UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Scienze Medico-Veterinarie

Ciclo XXVIII

Intermediate hosts of *Toxoplasma gondii* and food safety: epidemiology, genetics and parasite survival in meat-producing animals in Italy

Coordinatore:
Chiar.mo Prof. Franco Brindani

Tutor:
Gent.ma Dott.ssa Cristina Bacci

Co-tutor:
Chiar.ma Prof.ssa Laura Helen Kramer

Dottorando: Alice Vismarra

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1. Abstract

Toxoplasma gondii is a coccidian parasite with a global distribution. The definitive host is the cat (and other felids). All warm-blooded animals can act as intermediate hosts, including humans. Sexual reproduction (gametogony) takes place in the final host and oocysts are released in the environment, where they then sporulate to become infective. In intermediate hosts the cycle is extra-intestinal and results in the formation of tachyzoites and bradyzoites. Tachyzoites represent the invasive and proliferative stage and on entering a cell it multiplies asexually by endodyogeny. Bradyzoites within tissue cysts are the latent form.

T. gondii is a food-borne parasite causing toxoplasmosis, which can occur in both animals and humans. Infection in humans is asymptomatic in more than 80% of cases in Europe and North-America. In the remaining cases patients present fever, cervical lymphadenopathy and other non-specific clinical signs. Nevertheless, toxoplasmosis is life threatening if it occurs in immunocompromised subjects. The main organs involved are brain (toxoplasmic encephalitis), heart (myocarditis), lungs (pulmonary toxoplasmosis), eyes, pancreas and parasite can be isolated from these tissues. Another aspect is congenital toxoplasmosis that may occur in pregnant women and the severity of the consequences depends on the stage of pregnancy when maternal infection occurs. Acute toxoplasmosis in developing foetuses may result in blindness, deformation, mental retardation or even death.

The European Food Safety Authority (EFSA), in recent reports on zoonoses, highlighted that an increasing numbers of animals resulted infected with *T. gondii* in EU (reported by the European Member States for pigs, sheep, goats, hunted wild boar and hunted deer, in 2011 and 2012). In addition, high prevalence values have been detected in cats, cattle and dogs, as well as several other animal species, indicating the wide distribution of the parasite among different animal and wildlife species.

The main route of transmission is consumption of food and water contaminated with sporulated oocysts. However, infection through the ingestion of meat contaminated with tissue cysts is frequent. Finally, although less frequent, other food products contaminated with tachyzoites such as milk, may also pose a risk.

The importance of this parasite as a risk for human health was recently highlighted by EFSA's opinion on modernization of meat inspection, where *Toxoplasma gondii* was identified as a relevant

hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer (Call for proposals -GP/EFSA/BIOHAZ/2013/01). The risk of infection is more highly associated to animals reared outside, also in free-range or organic farms, where biohazard measure are less strict than in large scale, industrial farms. Here, animals are kept under strict biosecurity measures, including barriers, which inhibit access by cats, thus making soil contamination by oocysts nearly impossible. A growing demand by the consumer for organic products, coming from free-range livestock, in respect of animal-welfare, and the desire for the best quality of derived products, have all led to an increase in the farming of free-range animals. The risk of *Toxoplasma gondii* infection increases when animals have access to environment and the absence of data in Italy, together with need for in depth study of both the prevalence and genotypes of *Toxoplasma gondii* present in our country were the main reasons for the development of this thesis project.

A total of 152 animals have been analyzed, including 21 free-range pigs (Suino Nero race), 24 transhumant Cornigliese sheep, 77 free-range chickens and 21 wild animals. Serology (on meat juice) and identification of *T. gondii* DNA through PCR was performed on all samples, except for wild animals (no serology). An in-vitro test was also applied with the aim to find an alternative and valid method to bioassay, actually the gold standard. Meat samples were digested and seeded onto Vero cells, checked every day and a RT-PCR protocol was used to determine an eventual increase in the amount of DNA, demonstrating the viability of the parasite. Several samples were alos genetically characterized using a PCR-RFLP protocol to define the major genotypes diffused in the geographical area studied.

Within the context of a project promoted by Istituto Zooprofilattico of Pavia and Brescia (Italy), experimentally infected pigs were also analyzed. One of the aims was to verify if the production process of cured "Prosciutto di Parma" is able to kill the parasite. Our contribution included the digestion and seeding of homogenates on Vero cells and applying the Elisa test on meat juice.

This thesis project has highlighted widespread diffusion of *T. gondii* in the geographical area taken into account. Pigs, sheep, chickens and wild animals showed high prevalence of infection. The data obtained with serology were 95.2%, 70.8%, 36.4%, respectively, indicating the spread of the parasite among numerous animal species. For wild animals, the average value of parasite infection determined through PCR was 44.8%.

Meat juice serology appears to be a very useful, rapid and sensitive method for screening carcasses at slaughterhouse and for marketing "Toxo-free" meat. The results obtained on fresh pork meat (derived from experimentally infected pigs) before (on serum) and after (on meat juice) slaughter showed a good concordance.

The free-range farming put in evidence a marked risk for meat-producing animals and as a consequence also for the consumer.

Genotyping revealed the diffusion of Type-II and in a lower percentage of Type-III. In pigs is predominant the Type-II profile, while in wildlife is more diffused a Type-III and mixed profiles (mainly Type-II/III). The mixed genotypes (Type-II/III) could be explained by the presence of mixed infections. Free-range farming and the contact with wildlife could facilitate the spread of the parasite and the generation of new and atypical strains, with unknown consequences on human health.

The curing process employed in this study appears to produce hams that do not pose a serious concern to human health and therefore could be marketed and consumed without significant health risk.

Little is known about the diffusion and genotypes of *T. gondii* in wild animals; further studies on the way in which new and mixed genotypes may be introduced into the domestic cycle should be very interesting, also with the use of NGS techniques, more rapid and sensitive than PCR-RFLP. Furthermore wildlife can become a valuable indicator of environmental contamination with *T. gondii* oocysts.

Other future perspectives regarding pigs include the expansion of the number of free-range animals and farms and for Cornigliese sheep the evaluation of other food products as raw milk and cheeses. It should be interesting to proceed with the validation of an ELISA test for infection in chickens, using both serum and meat juice on a larger number of animals and the same should be done also for wildlife (at the moment no ELISA tests are available and MAT is the reference method for them).

Results related to Parma ham do not suggest a concerning risk for consumers. However, further studies are needed to complete the risk assessment and the analysis of other products cured using technological processes other than those investigated in the present study. For example, it could be

interesting to analyze products such as salami, produced with pig meat all over the Italian country, with very different recipes, also in domestic and rural contexts, characterized by a very short period of curing (1 to 6 months).

Toxoplasma gondii is one of the most diffuse food-borne parasites globally. Public health safety, improved animal production and protection of endangered livestock species are all important goals of research into reliable diagnostic tools for this infection. Future studies into the epidemiology, parasite survival and genotypes of *T. gondii* in meat producing animals should continue to be a research priority.

