol-fixed, mounted and stained *E. granulosus* parasites. The comic educational booklet (Additional file 1: Text S1) and the cartoon video (Additional file 3: Movie files S1) were shown and discussed on the multi-media whiteboard. Interventions were supplemented by a drawing session on CE for positive message reinforcement [31]. Some themes or nuclei (i.e., morphology of the *E. granulosus*, biological cycle in intermediate and definitive hosts, characteristics of eggs and their strategies for dissemination in the environment) were presented by means of brief dramatizations [32] and colorful cardboards highlighting key health education messages.

On-site edutainment tour in primary schools of an endemic region for CE

The edutainment tour was carried out in Sardinia (Italy), a region considered hyperendemic for CE due to the annual incidence rate of hospitalized patients being four times higher than the national average ($6.9/10^5$ *vs* $1.6/10^5$ inhabitants) [33]. Sardinia is the second largest Mediterranean island and hosts > 40 % of the whole Italian sheep population [34]. Studies on the intermediate hosts have revealed infection rates in sheep ranging from 65.3% to 75% [7, 35], followed by cattle (41.5%), pigs (9.4%) [36] and wild boars (3.7%) [37]. Despite numerous control campaigns carried out over the past 50 years, CE is still one of the most widespread parasitic diseases in Sardinia [35] with serious social and economic consequences [7]. Between March 2022 and June 2022, the tour covered 14 municipalities located in seven historical regions of Sardinia, i.e., Romangia, Nurra, Goceano, Barbagia, Mandrolisai, Ogliastra and Campidano, including urban areas, rural areas, and peri-urban settings where a previous survey had reported CE infections in animals and humans [7]. In collaboration with local educators, as well as school principals and the teaching staff, 14 primary schools were selected for this campaign (Table1).

Post-intervention evaluation of CE knowledge

Assessment of schoolchildren knowledge post-intervention was carried out by administering a follow-up questionnaire, identical to the pre intervention and delivered immediately after the participatory classroom meeting (Additional file 4: Text S3).

Results

The project involved 14 primary schools and a total of 896 schoolchildren in 67 classrooms (mean 13.6 ± 4.8 students per classroom) (Table1). The mean age of the schoolchildren was 8.7 years (±1.2).

Comparative analyses of pre- and post-intervention questionnaire answers revealed that the intervention significantly improved general knowledge of CE, as shown by the percentage of correct answers increasing from 65% (pre-intervention questionnaire) to 87.9% (post-intervention) ($\chi2$= 648.12, *df* = 1, *P* < 0.0001) (Fig. 1). Prior to the intervention, the schoolchildren displayed little to no knowledge of the cause of CE (cf. Additional file 4: Text S3). After the intervention, correct answers to the statement "Dogs become infected by *E. granulosus* by eating cooked sheep offals" increased from 42.2% to 75.4% ($\chi2$= 204.58; *df* = 1, *P* < 0.0001), from 65.8% to 89.7% for the statement "You could get infected by touching your dog's feces " ($\chi2$= 147.91; *df* = 1, *P* < 0.0001), and from 81.7% to 95.3% for "Washing fruits and vegetables well keeps you protected from the parasite" ($\chi2$= 81.63; *df* = 1, *P* < 0.0001). Similarly, correct answers increase from 46.3% to 82.7% for the statement "There is no medicine for dogs against *E. granulosus*" ($\chi2$= 259.03; *df* = 1, *P* < 0.0001), and from 88.9% to 96% for "Washing hands after touching a dog can help you not get the parasite" ($\chi2$= 31.79; *df* = 1, *P* < 0.0001) (Table 2).

Schoolchildren clearly associated CE infection with poor eating hygiene, and particularly to inaccurate washing of fruit and vegetables. The percentage of "I don't know" answer decreased from 23% in the pre-intervention questionnaire to 5% post-intervention ($\chi2$= 603.44; *df* = 1, *P* < 0.0001) (Fig.1).

The educational research and assessment of CE risk factors led to the following key messages being selected [15] for inclusion in the educational tool (see Additional file 1: Text S1, Additional file 2: Text S2, Additional file 3: Movie files S1):

- Wash your hands with soap and warm water after handling dogs and before handling food.

- Wash adequately fruit and vegetable with clean water.
- Feed your dog well-cooked meat.
- Prevent home slaughtering of livestock, especially sheep.
- Prevent dogs from feeding on the carcasses of infected sheep.
- Treat dogs with effective anthelmintics.

These key messages were identified according to the Health Belief Model [38, 15], ESCCAP guidelines [39] and CDC prevention measures [40] and incorporated as drawings into key scenes of the educational video cartoon. The video cartoon is completely produced by the researcher team and can be accessed online (see Additional file 3: Movie files S1).

In several classes we assessed the absorption of these messages through CE-themed drawings post-intervention. In particular, the schoolchildren were able to represent the complete cycle of the parasite, and the main risk factors and behaviours that may lead to infection (see Additional file 5: Movie files S2). Children mainly referred to the images observed under the optical microscope and displayed on the card boards provided during the participatory lesson and reproduced them as faithfully as possible through mnemonic or fantasy images. The teachers' instructions manual (Additional file 2: text S2, Additional file 6: text S4) assisted to facilitate the role of educators during the meeting and represents a resource to further explore the topic with schoolchildren during teacher-student contact hours.

