Trichinella spp. detection in hunted wild boar (*Sus scrofa*) diaphragm biopsies in Central Italy

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Abstract

Trichinellosis is a globally diffused foodborne parasitic disease caused by nematodes of the *Trichinella* complex. During evolution, guided by ecological interactions, natural selection, and biochemical "intelligence", these parasites developed admirable strategies to infect the host's organism. One of the most fascinating is represented by the nurse cell formation in muscular tissue (*e.g.*, diaphragm, skeletal muscle, extrinsic ocular muscles, *etc*.). This strategy allowed the parasite to adapt and conquer the wider host species spectrum, including ungulates and humans. Consumption of undercooked meat from infected wild ungulates constitutes the most important source of infection for the human species. In this study, we show the prevalence of *Trichinella* spp. in hunted wild boars (*Sus scrofa*) in Central Italy. During the hunting season 2021/2022 in the province of Rieti, 554 wild boar diaphragm biopsies were collected for *Trichinella* spp. screening, in accordance with Regulation EU 1375/2015. An artificial digestion method was used for the detection of *Trichinella* spp. larval forms. The results revealed a positivity of 0.18% (1/554), and molecular biology identification demonstrated the presence of *Trichinella britovi* in the positive sample.

This species is the most diffused in wild ungulate populations in Central Italy and the most frequently isolated in human patients with trichinellosis from this area, showing a close epidemiological relation between *Homo sapiens* and *Sus scrofa* for *Trichinella* spp. diffusion in an ecosystem. Epidemiological surveillance, in receptive animal species destined for human consumption and at any One Health level, represents the main "winning" strategy in the control of this worldwide, widespread foodborne parasitic disease.

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Introduction

Trichinellosis is a globally diffused foodborne parasitic disease caused by nematodes of the *Trichinella* complex (Korhonen *et al*., 2016). During evolution, guided by biochemical "intelligence", natural selection, and ecological interactions, these parasites developed admirable strategies to infect the host's organism.

One of the most fascinating is represented by the nurse cell formation in the striated muscle cell, the main cytotype of the muscular tissue (*e.g.*, diaphragm, skeletal muscle, extrinsic ocular muscles, *etc*.). Through a biomolecular approach, scientists have reconstructed and retraced the phylogenetic path and the main evolutionary steps that led this parasite from a common ancestor, characterized by an exclusive intestinal (enterozoic) parasitism, to the conquest of the intracellular world, the myocyte (Korhonen *et al*., 2016).

This strategy allowed the parasite to conquer the wider host species spectrum, including omnivorous species (*e.g.*, *Sus scrofa*, *Ursus arctos*, *Homo sapiens*) and carnivorous ones (*e.g.*, *Lupus lupus*, *Gyps fulvus*), increasing the possibilities of diffusion within the ecosystems of the 6 continents (Antarctica excluded) of the Earth (Korhonen *et al*., 2016). The *Trichinella* complex comprises 12 species and genotypes identified on a phylogenetic and biogeographic basis (Korhonen *et al*., 2016).

All *taxa* are characterized by an adult intestinal stage (enterozoic) and a larval (L1) muscular (myocytozoic) infective stage. Both stages develop within a single vertebrate host, and an infected animal represents a definitive and potential intermediate host (Centers for Disease Control and Prevention, 2019).

New-born *larvae*, oriented by chemotactic signals, start a migration from the intestinal lymphatic system to the target cytotype (striated myocyte) and associated organs. This causes *larva migrans* to disseminate multiple lesions (*e.g.*, interstitial eosinophilic myositis), with reparative, cicatricial phenomena and massive immune system activation, with special regard to eosinophil chemotaxis, until the larva enters the myocyte "shelter", starting the nurse cell "barrier" formation (Marcato, 2002; Kumar *et al*., 2014; Nuzzolo-Shihadeh *et al*., 2020).

The epidemiological diffusion of *Trichinella* spp. in a receptive species population is the result of a complex system of "equations" determined by ecological, biological, and ethological, anthropological included, variables intertwined together in an ecosystem scenario (Korhonen *et al*., 2016). Consumption of undercooked meat from infected wild ungulates constitutes the most important source of infection for the human species. This determines a higher risk of trichinellosis in human populations characterized by anthropological and cultural systems hunting receptive species (*e.g.*, wild ungulates) with consumption of their meats in traditional products whose processing and preparation do not provide and guarantee any sanitizing larvicidal treatment (Santos Durán-Ortiz *et al*., 1992). Moving from these considerations and investigating with an epidemiological and ecological approach from the One Health perspective, here we show the prevalence of *Trichinella* spp. in hunted wild boars (*Sus scrofa*) in Central Italy.