2. Introduction

2.1Biology of Toxoplasma gondii

2.1.1 Classification

Toxoplasma gondii is a coccidian parasite with a worldwide distribution. The cat (and wild felids) is the definitive host, while all warm-blooded animals can act as intermediate hosts. It belongs to:

Phylum	Apicomplexa	Levine, 1970
Class	Sporozoasida	Leukart, 1879
Subclass	Coccidiasina	Leukart, 1879
Order	Eimeriorina	Leger, 1911
Family	Toxoplasmatidae	Biocca, 1956
Genus	Toxoplasma	Nicolle and Manceaux, 1909
Species:	only one, *Toxoplasma gondii*	named "gondii" after the African rodent (Ctenodactylus gundi) from which it was first isolated in the early 1900s. Nicolle and Manceaux, 1909

Coccidia are among the most important parasites of animals, and they were the first protozoa discovered (Dubey, 2010a). The oocyst is the key stage of all coccidians, and their classification was based on the structure of oocyst. Oocysts with four sporocysts, each with two sporozoites (total: eight sporozoites), are classified as *Eimeria.* Oocysts containing two sporocysts, each with four sporozoites, historically were classified as *Isospora* (Dubey, 2010a).Unlike *Eimeria*, the life cycle of which has been known for many years, little was known of the complete life cycle of most *Isospora* species until 1970, when the life cycle of *Toxoplasma gondii* was discovered. In that year, *T. gondii*, a parasite previously known to parasitize extra-intestinal tissues of virtually all warm-blooded hosts, was found to be an intestinal coccidium of cats and to have an isosporan-like oocyst. This finding was a major breakthrough in medical and veterinary science and eventually led to the recognition of several new taxa of economically important *Toxoplasma*-like parasites (e.g., *Neospora*, *Sarcocystis*) and discovery of their cycles.

Historically, *T. gondii* originated probably as a coccidian parasite of cats with a fecal-oral cycle. With domestication, it adapted its transmission by several modes, including transmission by fecal-

oral cycle, by carnivorism, and transplacentally. There are three infectious stages of *T. gondii*: the tachyzoites, the bradyzoites, and oocysts. These stages are linked in a complexan life cycle (Fig. 1).

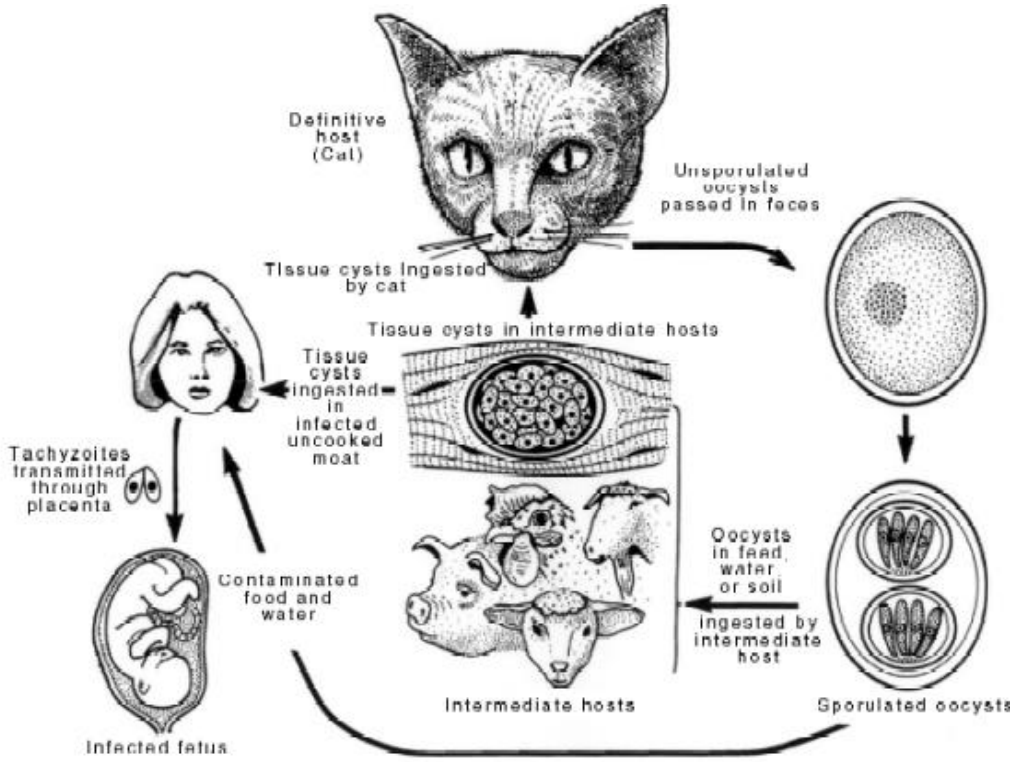


Figure n. 1: *Life cycle of T. gondii described by Dubey J.P in 1970. (taken from: Dubey, 2010a).*

2.1.2 Structure and life cycle

The tachyzoite is often crescent-shaped and is approximately the size (2 mm x 6 mm) of a red blood cell. The anterior end of the tachyzoite is pointed (conoidal), and the posterior end is round. It has a pellicle (outer covering), several organelles including sub-pellicular microtubules, mitochondrium, smooth and rough endoplasmic reticulum, a Golgi apparatus, apicoplast, ribosomes, a micropore, and a well-defined nucleus. The nucleus is usually situated toward the posterior end or in the central area of the cell.

The tachyzoite enters the host-cell by active penetration of the host-cell membrane. The mechanical events involved in zoite attachment and penetration include (i) gliding of the zoite; (ii) probing of the host cell with the zoite's conoidal tip; (iii) indenting the host cell plasmalemma; (iv) forming a moving junction that moves posteriorly along the zoite as it penetrates into the host cell; and (v)