Discussion

The results of the present survey indicate that our strategy, which combines a digital educational toolkit, the use of video comic cartoon as a model for science communication, and other participatory teaching methods [9, 15], enabled children to retain newly acquired knowledge of CE. Comparative analyses of questionnaire results prior to and following the intervention revealed that schoolchildren knowledge of CE improved by 22.8%, and that establishing compliance with all schools was beneficial. The overall percentage of correct answers prior to the intervention (65%) was quite high considering the young age, but it was to be expected considering the long-time presence of CE in Sardinia affecting the livestock and the human population for decades. Indeed, during the last sixty years there have been

several actions aiming to eradicate echinococcosis, including information campaigns also supported by the local broadcasters and in the late 1980s, a comprehensive “Action plan for the eradication of Echinococcosis / Hydatidosis”. For these reasons it is quite possible that schoolchildren have heard about echinococcosis in their family environment, from the previous generations.

The significant decrease in uncertain questionnaire answers (“I don't know”) from 23% to 5% is a testimony of student acquired self-confidence toward a new topic, and of developing critical thinking (yes or no). Health education aims to improve health literacy [41], empower end users to access health information use it effectively, and increase autonomy [41]. This, in turn, enhances opportunities for educational attainment and self-reliance, and assists independent citizens with making informed decisions on health and disease, and with evaluating the consequences and ethics of their actions towards themselves and society [42].

Understanding of fundamental hygiene practices to minimize risks of CE transmission, such as thoroughly washing fruits and vegetables, washing hands prior to eating and after handling a dog or touching soil, improved substantially post-intervention. This was likely aided by increased awareness of hygiene procedures implemented during the COVID 19 pandemic. However, the intervention helped reinforce these positive behaviours: these are not issues to be overlooked as habits such as picking vegetables and herbs in the countryside or consuming produce from home gardens without washing them, even they believed to be safe because they have not been treated with pesticides, unfortunately persisting.

In line with previous studies, schoolchildren were unaware of the life cycle of *E. granulosus* [43]. Nevertheless, children reported knowledge of and/or direct experience with other parasites of companion animals, such as ticks, fleas and roundworms. This serves as a proxy indicator of the importance a given community places on a particular subject [44]. In addition, this emphasizes the importance of conducting surveys during primary prevention projects, as these it can help delineate the school-age population perception of disease in endemic areas by raising community awareness and/or developing such awareness from childhood [45] which, in turn, encourages active prevention [43]. Prior to the intervention, children were not fully aware of routes of CE transmission, and in particular of consumption of raw offal as a risk factor for dogs (question 1) and thus humans. This lack of knowledge could be justified by the long latency between infection and symptoms in humans (which also appear nonspecific) [46]. Furthermore, children were unaware of the existence of effective drugs against *E. granulosus* in dogs at the pre-intervention questionnaire (46.3%

of correct answers), but at the post-intervention questionnaire the correct responses significantly increase (82.7%) and children became conscious that there are effective drugs against the tapeworm in dogs and that their administration can prevent CE transmission to sheep and humans.

Addressing the issue of home slaughter without official supervision could be helpful, particularly for children in rural areas. Indeed, these children are usually equipped with a wealth of knowledge of local livestock husbandry, coupled with direct experience of home slaughter and carcass disposal (i.e., abandoned in the countryside, buried, incinerated, or fed to dogs either raw or cooked). After children have been made aware of proper health behaviors, they can subsequently be entrusted with the role of health messenger [29] for their friends and family members. The acquisition of these skills has high potential [47] for the future of young generations from high agricultural and pastoralist settings, particularly in endemic areas where community-based risk is present [46]. Improved health and educational outcomes in school increases the potential for greater economic benefits for children during adulthood as a result of enhanced career opportunities as well as better physical and emotional health; these effects can be passed down to future generations [48]. Teachers are fundamental to the success of school-based health promotion initiatives, given their roles in educating children about health issues and facilitating the development of health literacy skills through classroom and school activities [49]. Pursuing the goal of improved health literacy will also require more overt alliances between health and education sectors at local, national and international levels emphasizing, e.g., the need for improved alliances between WHO and UNESCO, at an international level, and clearer understanding between agencies at the local level [41].

Conclusions

The authors believe that it is vital to promote broad and multidisciplinary educational approaches to raise awareness of zoonotic diseases, as human beings are built on words, in work, and in action-reflection [32]. Health education programmes in schools should be designed and carried out with input from experts such as veterinarians, in accordance with the new European animal health law that emphasizes the active role that these professionals should play in raising awareness of the importance of animal and human health [50]. The results also encourage the establishment of an echinococcosis HE system, the launching and promotion of school-based health education interventions on CE, and the implementation of

a gradual and dynamic education model for students [43]. The current framework for the control of CE is mainly focused on prophylaxis, chemotherapy-based control, vaccination and health education. The added benefits of including edutainment in programs for the prevention of CE as part of an integrated multicomponent approach for sustainable control must be emphasized. It has been repeatedly shown that additional public health measures, including novel and effective health educational interventions are needed to establish an atmosphere conducive to sanitation development [31]. Edutainment has long been an internationally recognized tool successful in delivering complex ideas and raising awareness of a wide range of topics, particularly among the younger generations, whose actions and choices can greatly reduce the spread of CE. However, short-term interventions are not intended for single use. Healthy behaviors need to be sustained over long time periods to achieve health advantages [18] therefore, it would be desirable to implement such programmes throughout the school years, in order maximize benefits and monitor long-term results.

Supplementary information

Additional file 1: Text S1, English and Spanish versions. Comic educational booklet featuring educational games and activities on CE, edited in English and Spanish.

13071_2022_5575_MOESM1_ESM.pdf (springer.com)

Additional file 2: Text S2, English and Spanish versions. Teacher's guide; a digital information tool for education and training on CE, edited in English and Spanish.