Materials and Methods

Diaphragm biopsy sampling

During the hunting season 2021/2022 in the province of Rieti, 554 wild boars (*Sus scrofa*) were screened for *Trichinella* spp. by diaphragm biopsy sampling, following Regulation 1375/2015 (European Commission, 2015). The diaphragm biopsy sampling was performed by the incisional surgical method, biopsying from each hunted wild boar a 5 g single aliquot of diaphragmatic muscle at the pillars (left and right *crura*) of the diaphragm in the transition zone between muscle and tendon (myotendinous junction).

After sampling, diaphragm biopsies were transported under refrigerated conditions and stored at -20°C until their processing for the artificial digestion method.

Artificial digestion method

In accordance with Regulation 1375/2015 and Regulation 1478/2020 (European Commission, 2015, 2020), diaphragm biopsies were screened for *Trichinella* spp. by the artificial digestion method, defined by the International Standardization Organization and described in ISO 18743 (2015).

The method allows for the detection of *Trichinella* spp. L1 *larvae* by their release from muscle tissues after artificial enzymatic digestion, followed by filtration, sedimentation, and detection of recovered *larvae* by microscopy (ISO, 2015). The artificial digestion of diaphragm biopsy tissues was conducted using the proteolytic enzyme pepsin, at a temperature and pH *optimum* of 46-48°C and 3.0-5.0, respectively, in stirring conditions. The resulting fluid was filtered and sedimented. Then the sediment was collected and observed by microscopy.

Stereo and optic microscopy

Previously obtained sediment was observed by stereomicroscopy to detect isolated *Trichinella* spp. L1 *larvae*, possibly present for morphobiometric observations and genus identification. *Trichinella* spp. positive diaphragm biopsies were also observed by stereo and optic microscopy for *in situ* larval and nurse cell morphobiometric characterization.

Multiplex polymerase chain reaction

Isolated *Trichinella* spp. L1 *larvae* were sent to the European Union Reference Laboratory for Parasites (Italian National Institute of Health, Rome, Italy) for species or genotype identification by molecular biology multiplex polymerase chain reaction (PCR) (Pozio and La Rosa, 2003).

Results

Diaphragm biopsy sampling

A total of 554 wild boar (*Sus scrofa*) diaphragm biopsies were obtained by biopsy sampling for *Trichinella* spp. screening.

Artificial digestion method

Artificial digestion of the diaphragm biopsies samples, followed by filtration and sedimentation, revealed the presence of cylindrical helminthic bodies, morphologically referable to nematode larval forms, in one sample sediment derived from an adult male wild boar's diaphragm biopsy.

Stereo and optic microscopy

Microscopic observation of sediment by stereomicroscopy confirmed the presence of isolated *larvae* with cylindrical bodies characterized by 0.03 mm in width and 0.70-1.00 mm in length with nematode anatomical structures, morphobiometrically congruent with L1 larval forms ascribable to the genus *Trichinella*. Histopathological observation of the *Trichinella*-positive diaphragm biopsy unfixed, unstained tissues by stereo and optic microscopy revealed the presence of numerous parasitic lesions characterized by intramyocytic, encapsulated, spiralized, single or double nematode *larvae* (Figures 1 and 2). Myocytes infected with intrazoic larval forms presented cytomorphometric modifications compared with the normal ones. In particular, an increase in myocyte's transverse diameter was observed (Figure 2). A capsule defined the limits between the larval vacuolar compartment and the myocyte's extracapsular sarcoplasm, originating the structural and functional wall of the nurse cell complex (Figure 2). Nurse cells mean estimated biometrics were 500 μm in length and 250 μm in height.

Multiplex polymerase chain reaction

Molecular biology species identification by multiplex PCR demonstrated the presence of *Trichinella britovi* in the positive sample. The described results demonstrated and revealed in samples a positivity of 0.18% (1/554) for *Trichinella* spp. with the presence of *T. britovi* in the positive one.