partially exocytosing micronemes, rhoptries, and dense granules. *Toxoplasma gondii* can penetrate a variety of cell types from a wide range of hosts, indicating that the biochemical receptors involved in the attachment and penetration are probably common to most animal cells. After entering the host cell, the tachyzoite becomes ovoid in shape and becomes surrounded by a parasitophorous vacuole. *Toxoplasma gondii* in a parasitophorous vacuole is protected from host defense mechanisms. The tachyzoite multiplies asexually within the host cell by repeated divisions in which two progenies form within the parent parasite, consuming it. Tachyzoites continue to divide until the host cell is filled with parasites. After a few divisions, *T. gondii* forms tissue cysts and remains intracellular. Tissue cysts may vary in size: young ones may be small as 5 µm and contain only two bradyzoites, while older ones may contain thousands of organisms. The tissue cyst wall is elastic, thin (<0.5 mm), and may enclose hundreds of the crescent-shaped, slender *T. gondii* stage known as bradyzoites. The bradyzoites are approximately 7 mm x 1.5 mm and differ structurally only slightly from tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. Bradyzoites are more slender and less susceptible to destruction by proteolytic enzymes than tachyzoites. Although tissue cysts containing bradyzoites may develop in visceral organs, including lungs, liver, and kidneys, they are more prevalent in muscular and neural tissues, including the brain, eye, skeletal, and cardiac muscle. Intact tissue cysts probably do not cause any harm and can persist for the life of the host. After the ingestion of tissue cysts by cats, the tissue cyst wall is dissolved by proteolytic enzymes in the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the small intestine and initiate development of numerous generations of asexual and sexual cycles of *T. gondii*. *Toxoplasma gondii* multiplies profusely in intestinal epithelial cells of cats (enteroepithelial cycle) and these stages are known as schizonts. Organisms (merozoites) released from schizonts form male and female gametes. The male gamete has two flagella and it swims to and enters the female gamete. After the female gamete is fertilized by the male gamete, oocyst wall formation begins around the fertilized gamete. When oocysts are mature, they are discharged into the intestinal lumen by the rupture of intestinal epithelial cells. Oocysts are the environmentally resistant stage in the life cycle of coccidia. Oocysts of *T. gondii* are formed only in felids, probably in all members of the Felidae. Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*, i.e., tachyzoites, bradyzoites, and sporozoites. Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. Prepatent periods are 3–10 days after ingesting tissue cysts and 19 days or more after ingesting

tachyzoites or oocysts. Less than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts (Dubey and Frenkel, 1972). In freshly passed feces, oocysts are unsporulated (non-infective). Unsporulated oocysts are subspherical to spherical and are 10 mm x 12 mm in diameter. They sporulate (become infectious) outside the cat within 1–5 days depending upon aeration and temperature. Sporulated oocysts contain two ellipsoidal sporocysts. Each sporocyst contains four sporozoites. The sporozoites are 2 mm x 6 mm to 2 mm x 8 mm in size. As the enteroepithelial cycle progresses, bradyzoites penetrate the lamina propria of the feline intestine and multiply as tachyzoites. Within a few hours after infection of cats, *T. gondii* may disseminate to extraintestinal tissues. *Toxoplasma gondii* persists in intestinal and extraintestinal tissues of cats for at least several months, and possibly for the life of the cat.

2.1.2a. Life cycle in the definitive host: the cat

Cats, not only the domestic, but nearly all the species of felids, can shed *T. gondii* oocysts. Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*. Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. They can go from three to ten days after ingesting tissue cysts, and more than 18 days after ingesting oocysts, irrespective of the dose. The prepatent period after ingesting tachyzoites may vary. Fewer than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts. *T. gondii* is adapted to be transmitted biologically by carnivorism in cats and transmission by the oocysts is more efficient in non-feline hosts; bradyzoites are more infective to cats and oocysts are more infective to mice. After the ingestion of tissue cysts the tissue cyst wall is dissolved by proteolytic enzymes in the stomach and small intestine and initiates the development of numerous generations of *T. gondii*. Five morphologically distinct types of *T. gondii* develop in intestinal epithelial cells before gametogony begins. These asexual stages in the feline intestine are structurally distinct from tachyzoites that also develop in the lamina propria. The entroepithelial stages (A-E gamonts) are formed in the intestinal epithelium and the development of types B-E schizonts in enterocytes has been confirmed ultrastructurally. Occasionally, type B and C schizonts develop within enterocytes that are displaced beneath the epitelium into the lamina propria. Tachyzoites occur exclusively within the lamina propria. Schizonts and gamonts develop exclusively in enterocytes. Types C, D, E multiply by schizogony; in this process the nucleus divides two or more times without cytoplasmic

division. Before or simultaneous with the last nuclear division, merozoite formation is initiated near the center of the schizont. The merozoites eventually move towards the periphery of the schizont and the schizont plasmalemma invaginates around each merozoite forming the plasmalemma of the merozoite. The merozoites separate from the schizont at their posterior ends. The sexual cycle starts two days after ingestion of tissue cysts by the cat. The origin of gamonts has not been determined, but the merozoites released from schizont type D and E probably initiate gamete formation. Gamonts occur throughout the small intestine but most commonly in the ileum, 3-15 days after inoculation. They occur above the nucleus of the host epithelial cell near the tips of the villi of the small intestine. Female (macro) gamonts are subspherical and each contains a single centrally located nucleus and several PAS-positive granules. Mature male gamonts (micro) are ovoid to ellipsoidal in shape. During microgametogenesis, the nucleus if the microgamont divides and ultrastructurally, after several nuclear division, it migrates to the periphery of the gamont. Microgametes are biflagellate and contain a perforatium, two flagella (up to 10 µm long) that arise from two basal bodies immediately behind the perforatorium. Microgametes use their flagella to swim to penetrate and fertilize mature macrogametes to form zigotes. After fertilization, an oocyst wall is formed around the parasite. Infected epithelial cells rupture and discharge oocyst into the intestinal lumen. Unsporulate oocysts are subspherical to spherical and are 10 x 12 µm in diameter. Sporulation occurs in the environment, within 1 to 5 days depending upon aeration and temperature. Sporulated oocysts are subspherical to ellipsoidal and are 11 x 13 µm in diameter. Each sporulated oocyst contains two ellipsoidal sporocysts without Stieda bodies. Sporocysts measure 6 x 8 µm. Each sporocyst contains four sporozoites. During sporulation the nucleus divides twice giving rise to four nuclei that are situated at the periphery of the zygote; at this stage, a second limiting membrane is formed. After the cytoplasm divides, two spherical sporoblasts areformed, each with two nuclei. As sporulation continues, the sporoblasts elongate and sporocysts are formed. Ultrastructurally, the sporozoite is similar to a tachyzoite except that there is an abundance of micronemes, rhoptriess and amylopectin granules in former. They are 2 x 6-8 µm with a subterminal nucleus (Dubey, 2010a).

2.2 Population structure of T. gondii

2.2.1 Genotypes

Toxoplasma gondii has a clonal population structure and the genotypes are divided into types I, II, III. This distinction was originally made based on the different virulence patterns observed in mice.