Annex 2 Fight the parasite teacher's guide (springer.com)

Additional file 3: Movie files S1. “Fight the parasite” cartoon video, link and QR Code, showing the CE transmission and prevention in an entertaining narrative form.

https://youtu.be/XTf3fTtmfA8

Additional file 4: Text S3, English and Spanish versions. Questionnaires to assess students' knowledge of CE and quiz solutions with the right answers, edited in English and Spanish.

Fight the parasite Quizzes (springer.com)

Additional file 5: Movie files S2. Link to slideshow of artwork made by children.

https://youtu.be/9kWQ7JzIlLM

Additional file 6: Text S4. Instructions for teachers to carry out the project in the classroom.

https://static-content.springer.com/esm/art%3A10.1186%2Fs13071-022-05575-2/MediaObjects/13071_2022_5575_MOESM6_ESM.docx

Abbreviations

CDC: Centers for Disease Control and Prevention; CE: Cystic Echinococcosis; CNSA: National food safety committee; DALY's: Disability adjusted life years; ESCCAP: European Scientific Counsel Companion Animal Parasites; HE: Health education; SCI: State Comprehensive Institute; SP: School Principal; TFD: True False Don't Know; UNESCO: United Nations Educational, Scientific and Cultural Organization; WHO: World Health Organization.

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Figure captions

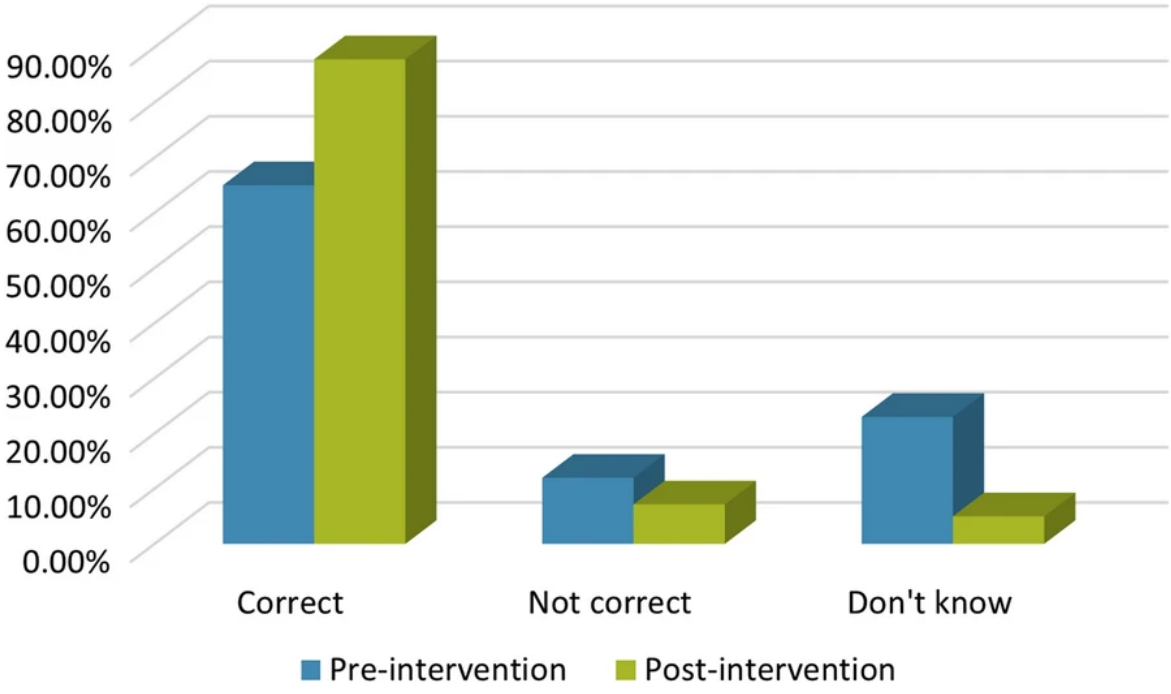

Fig. 1 Graphical representation of the improvement on the questionnaire answers in pre and post-intervention administration. Significant differences were found in the percentage of correct answers, that increased from 65% of pre-intervention questionnaire to 87.9% post-intervention ($\chi 2 = 648.12$; $df = 1$, $P < 0.0001$), but also in the percentage of not correct answers, that decreased from 12% of pre-intervention questionnaire to 7.2% post-intervention ($\chi 2 = 60.13$; $df = 1$, $P < 0.0001$), and in the percentage of "I don't know" answer that decreased from 23% in the pre-intervention questionnaire to 5% post-intervention ($\chi 2 = 603.44$; $df = 1$, $P < 0.0001$).

Tables

Table 1 Details and location of the Sardinian Primary Schools involved in the project

State Comprehensive Institute (SCI). Didactic Direction (DD)	School plexus (SP)	Number of schoolchildren involved per plexus	Municipality	Province	Google Maps
DD Is Mirrionis	C. Collodi SP	151	Cagliari	CA	64H7+HV
SCI 1 Alghero	Sacro Cuore SP	51	Alghero	SS	H858+HM
SCI Bono	Bono SP	97	Bono	SS	C26J+JF
SCI Bono	Nule SP	30	Nule	SS	F57R+22
SCI Bono	Benetutti SP	24	Benetutti	SS	F549+FX
CI Brigata Sassari	Via Togliatti SP	135	Sassari	SS	PHCM+M4H
SCI Atzara	Sorgono SP	39	Sorgono	NU	24H4+HJ
SCI Baunei	Urzulei SP	15	Urzulei SP	NU	3GR5+QV
SCI Bitti	Bitti SP	53	Bitti	NU	F9HP+CQ
SCI Bitti	Orune SP	40	Orune	NU	C949+6J
SCI Bitti	Lula SP	22	Lula	NU	FFCP+F7
SCI 2 Monte Attu	Tortolì SP	125	Tortolì	NU	WMG2+WR
SCI 2 Monte Attu	Lotzorai SP	69	Lotzorai	NU	XMC7+52
SCI 2 Monte Attu	Girasole SP	45	Girasole	NU	XM36+5F

Table 2 Comparison of changes in knowledge among school children based on the percentage of correct responses to the questionnaire administered before and after the edutainment activity.