Discussion

Indagating with an epidemiological and ecological approach from the One Health perspective, the obtained results show a prevalence of 0.18% (1/554) for *Trichinella* spp. in hunted wild boars (*Sus scrofa*) in Central Italy. Based on diaphragm biopsy samples, artificial digestion, microscopic observation of isolated and *in situ larvae*, and molecular biology species identification, our results demonstrated a positivity of 0.18% (1/554) for *Trichinella* spp. with the presence of *T. britovi* in the positive sample. Histopathological observation permitted the *in situ* detection of numerous parasitic lesions characterized by intramyocytic, encapsulated, spiralized, single- or double-nematode *larvae*.

A capsule defines limits between the larval vacuolar compartment and myocyte's extracapsular sarcoplasm, originating the structural and functional wall of the nurse cell complex. This mirable structure, guaranteeing the absence of direct contact between larval cuticular surface antigens and extracellular immunosurveillance, acts as an anatomical and functional immunoevasion strategy (Bruschi *et al*., 2022).

Myocytes infected with intrazoic larval forms presented cytomorphometric modifications compared with the normal ones. In particular, an increase in myocyte's transverse diameter was observed. The described cytomorphological observation corresponds to a metabolic modification induced by the parasite: infected myocytes' mitochondria degenerate, causing an anaerobic shift of the nurse cell metabolism and consequent induction of the anaerobic glycolysis pathway with lactic acid production. This molecule causes immune cell functions suppression and differentiation of monocytes into dendritic cells with an immunosuppressive phenotype (Wang *et al*., 2021). This mechanism, similar to that observed in neoplastic cell populations and known as the Warburg effect, may represent another immunoevasion strategy performed through immunomodulation (Wang *et al*., 2021). Furthermore, the anaerobic shift in the nurse cell metabolism also represents a survival mechanism for *larvae* in decaying carcasses.

T. britovi and *T. spiralis* are the 2 most common species of the *Trichinella* complex circulating in Europe (Pozio *et al*., 2009). In particular, *T. britovi* is the most common species within the *Trichinella* complex in wild ungulate populations in Central Italy and the most frequently isolated in human patients with trichinellosis from this area, showing a close epidemiological relation between *Homo sapiens* and *Sus scrofa* for *Trichinella* spp. diffusion in an ecosystem (Troiano and Nante, 2019). This is the result of a fascinating interconnection between host species' and parasites' ecological, biological, and ethological variables, intertwined together in an ecosystem scenario. Human populations characterized by anthropological and cultural systems hunting receptive species (*e.g*., wild ungulates) with consumption of their meats in traditional products whose processing and preparation do not provide or guarantee any sanitizing larvicidal treatment present a higher risk of trichinellosis. The single positive hunted animal, identified in our study and foiled by sanitary inspection surveillance, could have caused this parasitic disease in tens of human beings and their families. According to the European Food Safety Authority and the European Center for Disease Prevention and Control (2022), as reported in the European Union One Health 2021 Zoonoses Report, 117 cases of human trichinellosis were diagnosed in 2020. We can think of ecosystems as the mirable result of a complex system of equations determined by a continuously interacting multitude of variables (Cammarota and Marinari, 2022).

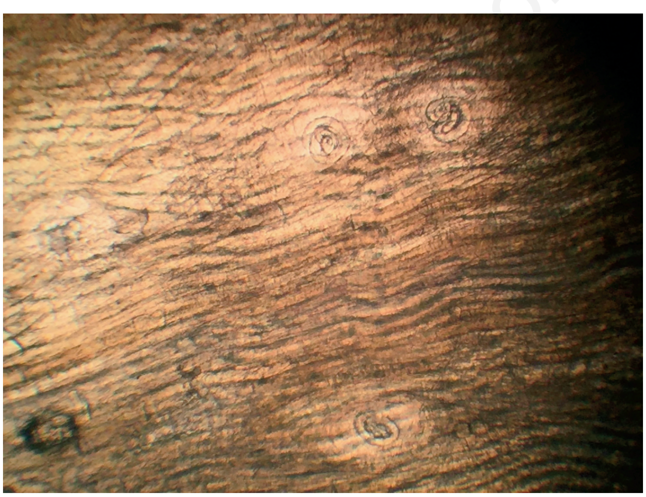

Figure 1. Parasitic lesions containing spiralized *larvae* (L1) of *Trichinella* spp. (*Sus scrofa*, diaphragmatic muscle biopsy, unfixed, unstained tissues, contrast-enhanced optical microscopy). Diaphragm biopsies, obtained by the incisional surgical method, revealed the presence of numerous parasitic lesions, characterized by intramyocytic, encapsulated, spiralized nematode *larvae*, in one sample.