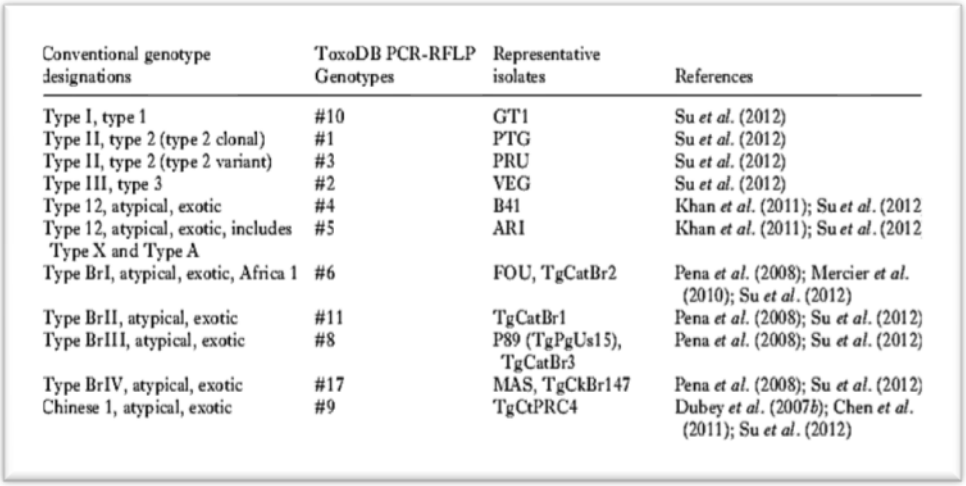
Despite the presence of a sexual stage in the life cycle of the parasite and a worldwide distribution, the population structure, in isolates genotyped and sequenced in USA and Europe, appears highly clonal with low genetic diversity. However, multilocus and multichromosome genotyping of isolates from other continents have revealed a much more complex population structure. The majority of isolates in South America, Africa or Asia do not fit into the three major lineages.

Several molecular techniques including polymerase chain reaction – restriction fragment length polymorphism analysis (PCR-RFLP) (Howe and Sibley, 1995; Su et al. 2006, 2010), microsatellite DNA analysis (Ajzenberg et al. 2002, 2010) and multilocus DNA sequence typing of introns (Khan et al., 2011) have been used to study the genetic makeup of *T. gondii* strains.

Up to now, 12 different haplogroups have been described (including the initially 3 lineage I, II, III), based on sequence-based analysis and they are not completely homogeneous. There are isolates that are defined as "atypical" for the presence of unique polymorphisms which cannot be clustered into one of the twelve haplogroups (Mercier et al., 2011).

There is currently no precise nomenclature for *T. gondii* genotypes. Conventional designation defined type I, II, III and lumped together all the others as atypical or exotic. The subsequently identified major genotypes were added to the list including Type BrI, BrII, BrIII and BrIV, Type 12, Africa 1 and Chinese 1 (Pena et al. 2008; Mercier et al. 2010; Chen et al. 2011; Khan et al., 2011). However, this scheme of genotype designation is too cumbersome to define the hundreds of genotypes identified by the multilocus PCR-RFLP method. To overcome this cumbersome designation, a scheme has been adopted in which each genotype is designated as a 'ToxoDB PCR-RFLP genotype' followed by a specific numeral. A comparison of conventional nomenclature and ToxoDB PCR-RFLP designations for major genotypes is provided in figure 2.
The 10 most frequently identified are, in order, genotypes #2, #3, #1, #5, #4, #9, #6, #7, #8 and #10. Genotypes #1 and #3, which differ only at the Apico locus, together compose the conventional Type II lineage and accounted for 24.8% (362/1457) of the population. Genotype #1 is also referred to as Type II clonal, whereas #3 as Type II variant (Figure 2). As noted, genotype #2, also known as Type III, accounted for 13.8% (201/1457) of the samples. Genotypes #4 and #5, which differ only at the SAG1 locus and are collectively known as Type 12, accounted for 9.5% (139/1457) of the population. The results showed that genotype #1, #2 and #3 (Type II clonal, Type III and Type II variant) are identified worldwide. These three genotypes are highly prevalent in Europe. Genotypes

#1, #2, #3, #4 and #5 dominate in North America. Genotypes #2 and #3 (Types III and II variant) dominate in Africa, and genotypes #9 and #10 (Chinese 1 and Type I) are prevalent in East Asia.



Conventional genotype designations	ToxoDB PCR-RFLP Genotypes	Representative isolates	References
Type I, type 1	#10	GT1	Su *et al.* (2012)
Type II, type 2 (type 2 clonal)	#1	PTG	Su *et al.* (2012)
Type II, type 2 (type 2 variant)	#3	PRU	Su *et al.* (2012)
Type III, type 3	#2	VEG	Su *et al.* (2012)
Type 12, atypical, exotic	#4	B41	Khan *et al.* (2011); Su *et al.* (2012
Type 12, atypical, exotic, includes Type X and Type A	#5	ARI	Khan *et al.* (2011); Su *et al.* (2012
Type BrI, atypical, exotic, Africa 1	#6	FOU, TgCatBr2	Pena *et al.* (2008); Mercier *et al.* (2010); Su *et al.* (2012)
Type BrII, atypical, exotic	#11	TgCatBr1	Pena *et al.* (2008); Su *et al.* (2012)
Type BrIII, atypical, exotic	#8	P89 (TgPgUs15), TgCatBr3	Pena *et al.* (2008); Su *et al.* (2012)
Type BrIV, atypical, exotic	#17	MAS, TgCkBr147	Pena *et al.* (2008); Su *et al.* (2012)
Chinese 1, atypical, exotic	#9	TgCtPRC4	Dubey *et al.* (2007*b*); Chen *et al.* (2011); Su *et al.* (2012)

Figure n. 2 : *Genotypes designations for common genotypes (source: Shawb et al., 2013)*

Clonal type	Strain
I	RH, GT1, ENT, VEL
II	PTG, DEG, PIH, ME49, PLK
III	CTG, VEG, STRL
ATYPICAL ISOLATES	CAST, COUGAR, MAS, CASTELLS, VAND, GUYDOS, GUYMAT, GPHT(I/II recombinant isolate)

Table n. 1: *some of the most common strains and isolates of T. gondii divided in clonal types I, II, III and atypical isolates.*

2.2.2 Genotypes and their geographic distribution

From Northern Europe to Southern Europe the population structure of *T. gondii* is highly clonal, with a predominance of strains belonging to the type II lineage. Type III is more diffused in Southern Europe and atypical strains have been rarely isolated. In North America the population structure is very similar to that one described for Europe, with a strong predominance of type II strains. Recent data has reported the presence of atypical strains isolated both from domestic and

wild animals (Dubey et a., 2008a, Dubey et al., 2008b). A high level of diversity for *T. gondii* was recorded in Central and South America; in Brazil in particular numerous studies have highlighted the presence of atypical and new strains isolated from animals and also from humans with acute toxoplasmosis. Eighty-eight genotypes (defined with 11 genetic PCR-RFLP markers) have already been identified in Brazil, and new genotypes are continuously being identified in different animal species (Pena et al., 2011). The high level of genetic diversity was recorded in many species in the Amazonian area (Ajzenberg et al., 2004). Most samples identified and genotyped (118/141) in Africa were from Egypt. Overall, genotype #3 and #2 are the two dominant types. Genotype #6 was identified for several samples. The limited data that are available from sampling in Western Africa seem to suggest a high level of diversity, with a relatively low frequency of common genotypes (Shwab et al., 2013).