	Schoolchildren responses to the questionnaire					
	Pre-intervention (%)			Post-intervention (%)		
	Correct	Not correct	Don't know	Correct	Not correct	Don't know
General knowledge of parasite biology and transmission routes						
Dogs become infected by *Echinococcus* by eating cooked sheep offals	42.2%	19%	38.8%	75.4%	17.9%	6.7%
You could get infected by touching your dog's feces	65.8%	15.3%	18.9%	89.7%	5.8%	4.5%
Prevention strategies						
Washing fruits and vegetables well keeps you protected from the parasite	81.7%	7.3%	11%	95.3%	2.1%	2.6%
There is no medicine for dogs against *Echinococcus*	46.3%	14.9%	38.8%	82.7%	8%	9.3%
Washing hands after touching a dog can help you not get the parasite	88.9%	3.6%	7.5%	96%	2%	2%

Chapter 4

Description of the website as exploitation of the result of the project

The advancement of information systems and communication technologies (ICTs) has significantly transformed the way we communicate, live our lives, and conduct business across various industries (Ryu, 2012) Medical activities have significantly benefited from the development and application of the internet. The internet serves as a free, real-time, and open platform for the exchange and transfer of information (Su et al., 2024). In recent years, the integration of healthcare with technological innovation has led in a new era, characterized by enhanced accessibility and efficiency in medical services. The introduction of telemedicine applications has revolutionized healthcare delivery, bridging geographical gaps and providing timely interventions (Parmar et al., 2014). The potential of telemedicine and digitalization in the ongoing battle against zoonotic diseases could be significant.

Telemedicine and digitalization have played a crucial role in connecting professionals and ordinary citizens in the field of zoonotic diseases, particularly parasitosis. In countries like India and South Africa, telehealth and mobile phones have been utilized to improve healthcare access, research, and early detection of zoonotic diseases (Pathak & Kumar, 2017; Thinyane, 2010) In parasitology, digitalization has been applied to information processing and research methods, with successful implementation in various countries (Delgado-Noguera et al., 2022; Holston et al., 2021) In animal care, telemedicine has been proposed as a tool to improve access and quality of veterinary care in rural communities.(Johnson et al., 2018)

In the context of zoonotic diseases, Citizen Science (CS) has emerged as a pivotal approach, integrating the scientific contributions of non-specialized individuals into research actions through data collection and dissemination. (Ashepet et al., 2021). Digital Citizen Science systems have become widely adopted, particularly in communities grappling with Neglected Tropical Diseases (NTDs), facilitating efficient tracking and reporting on a global scale (Gardiner & Roy, 2022). Neglected tropical diseases are a prominent cause of morbi-mortality in middle to lower-income countries throughout the world (Aagaard-Hansen & Chaignat, 2010)(Aagaard-Hansen and Chaignat, 2010). In Sardinia, as other mediterranean region, *Echinococcus granulosus* is listed by the World Health Organization (WHO) as one

of the seven endemic and neglected zoonotic diseases (NZDs) worldwide(Eckert & Deplazes, 2004; Varcasia et al., 2020).

The project idea underlying this research is to create an interactive graphical interface on digital support that allows, on one hand, the knowledge of parasitological risk based on the characteristics of the location and farms, and on the other hand, a real-time health education tool and support for the main involved categories (farmers, producers, veterinarians, mountain communities) (fig 1)

To implement this project specifically, the development and realization of a platform containing four main diagnostic-educational tools was required, which will compose a single network platform that may eventually have access from portable multimedia devices:

- Multimedia informative sheets for each parasite of zootechnical and zoonotic interest, which will support owners, breeders, producers, veterinarians with texts and multimedia materials (photos and videos) on biology, life cycle, epidemiology, pathogenesis, clinical aspects, prophylaxis, and therapy (fig 2, fig 3 fig, 4).
- Interactive epidemiological tool on the main zoonoses present in the area, such as Cystic Echinococcosis. The idea is the development of a Monitoring and Epidemiological Surveillance System through a map showing the spread and degree of risk for the various parasitic diseases present in the region to track the spread, powered by a database provided by the research group and managed by an algorithm to be defined with the company that created the platform.
- Interactive diagnostic tool that can provide to owners, farmers, veterinarians or ordinary citizen a telemedicine consultation, with booking system, chat, and video image sharing with the client for Veterinary Parasitology and parasitic zoonoses (fig 5, fig 6).

The website is designed to be responsive. Responsive web design ensures that the site's layout and content adapt seamlessly to the user's behaviour and environment, taking into account factors like screen size, platform, and device orientation. This approach involves using flexible grids, layouts, and images, along with CSS media queries. For instance, when a user switches from a desktop PC to an iPad, the site automatically adjusts to the new resolution, resizing images and modifying script-based interactions. Essentially, the site

integrates technologies that enable it to automatically adapt to the user's preferences, providing an optimal viewing experience across different devices (Shanthi et al., 2022).