Figure 2. Nurse cell (*Sus scrofa*, diaphragmatic muscle biopsy, unfixed, unstained tissues, contrast-enhanced optical microscopy). Myocyte infected with 2 spiralized *Trichinella* spp. L1 *larvae.* Myocytes infected with intrazoic larval forms presented cytomorphometric modifications compared with the normal ones. In particular, an increase in myocyte's transverse diameter was observed. A capsule defines limits between the larval vacuolar compartment and myocyte's extracapsular sarcoplasm, originating the structural and functional wall of the nurse cell complex. Nurse cells mean estimated biometrics were 500 μm in length and 250 μm in height.

Determining ecosystem evolution and transition over time in terms of environmental changes and species' population dynamics represents a decisive challenge, whose implications are directly projected from public health issues to the global climate change level (Cammarota and Marinari, 2022).

A great contribution to the understanding of complex systems, ecosystems included, was made by the Nobel Prize winner Giorgio Parisi with his "discovery of the interplay of disorder and fluctuations in physical systems from atomic to planetary scales" (Parisi, 1980; Cammarota and Marinari, 2022).

Complex systems represent a wide variety of phenomena in which numerous units are linked together through disordered, highly complex interactions. These systems, which present high levels of disorder, during their evolution, reach different possible states of equilibrium (Cammarota and Marinari, 2022). What we observe in an ecosystem represents the description of one of those states of equilibrium reached by the system, like a "photogram" of the evolution "trailer".

With a translational, interdisciplinary approach, the paradigm of Parisi can be applied to "eco-complex" systems, shedding light and explaining many of the intimate interspecific dynamics, as well as those intertwined by host-parasite species. The paradigm shows high potential in the prediction of epidemiological dynamics implicated in disease diffusion, such as trichinellosis and other zoonotic diseases (Ferri *et al*., 2022; Ferri *et al*., 2022; Ferri *et al*., 2023).

Climate change, with special regard to global warming, represents one of the deepest challenges for ecosystems and biodiversity, parasites and host species included (Korhonen *et al*., 2016). Maintaining a wider perspective on ecosystems and biodiversity, are there any effects on host-parasite population dynamics, their ecological equilibrium, and public health implications?

Muscle stage *larvae* survival is guaranteed by the host's organism homeostasis, determining a microenvironment characterized by constant temperature and humidity. By the death of the host, "free-living" stage *larvae* survival in carcasses is entrusted to the parasite's ability, shaped by evolution over millions of years, to resist environmental temperature and humidity variations during seasons. *T. britovi* survives in frozen muscle for months, up to several years. Survival is higher at temperatures between 0 °C and -20°C, the thermal range characterizing the environment under the snow. Survival time is rapidly reduced under this range (Pozio, 2016). This evolutive thermal parasite's adaptation has direct food safety implications for game meat and derived products, potentially limiting the sanitizing larvicidal efficacy of low-temperature (freezing) meat exposure (Pozio, 2016).

Furthermore, climate change and the related increasing temperatures may cause a higher survival rate of "free-living" *larvae* during the winter season, determining a larger circulation of the parasite's infective forms in the environment and diffusion in new ecosystems through colonization of northern latitudes, with ecological, epidemiological, and public health implications.

Conclusions

During evolution, guided by ecological interactions, natural selection, and biochemical "intelligence", these parasites developed admirable strategies to infect hosts, spreading in biodiversity, human species included, through ecosystems' food chain connections and exerting a high potential impact on public health. From Virchow's first cycle description to molecular immunoevasion strategies, most of the host-parasite microenvironmental pathogenetic interactions remain unveiled.

Our results confirm how epidemiological surveillance, in receptive animal species destined for human consumption and at any One Health level, in synergism with inspective anatomopathological examination (Piccinini *et al*., 2022), represents the main "winning" strategy in the control of this worldwide widespread foodborne parasitic disease and human health protection.

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