In Asia, there also appears to be a high degree of genetic uniformity. Genotype #9 (Chinese 1) is by far the most commonly found, and it is present in China, Vietnam and Sri Lanka, indicating a widespread distribution in Eastern Asia. Genotype #10 is also common in China, unlike in most other countries. Genotypes #4, #18 and #20 have relatively high frequencies among the samples in Asia. Genotype #20 has been identified in a wide range of areas, from Sri Lanka in south Asia to Egypt in North Africa. Thus far, most of the sampling from Asia has been from the more populous eastern regions (Shwab et al., 2013).

2.2.3 Genotypes and virulence

Experimental virulence is defined by the mouse reaction after the inoculation of the parasite intraperitoneally. Type I isolates are highly virulent, leading to the death of mice in less than 10 days after the inoculation of less than 10^3 tachyzoites, while type II and III are considered avirulent strains, allowing survival after the inoculation of the same dose. Also genotypes with a majority of type I alleles are usually more virulent (Mercier et al., 2010). The virulent strains display several characteristics that may explain the rapid dissemination and virulence. Type I strains are more rapid in destroying a cell monolayer than type II or III because they multiply rapidly and show a lower rate of tachyzoite- to- bradyzoite interconversion (Saeij et al., 2006).

In vivo infection with type I strains display a higher ability to penetrate the epithelia, lamina propria and submucosa (Barragan and Sibley, 2002).

It has been demonstrated that the rhoptry protein family, in particular the association between ROP5/ROP18, plays a role in the infection process. This allele association is always present in virulent strains and the deletion of ROP18 made the stains completely avirulent.

2.3 Virulence factors of T. gondii and host-parasite relationship

2.3.1 Virulence loci

During invasion of host cells, secretory organelles known as rhoptries discharge their contents into the host cell, making these primary candidates for modulating host signalling (Hakansson et al., 2001). **ROP18** is an active serine/ threonine (S/T) protein kinase that is secreted into the host cell where it decorates the surface of the parasitophorous vacuole membrane (PVM). It is a polymorphic protein and it was identified as a major factor that contributed to strain-specific differences in virulence (Taylor et al., 2006; Saeij et al., 2006). Transgenic expression of ROP18 from type I or type II strains in the type III background greatly enhanced virulence confirming that this locus was responsible for the observed differences in virulence between strains. The role of **ROP16** in altering host gene transcription was initially identified by analysing the differences in host gene expression induced by different parasite strains. These studies led to a focus on genes involved in IL-4 and IL-6 responses and implicated changes in the activity of the transcription factors STAT3 and STAT6 (Saeij et al., 2007). Although all three strains initially induce STAT3 and STAT6 activity, only the type I or III strains (which share the same ROP16 variant, ROP16I/III) sustain this response. $ROP16_{I/III}$ directly phosphorylates Tyr705 in STAT3 (Yamamoto et al., 2009) and Tyr641 in STAT6 (Ong et al., 2010), residues that are required for activation of these transcription factors (Saeij et al., 2007). Prolonged STAT3/6 activation by $ROP16_{I/III}$ down-regulates the induction of IL-12, thus limiting the protective TH1 cytokine responses (Saeij et al., 2007), which might lead to less inflammation and reduced pathology but also enhanced parasite survival .

Genetic studies show that the pseudokinase **ROP5** is essential for acute virulence. ROP5 is an inactive member of protein kinase family that controls virulence by blocking IFN-γ mediated clearance in activated macrophages. It is able to regulate the active protein kinase ROP18, which normally prevents clearance of the parasite in interferon-activated macrophages phosphorylating host immunity related GTPases (IRGs). Additionally, ROP5 has other functions that are also Irgm3 and IFN-γ dependent, indicting it plays a general role in governing virulence factors that block immunity (Behnke et al., 2012; 2015).

Another virulence factor that stimulates a high production of IL-12, is a dense granule protein named **GRA15**. The molecular mechanism of GRA15 function is uncertain as it has no homology to any other proteins in the database, nor does it contain any conserved domains. Localization studies revealed that GRA15 is secreted into the host cell together with ROP proteins in small cytoplasmic inclusions known as evacuoles (Rosowski et al., 2011). Deletion of GRA15 in the type II strain ME49 prevents nuclear translocation of NFκB but the mechanism by which GRA15 activates NFκB is incompletely understood. $GRA15_{II}$ activates NFκB resulting in high levels of IL-12 production, further enhancing the classical activation pathway in cells infected by type II parasites. Classically, activated macrophages express chemokines and cytokines that activate cells with antimicrobial activity, while alternatively activated macrophages secrete anti-inflammatory molecules that can down-regulate Th1 responses (Goerdt et al., 1999). Hence, different variants of the effector proteins drive antagonizing responses in infected macrophages, which can have broad effects on antimicrobial effector pathways, inflammation and parasite control. High production levels of IL-12 are also modulated by ROP16 since the allele expressed in type II strains is incapable of sustained phosphorylation of STAT3, and the down-regulation of the IL-12 induction as discussed above.

2.3.2 Mechanism of cell invasion

Toxoplasma gondii usually parasitizes the host, definitive and intermediate, without producing clinical disease. Only rarely does it produce severe clinical manifestations. The majority of natural infections are probably acquired by ingestion of tissue cysts in infected meat or oocysts in food or water contaminated with cat feces. The bradyzoites from the tissue cysts or sporozoites from the oocyst penetrate intestinal epithelial cells and multiply in the intestine as tachyzoites within 24 h of infection. *Toxoplasma gondii* may spread first to mesenteric lymphnodes and then to distant organs by invasion of lymphatic system and blood, and can multiply in virtually any cell in the body. All extracellular forms of the parasite are directly affected by antibody but intracellular forms are not. It is believed that cellular factors, including lymphocytes and lymphokines, are more important than humoral factors in immune mediated destruction of *T. gondii*. Immunity does not eradicate infection. *Toxoplasma gondii* tissue cysts persist several years after acute infection. The fate of tissue cysts is not fully known. Whether bradyzoites can form new tissue cysts directly without transforming into tachyzoites is not known. It has been proposed that tissue cysts may rupture during the life of the host. The released bradyzoites may be destroyed by the host's immune

responses or there may be formed new tissue cysts. In immunosuppressed patients a rupture of a tissue cyst may result in transformation of bradyzoites into tachyzoites and renewed multiplication. The immunosuppressed host may die from toxoplasmosis unless treated.