This web app features a simple, intuitive, and easily manageable Content Management System (CMS) called Apollo®, developed by MTN® Company S.r.l. . Apollo® is a software tool installed on a web server designed to facilitate content management, allowing clients to manage their content autonomously without needing specialized IT skills. It offers managers straightforward and efficient tools for adding and modifying content.

The system is web-based, meaning it can be accessed anytime through any internet browser. An authorized user can connect remotely, and after authentication, they can manage content on the platform without any additional installation. Apollo®'s technology is reliable, available, manageable, secure, and provides the necessary service levels. The interface used by Apollo® is a standard internet browser, which, by using server-side applications integrated with one or more databases, allows for information processing and quality evaluation of planned interventions.

The operating system underpinning this communication is UNIX/Linux, leveraging cutting-edge technology that enables various interfaces and operating systems, providing Apollo® with exceptional flexibility and reliability. One significant advantage is that Apollo® is entirely internet-based, requiring no software installation on the organization's computers. This approach eliminates licensing costs and simplifies hardware updates without disrupting software functionality.

Apollo®'s standard suite includes these features, but its great versatility allows for extensive customization based on business needs. New modules can be seamlessly integrated into the core suite in addition to already developed modules. As an information system applied to the web, Apollo® is useful for publishing and managing any type of information or service, enabling extremely simple and immediate content management without requiring HTML or programming knowledge from operators.

Furthermore, Apollo® is structured to interface with almost all management software on the market, allowing connections to any data archive, such as registry archives. Upon authorized request, it can provide information from these archives without preloading it into the Apollo® database. It also allows for integrating incomplete information from existing archives by adding new data.

In conclusion, the proposed website will represent a significant advancement in healthcare delivery, enhancing patient outcomes, and providing valuable educational opportunities in both veterinary and human medicine. This is particularly crucial in remote areas such as Gennargentu Mandrolisai, where the uneven distribution of medical and veterinary resources has led to critical conditions and low-quality services. Difficulty accessing medical care remains a pressing issue for many individuals in these regions.

Thanks to the technology employed, the website can be easily updated and improved by the client. This capability allows for the continuous expansion of the epidemiological database over time, encompassing not only zoonotic parasites but also other parasitological concerns affecting animal health. This flexibility enables the website to be tailored for future users, opening up numerous possibilities for new projects that incorporate a citizen science approach using this innovative technology.

The website can be consulted by scanning the QR code provided in figure 7.

Figures

Fig 1 Main page of Parassitologia.it website

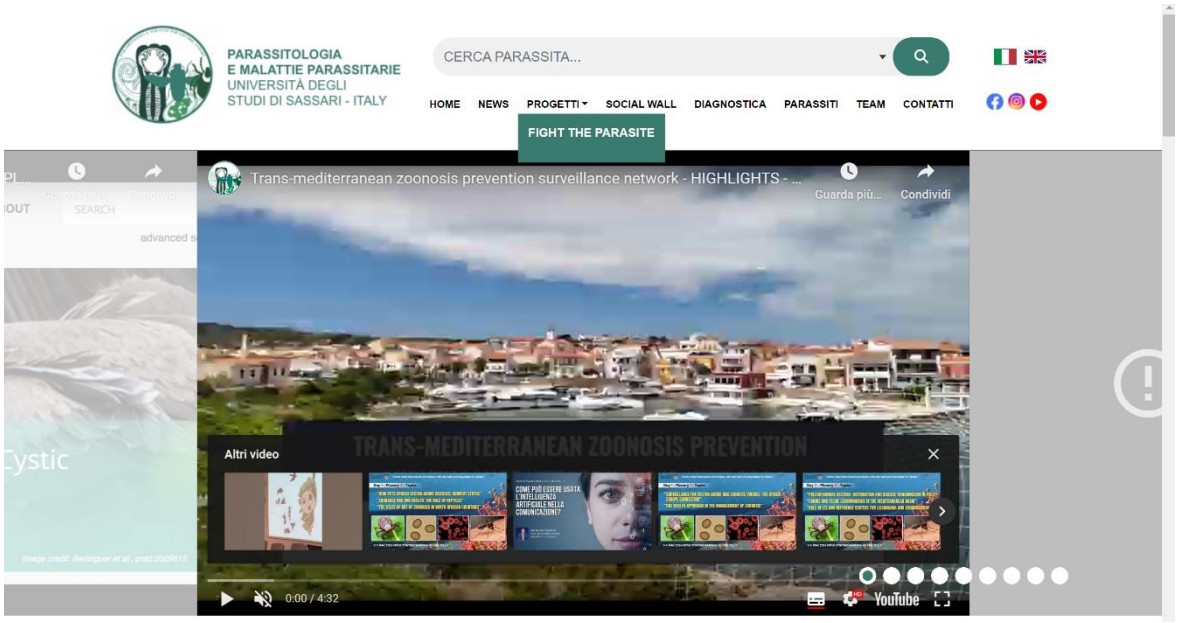

Fig 2 Main page where is possible to search the information of different parasites through the search bar

Fig 3 Informative sheet of *Echinococcus granulosus*

Fig 4 Informative sheet of *Toxoplasma gondii*

Fig 5 Interactive diagnostic tool linked to price list of different services

Fig 6 Telemedicine consultation

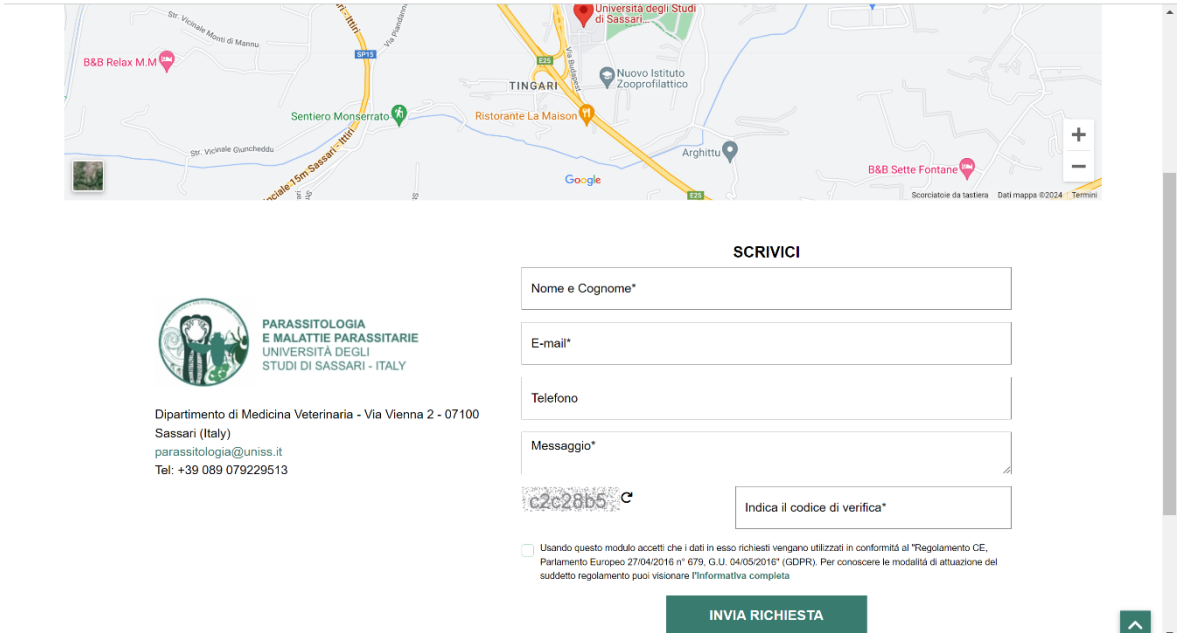

Fig.7 QR code remanding at Parassitologia website

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Conclusions

This PhD thesis provides a comprehensive description and epidemiological data on two main zoonoses in Sardinia, namely Toxoplasmosis and Echinococcosis. The ultimate goal is to provide a useful interactive website, to promote public health, education, and environmental protection with a One Health perspective.

In **Chapter 1**, a comprehensive overview of the main zoonotic parasites relevant to the Parasitology Laboratory has been provided. This includes their description, epidemiology based on Laboratory Associated Infections (LAIs), and the associated risks and hazards in the laboratory environment. Laboratory workers are at a heightened risk of acquiring infections due to their handling of infectious materials, some of which may serve as potential vehicles for dangerous zoonotic infections, including parasitic zoonoses (Harding & Byers, 2006). Common pathogenic/zoonotic species such as *Cryptosporidium* spp., *Giardia duodenalis*, *Entamoeba* spp., *Toxoplasma gondii*, *Leishmania* spp., *Echinococcus* spp., *Schistosoma* spp., *Toxocara canis*, *Ancylostoma caninum*, and *Strongyloides stercoralis* were reviewed for occupational infection risks. Specific disinfection methods for each parasite were discussed in order to prevent infections in the laboratory setting.

From this review, it is apparent that parasites with greater environmental resistance, such as foodborne and waterborne protozoa like *Cryptosporidium* spp., *Entamoeba* spp., *G. duodenalis*, and *T. gondii*, underscore the importance of hand hygiene in reducing the chance of transmission, along with the proper disposal of waste(Bartelt, 2020). Specifically, both *G. duodenalis* and *Entamoeba* spp. are sensitive to a 5% chlorine concentration solution, recommended for disinfecting lab tools and benches (Herwaldt, 2001). For *Cryptosporidium*, disinfection with 3% undiluted hydrogen peroxide for 30 minutes is advised, while sterilization with an autoclave is recommended for T. *gondii*, as only ammonium hydroxide may be effective (Dubey et al., 1970; Herwaldt, 2017; Meechan & Potts, 2020; National Centre of Biotechnology Information, 2022). Other zoonotic parasites are more sensitive to commercial bleach and 70% ethanol, emphasizing the importance of following good laboratory practices (Herwaldt, 2017; Meechan & Potts, 2020; Von Dohlen et al., 2017).

The study also emphasizes the need for updated epidemiological data on infections acquired by laboratory workers and the importance of accurate risk indicators. Biological risk for

researchers is significantly higher compared to hospital and public health laboratory workers Due to the fact that Laboratory Associated Infections (LAIs) can be symptomatic or asymptomatic, establishing their real incidence is challenging due to underreporting and lack of centralized reporting systems (Harding & Byers, 2006; Herwaldt, 2001). However, epidemiological studies are crucial for understanding the nature and extent of LAIs, especially regarding parasitological risks. Infections can occur through various routes, such as parenteral exposure and ingestion of infectious agents, highlighting the importance of preventive measures and accurate risk indicators.