Pathogenicity of *T. gondii* is determined by the virulence of the strain and the susceptibility of the host species. *Toxoplasma gondii* strains may vary in their pathogenicity in a given host. Certain strains of mice are more susceptible than others and the severity of infection in individual mice within the same strain may vary. Mice of any age are susceptible to clinical *T. gondii* infection. However, adult rats do not become ill, while young rats can die of toxoplasmosis. Adult dogs, like adult rats, are resistant, whereas puppies are fully susceptible to clinical toxoplasmosis. Certain species are genetically resistant to clinical toxoplasmosis. Cattle and horses are among the hosts more resistant to clinical toxoplasmosis, whereas certain marsupials and New World monkeys are highly susceptible to *T. gondii* infection (Dubey and Beattie, 1988).

2.3.3 The innate immune response to *T. gondii*

Most laboratory studies have used type II strains (intermediate virulence), which has facilitated the study of the immune response in mice during the acute and chronic phases of infection. They have shown that control of *T. gondii* requires the early production of the pro-inflammatory cytokine IL-12, which stimulates natural killer (NK) and CD4+ and CD8+ T cells to release IFN-γ (Johnson, 1992; Khan et al., 1994).

The importance of IL-12 induction in early infection is evident from studies reporting that monocytes (Robben et al., 2004) CD8α+ DCs (Reis e Sousa et al., 1997), plasmacytoid DCs (Bierly et al., 2008), and neutrophils (Bliss et al., 2000) all contribute to the production of this activation signal. Il-12 is also essential for controlling parasite growth. There has also been progress in defining the parasite molecules and host receptors involved in initial recognition, including TLR2 and TLR4 detection of glycosylphosphatidylinositol (GPI) anchored proteins (Debierre-Grockiego et al., 2007) and TLR11 mediated detection of parasite profiling (Yarovinsky et al., 2005). Consistent with these data,s idea, triple deficiency (3d) mice, which carry a point mutation in the UNC93B1 protein and are defective in TLR3/7/9 trafficking and TLR11 signalling, are highly susceptible to infection with *T. gondii* (Melo et al., 2010). IFN-γ is the major mediator of resistance to *T. gondii* and is crucial for the activation of a variety of antimicrobial mechanisms in

haematopoetic and non-haematopoetic cells that limit parasite replication (Suzuki et al., 1988), altering cell metabolism. This cytokine also stimulates professional phagocytes to produce reactive oxygen and nitrogen intermediates, which can lead to parasite damage and growth inhibition in macrophages (Murray et al., 1985). This pathway relies on immunity-related GTPases (IRGs) that are induced by IFN-γ and contribute to clearance of *T. gondii* in multiple cell types (Taylor et al., 2006). Although the precise mechanism is not understood, recruitment of IRGs to the parasite-containing vacuole leads to vesiculation, vacuole rupture and digestion of the parasite within the cytosol (Howard et al., 2011). A related family of GTPases known as the p67 guanylate binding proteins (GBPs) were recently shown to contribute to the control of toxoplasmosis in mice (Yamamoto et al., 2012). Although innate immune responses to *T. gondii* have been examined in detail, how these processes lead to the stimulation of adaptive immunity, including the ability of DCs to access antigens for priming of CD4+ and CD8+ T cells, are less well understood. Moreover, infection of host cells is associated with reduced expression of major histocompatibility complex (MHC) molecules (Luder et al., 1998). Despite these mechanisms of avoidance, infection with type II strains of *T. gondii* leads to the activation and expansion of DCs and a strong CD8+ T cell response, while infection with virulent type I strains induces a weaker response (Tait et al., 2010). Several prominent endogenous antigens that are presented on class I MHC molecules include the dense granule proteins GRA6 and GRA4, and the rhoptry protein ROP7 (Frickel et al., 2008). These antigens are polymorphic between strains, suggesting that they may be involved in strain-dependent evasion mechanisms that also influence adaptive immunity. Other studies have highlighted the importance of highly immunogenic surface antigens (SAGs) and SAG-related surface antigen (SRS) in stimulating the adaptive immune response (Lekutis et al., 2001).

2.3.4 Alterations in host signalling and immune evasion

Many innate immune effectors are under the control of transcription factors that enhance or regulate the overall immune response to invading microorganisms. In turn, successful pathogens have developed strategies to undermine important host cell immune pathways. Infection of mammalian cells with *T. gondii* induces many changes in host cell gene transcription, including those genes involved in energy metabolism, immune responses and signalling (Blader et al., 2001). Infection with *T. gondii* inhibits host cell signalling pathways involved in protective immunity, for example by blocking the transcription factors signal transducer and activator of transcription 1 (STAT1) (Luder et al., 2001) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

(Butcher et al., 2001). Infection with *T. gondii* also upregulates anti-inflammatory pathways including those involving the suppressor of cytokine signalling protein (SOCS) 1 (Stutz et al., 2011; Zimmerman et al., 2006), SOCS3 (Whitmarsh et al., 2011) and STAT3 (Butcher et al., 2005, 2011), potentially compromising host mechanisms of parasite control. Although IFN-γ promotes antimicrobial activities in many cell types, in macrophages it is not typically sufficient for the control of *T. gondii* as a second signal is required to fully activate killing. The best-characterized second signals that promote control of *T. gondii* in vitro are provided by TNF-α or signals through CD40 (Andrade et al., 2006; Reichmann et al., 2000), both of which utilize the NF-κB signalling pathway. Notably, the activation of NFκB by type II strains is associated with higher levels of IL-12 production (Robben et al., 2004), an effect that contributes to early control of infection.

2.4 Toxoplasmosis: sources of infection

The majority of horizontal transmission in human is caused by the ingestion of tissue cysts in infected meat or by the ingestion of soil, water, or food contaminated with sporulated oocysts, or less frequently, with faeces of infected cats.

2.4.1 Infection through tissue cysts

In Europe, meat consumption was estimated to be responsible for 30 to 63% of cases of infection, while soil contact represented 6 to 17% of cases (Cook et al., 2000). Outbreaks due to the consumption of raw or undercooked meat have been described but they usually involve only a few patients (Agence Française de Sécurité Sanitaire des Aliments. 2005).

Tissue cysts remain infectious in refrigerated carcasses (1°C to 6 °C) or minced meat for up to 3 weeks. However, the deep-freezing of meat at -12 °C or lower for at least 3 days is usually efficacious to kill cysts, although it may depend on the thickness of the piece of meat (Dubey et al., 1988).

Tissue cysts are killed immediately when heated at 67°C. Cooking for a prolonged period of time may be necessary under household conditions to achieve the temperature necessary the kill the cysts in all parts of the meat.

Commercial procedures of curing with salt, sucrose, or low-temperature smoking may kill tissue cysts, but this is strictly dependent on the concentration of the salt solution and with the temperature of storage. Solutions containing 2% sodium chloride or 1.4% potassium or sodium lactate are

effective within 8 h of injection for the killing of *T. gondii* tissue cysts (Dubey et al., 1988). Gamma irradiation was also found to be effective against tissue cysts in meat (Linsday et al., 2006).