Working with biological material in laboratories poses a risk of infection due to constant contact with biological agents, categorized into 4 risk groups by the WHO (WHO, 2004). While most zoonotic parasites fall into risk group 2, with common and available prophylactic measures, parasites like *E. granulosus*, *L. donovani*, and L. *braziliensis* fall into risk group 3 categories, requiring specific preventive measures and greater attention (Directive 2000/54/EC 2000) Containment measures for laboratory biological agents include workspace separation, air filtration, surface cleaning, and personnel restrictions. Universal precautions like hand washing, Personal Protective Equipment (PPE) use, and proper sample handling are crucial in preventing exposure to infectious agents (WHO, 2004)

Decontamination, disinfection, and sterilization procedures are essential in parasitology labs to prevent infections and infestations. Disinfection processes vary based on the nature of parasites, necessitating knowledge of effective agents for inactivation. Autoclaves using moist heat are reliable for sterilizing lab materials, with specific heating cycles ensuring effectiveness. Proper disposal of infectious waste, including hazardous medical waste and sharp objects, is crucial to prevent pathogen transmission. Developing a biological safety program based on transmission modes and pathogenicity helps minimize risks associated with handling pathogenic organisms. Training of laboratory staff is fundamental in ensuring biosafety measures are understood and followed effectively (Meechan & Potts, 2020).

In **Chapter 2**, epidemiological surveillance on *Toxoplasma gondii* is reported in sheep regularly slaughtered and in wild boar hunted during the hunting season 2021-2022.

The first study aims to evaluate co-infections with *Neospora caninum*, *Sarcocystis* spp., and *Toxoplasma gondii* in sheep slaughtered for human consumption in Italy. These protozoa are common causes of reproductive disorders in sheep (Lindsay & Dubey, 2020) According to current data, *N. caninum*, *Sarcocystis* spp., and *T. gondii* are widespread in sheep in Italy.

Seroprevalence for *N. caninum* ranges from 19% to 46% based on current scientific literature (Gazzonis et al., 2016; Tamponi et al., 2015). Pathogenic *Sarcocystis* spp. are detected in 78% to 100% of examined slaughterhouse samples (Bacci et al., 2016; Pagano et al., 2016; Pipia et al., 2016). Additionally, a *T. gondii* seroprevalence between 28% and 83% has been reported (Bacci et al,2016; Cenci-Goga et al., 2013; Fusco et al., 2007; Gazzonis et al., 2015; Masala et al., 2007; Natale et al., 2007; Vesco et al., 2007; Zedda et al., 2010). Co-infection with *T. gondii*, *N. caninum*, and *Sarcocystis* spp. was found in 66.7% of the examined sheep, indicating a high prevalence. In the present survey,. *Neospora caninum* DNA was found in 72.5% of the examined brain samples. *Sarcocystis* spp. DNA was detected in 92% and 52.2% of the heart and diaphragm samples, respectively. Antibodies against *T. gondii* were found in 36.2% of serum samples, highlighting exposure to the parasite. In addition, *T. gondii* DNA was detected in brain, heart, and diaphragm samples at varying rates (67.4%, 77.5%, and 21.7% respectively), emphasizing the risk of infection in different tissues. Finally, co-infection with all three parasites was found in 66.7% of the examined sheep. The study highlights the high risk of infection with these protozoa in sheep, underscoring the importance of controlling parasitic infections in ovine production. Adequate control programs and sanitary measures are recommended to prevent the spread of these parasitic infections and mitigate their clinical and economic impacts on ovine production. In conclusion, sheep may play a role in the zoonotic transmission of toxoplasmosis to humans, indicating a potential public health concern, with the suggestion that any ovine meat for human consumption should be cooked or prepared adequately in order to inactivate infective parasite stages.

The second study aims to assess the prevalence and seroprevalence of *Toxoplasma gondii* in wild boar hunted during the 2021-2022 hunting season. The growing numbers of wild boar worldwide, especially in Europe (Jori et al., 2021; Massei, 2023), alongside the increasing popularity of wild boar meat in various regions (Rostami et al., 2017), highlight the heightened risk of toxoplasmosis transmission through the consumption of meat from recreational hunting. Seroprevalence rates of *T. gondii* in wild boar vary globally, with rates ranging from 8.1% in Slovakia to 62% in Slovenia (Antolová et al., 2007; Bandelj et al., 2021). In Italy, seroprevalence rates of *T. gondii* in wild boar vary throughout the country, with rates ranging from 16% in Northern Italy to 39.6% in Southern Italy (Ferroglio et al., 2014; Santoro et al., 2019; Sgroi et al., 2020). Given the substantial wild boar population in Sardinia, Italy, this study presents an opportunity to investigate the distribution of

Toxoplasma in this species and its potential transmission risks to humans. The survey yielded a prevalence of *T. gondii* DNA in Sardinian wild boar was found to be 37.1% and a seroprevalence of 24.6% indicating a widespread occurrence of the parasite on the island. Comprehensive control programs, public education, regulatory legislations, and monitoring of the food chain are recommended to mitigate the risk of *T. gondii* transmission from wild boar to humans.

The **Chapter 3** delves into a survey exploring the use of edutainment to educate primary school students about Cystic Echinococcosis (CE), utilizing learning toolkit, video comic cartoons, and other interactive teaching methods. This approach serves as a innovative form of science communication, resulting in a significant increase in awareness ((Bieri et al., 2013; Porcu et al., 2022). Analysis of pre- and post- intervention questionnaires led to a 22.8% surge in CE knowledge among children. Initially, 65% of 139 participants answered all questions correctly, a commendable feat considering their youth, likely influenced by CE's longstanding presence in Sardinia, impacting both humans and livestock. Over six decades, numerous initiatives, including televised campaigns, have pursued to eradicate echinococcosis. Moreover, it is probable that children acquired knowledge about the disease from their grandparents, given its historical prevalence. Post-intervention, there was a notable decrease in responses of “I don’t know”, from 23% to 5%, indicating enhanced confidence and critical thinking. The primary educational goals included promoting public health and fostering independent hygiene practices, in order to improve students' ability to make informed health decisions (Nutbeam, 2000; Okan et al., 2020).

Following the intervention, students demonstrated a heightened understanding of hygiene practices crucial for preventing CE transmission, likely influenced by increased awareness during the COVID-19 pandemic. Moreover, they recounted learning about and addressing other pet parasites such as ticks, fleas, and roundworms, indicative of potential community attitudes toward specific issues. However, students lacked awareness of the *E. granulosus* life cycle. Pre-intervention responses revealed children's ignorance regarding the risks associated with feeding raw offal to dogs, a practice posing hazards not just to canines but also to humans (Eckert & Deplazes, 2004). The lengthy incubation period between infection and symptom onset in humans could explain this lack of awareness (Porcu et al., 2022). Additionally, pre-intervention questionnaire data indicated children's unfamiliarity with anthelmintic drugs for *E. granulosus* in dogs, with only 46.3% providing correct responses. Post-intervention, correct responses surged to 82.7%, demonstrating heightened awareness

of the role of such drugs in controlling CE transmission from dogs to sheep and subsequently to humans. This underscores the importance of surveys in primary prevention efforts, elucidating the perspectives of school-aged populations in endemic regions, thereby catalysing proactive prevention measures (Wang et al., 2022).

Addressing unsupervised home slaughter practices, especially in remote areas, is crucial given children's familiarity with livestock farming (Varcasia et al., 2020). Educating children about proper health practices can empower them to advocate for public health for their peers and families, (Ayi et al., 2010), a skillset with considerable potential, especially in high-risk regions like Sardinia (Dunkle & Mariner, 2013). Teachers play a vital role in promoting health education, and collaboration between healthcare and education sectors is essential for improving health literacy and disease control efforts, as demonstrated by enhanced partnerships between organizations like WHO and UNESCO, and improved coordination among local agencies, facilitating better understanding and control of diseases (Nutbeam, 2000).

In **Chapter 4,** as final outcome of the project, the website for the prevention of the zoonotic parasitosis has been described. Moreover, it is possible to get direct access to the website through scanning the QR code provided.

The rapid advancement of Information Systems and Communication Technologies (ICTs) has profoundly influenced various aspects of society, including healthcare. Specifically, the integration of the internet and digital technologies has facilitated unprecedented access to medical information and services. This has led to a paradigm shift in healthcare delivery, characterized by enhanced accessibility, efficiency, and innovation (Ryu, 2012; Su et al., 2024). Telemedicine, a key component of this digital transformation, has revolutionized healthcare by enabling remote diagnosis, consultation, and treatment (Parmar et al., 2014) By leveraging telecommunications technologies, telemedicine has effectively bridged geographical barriers, allowing healthcare professionals to reach patients in remote or underserved areas. This has resulted in improved healthcare access and outcomes, particularly in regions with limited medical (Delgado-Noguera et al., 2022; Holston et al., 2021). In the context of zoonotic diseases, such as parasitosis, telemedicine and digitalization have played a crucial role in connecting healthcare professionals and communities.

Citizen Science has emerged as a valuable approach in addressing zoonotic diseases, enabling the active participation of non-specialized individuals in research activities (Ashepet et al., 2021). Digital Citizen Science systems have been widely adopted, facilitating efficient tracking and reporting of Neglected Tropical Diseases (NTDs) on a global scale (Gardiner & Roy, 2022). This collaborative approach could contributed to the prevention and management of zoonotic diseases, such as *Echinococcus granulosus*, in regions like Sardinia and other Mediterranean areas.

The final chapter of the thesis outlines a project aimed at creating an interactive platform to address parasitological risks and enhance healthcare delivery. This project involves the development of diagnostic-educational tools and telemedicine consultation systems, providing real-time health education and support to stakeholders involved in managing zoonotic diseases. By leveraging digital technologies, the proposed platform represents a significant advancement in healthcare delivery, particularly in remote or underserved areas where access to medical and veterinary resources is limited, such as Gennargentu Mandrolisai.

In conclusion, zoonotic parasitosis continues to pose a threat to human and animal health, as well as to the economy of rural and internal areas such as Gennargentu-Mandrolisai. As detailed in this thesis and other publications, parasitic zoonotic diseases such as Echinococcosis and Toxoplasmosis are endemic and classified by the WHO as neglected tropical diseases. This study highlights the widespread presence of *T. gondii* in both livestock and wildlife, not only in the Gennargentu-Mandrolisai area but throughout the entire island of Sardinia. Prevention of these diseases is crucial, and emerging technologies can play a significant role in providing easily accessible information. Moreover, the availability of such information is essential for enhancing primary prevention programs like "Fight the Parasites," as described in Chapter 3. Web-app tools can serve to bridge the gap between professionals in the field (e.g., veterinarians and researchers) and farmers and ordinary citizens, all while adhering to the One Health Approach—an integrated approach to health focused on interactions between animals, humans, and the environment. Future perspectives could include the follow-up of the project's impact on primary prevention initiatives, such as "Fight the Parasite." This would involve re-evaluating the target population at different time points to assess whether they maintain appropriate levels of knowledge, attitude, and

practice (KAP). In addition, the ability to update the webapp by the client allows for continuous follow-up of the epidemiological database over time. This flexibility open up numerous possibilities for new projects that incorporate a citizen science approach using this innovative technology, tailoring the website for future research.

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