2.4.2 Infection through oocysts

A single cat may shed more than 100 million oocysts, which are non-sporulated. These need between 1 and 5 days to become infective for other hosts, which explains why the direct contact with cats is not thought to be a major risk for human infection. Unsporulated oocysts lose the ability to sporulate and to become infective, after freezing at -6°C for 7 days or after exposure to 37°C for 1 day. Once sporulated they remain viable in a moist environment for more than a year. Under laboratory conditions, they resist at 4 °C for up to 54 months. They survive freezing a -10°C for 106 days and heating at 35°C and 40°C for 32 days and 9 days, respectively. However, they are killed within 1 to 2 min by heating to 55-60°C (when cooking vegetables for example). They are also high impermeable and very resistant to disinfectants (Dumetre and Dardé, 2003).

All these characteristics makes oocysts very resistant and durable; the reason why we can find them in water and in soil and from there they can reach vegetables and fruits and finally infect humans and intermediate hosts. The water-borne route of infection is definitely the most common route of transmission of toxoplasmosis in human, as reported by several papers (Dubey 2004; Meireles et al., 2015; Vieira et al., 2015).

2.4.3 Infection through tachyzoites

Outside its host cell the tachyzoite is a fragile stage, and easily destroyed by digestive enzymes (10 min survival in pepsin-HCl). It is also very sensitive to environmental conditions and is usually dies rapidly outside the host. Therefore tachyzoites are not thought to be very important for horizontal transmission from an epidemiological point of view. However, there is evidence that infected goats may release tachyzoites in milk and in rare cases subjects who drink unpasteurized goat's milk may acquire toxoplasmosis (Tenter et al., 2000).

Tachyzoites are, however, responsible for congenital infection (mother-foetus) if the mother acquires primary toxoplasmosis during gestation. In developmented countries the frequency of congenital toxoplamosis was 3.3 per cent per 10,000 live births in France (Villena et al., 2007), 1 per 3,000 live births in Brazil (Villena et al., 2007) and 1 per 10,000 in USA (pilot study in Massachusetts) (Guerina et al., 1994).Tachyzoites may also be responsible for toxoplasmosis if blood or bone marrow, deriving from infected donors, are injected during transfusion or transplant.

Most cases of parental transmission are nevertheless attributed to inadvertent puncture with contamited needles or scratching while manipulating tachyzoites from the RH strain (Gangneux and Darde', 2012).

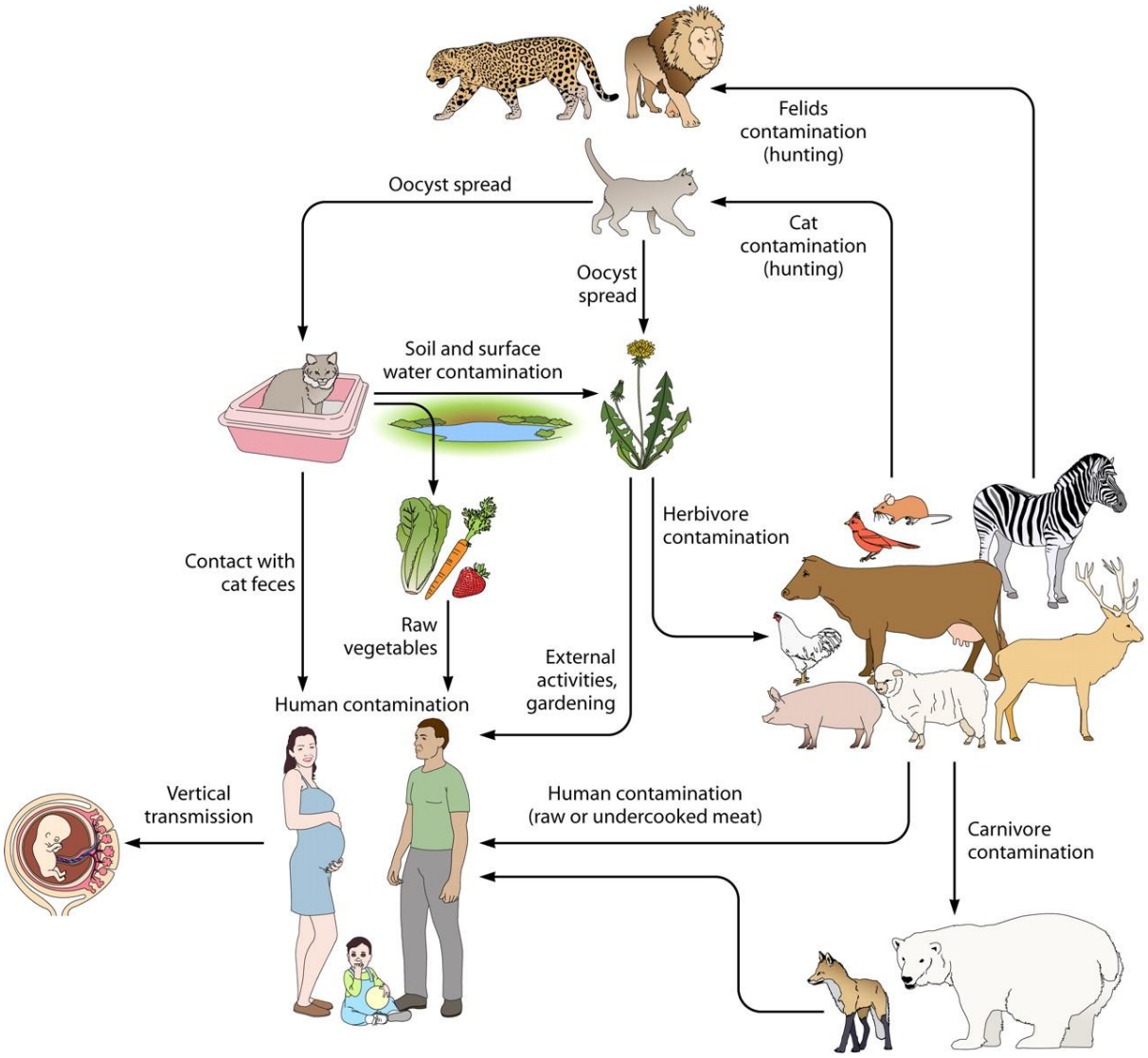


Figure n. 3*: *Sources of T. gondii infection in humans. The various sources of food-borne and environmental contamination of humans are represented (taken from: Epidemiology of and Diagnostic Strategies for Toxoplasmosi. Florence Robert-Gangneux and Marie-Laure Dardé, 2012)

2.5 Toxoplasmosis in animals and humans

2.5.1 Infection in domestic animals

Toxoplasma gondii is capable of causing infection and severe disease in animals other than humans.

Natural infection in cats can occur and the severity of the symptoms is correlated with the age, the stage of the parasite and the route of inoculation (in the case of experimental infections). Pneumonitis is the most common finding and it can be rapidly fatal. Infected cats can also show high fever, which is unresponsive to penicillin and streptomycin. Even if toxoplamosis is often included in the differential diagnosis of neurologic disease, cats rarely have obvious neurologic signs. Other organs affected by toxoplasmosis include the eyes (multifocal iridocyclochoroiditis) and pancreas (pancreatitis).

In livestock animals, toxoplasmosis causes marked losses in sheep and goats, and may cause embryonic death and resorption, foetal death and mummification, abortion, stillbirth, and neonatal death in these animals (Innes et al., 2009).

Outbreaks of toxoplasmosis in pigs have been reported from several countries, especially Japan (Dubey, 1986), China (Li et al., 2010), Korea (Kim et al., 2009). Mortality is more common in young pigs than in adult pigs. Pneumonia, myocarditis, encephalitis, and placental necrosis have been reported to occur in infected pigs. Sporadic and widespread outbreaks of toxoplasmosis occur in rabbits, mink, birds, and other domesticated and wild animals. Animals that survive infection harbour tissue cysts, and can therefore transmit *T. gondii* infection to human consumers.

Chickens do not usually present clinical signs. Confirmed severe toxoplasmosis was first reported in a flock of 40 hens from Norway (Erichsen and Harboe, 1953). In some rare cases it was recorded a neurologic symptomatology; Goodwin (1994), reported a peripheral neuritis in three chickens in USA diagnosed with immunohistochemistry (IHC), but also the Marek's disease could not be run out. One case of systemic toxoplasmosis (apathy, dyspnea and diarrhea and dead 18 hours after the onset of illness) in a quail from Brazil was recorded recently (Casagrande et al., 2015) with the presence of tachyzoites highlighted in a lot of organs through IHC and histology.

2.5.2 Infection in wild animals

Clinical and subclinical toxoplasmosis has been reported in many host species. Wild animals can be a source of *T. gondii* infection in humans, cats, and other carnivores. A recent Italian study conducted on red foxes, roe deers, wild boars, alpine chamois, red deers in Piemonte region (Northern Italy) revealed a higher prevalence of infection in carnivores and omnivores than in herbivores (no positive ones in this study). This reflects the higher probability of a carnivore or omnivore to consume tissues infected with *T. gondii* than the probability of an herbivore to ingest *T.*

gondii oocysts from the environment (Ferroglio et al., 2015). The idea that tissue cysts are important as oocysts in the transmission of *T. gondii* and that wild animals (especially wild boar and roe deer) could be an important source of foodborne toxoplasmosis in humans was the same expressed by Smith and colleagues in USA (Smith and Frenkel, 1995).

Sea mammals have also been found positive for *T. gondii*. *Toxoplasma gondii* is considered a significant cause of encephalitis in sea otters (Cole at al., 2000). In a study conducted in 2002 from Cole and colleagues (2000) in 67 sea otters found dead or killed because of their inability to survive due to advanced disease or trauma, 15 (22.4%) were positive by bioassay. Miller et al. (2002) has documented a high prevalence (42–62%) of infection of southern sea otters with *T. gondii* in areas along the Pacific coast of USA resulting from surface water run-off contaminated with *T. gondii* oocysts. Several cases of *T. gondii* infection were confirmed in DMV-affected striped dolphins from the 1990–'92 morbilliviral epidemic (Domingo et al., 1992), with lethal cases of *T. gondii* meningo-encephalitis being subsequently reported in striped dolphins stranded along the Ligurian Sea coast of Italy between 2007 and 2008 (Di Guardo et al., 2010).

2.5.3 Toxoplamosis in humans

It is generally assumed that approximately 25-30% of the world's population is infected by *Toxoplasma gondii* (Elbez-Rubinstein et al., 2009). The prevalence varies between countries and low seroprevalences (10-30%) are recorded in North-America, South East Asia, Northern Europe, Sahelian countries of Africa. Moderate prevalences (30- to 50%) have been found in countries of Central and Southern Europe, and high prevalences in Latin America and in tropical African countries (Gangneux and Darde', 2012).

Climate factors such as temperature and humidity influence the resistance of oocysts and as a consequence the level of infection. High prevelences are classically recorded in tropical countries (warm and humid) and lower ones in arid or colder countries. Other factors such as economic, social and cultural habits can explain the wide variations in human seroprevalence (dietary habits, methods of cooking meat, hand washing, water quality, risk knowledge associated with food consumption).

In a recent assessment of foodborne illnesses in the United States, toxoplasmosis was identified as the second leading cause of foodborne illness–related deaths and fourth leading cause of foodborne

illness–related hospitalizations (an estimated 327 deaths, and 4428 hospitalizations annually) (Scallan et al., 2011).

2.5.3a. *Toxoplasma gondii* and human health: pathogenesis

Humans can become infected with *Toxoplasma gondii* through the ingestion of sporulated oocysts (drinking contaminated water or eating contaminated vegetables for example), or through the ingestion of raw or undercooked meat containing tissue cysts.

Primary acquired infection in immunocompetent subjects is asymptomatic in more than 80% of cases in Europe and North-America. In the remaining cases patients show fever, cervical lymphadenopathy sometimes associated with myalgia or other no-specific clinical signs, very similar to a common flu (Montoya and Liesenfeld, 2004). Nevertheless, toxoplasmosis is life threatening if it occurs in immunocompromised patients. Patients with HIV/AIDS, transplants, those with cancer or with other diseases affecting the immune system are at risk for severe toxoplasmosis. A reactivation of a chronic infection may occur and this risk is related to the duration and degree of immunosuppression. HIV patients often develop toxoplasmic encephalitis that leads to lethargy, ataxia, loss of memory, dementia, but the heart (myocarditis), lungs, eyes and pancreas may also be involved and parasite can be isolated from these tissues. Pulmonary or disseminated toxoplasmosis is seen mostly in transplant patients (Pereira-Chioccola,Vidal and Su, 2009; Gangneux and Dardé, 2012; Barratt et al., 2010)

Congenital toxoplasmosis may occur in pregnant women and the severity of the consequences depends on the stage of pregnancy when maternal infection occurs. When a primary infection is acquired by a pregnant woman, tachyzoites can colonize plancental tissue during the dissemination process and from there gain the access to the fetal compartment. Acute toxoplasmosis in developing foetuses may result in blindness, deformation, mental retardation or even death (Montoya and Liesenfeld, 2004). The frequency of vertical transmission increases with the gestational age at maternal infection. If the infection occurs in the first trimester of pregnancy the risk of abortion or severe abnormalities in the brain and eyes is high.

During the second trimester, fetal infection can be of variable severity. Ecographical ultrasound reveals areas of hepatosplenomegaly, cerebral calcification or areas of hyperechogenic mesentery. At birth the baby may show epilepsy, anemia, thrombocytopenia-induced petechiae, rash, hepatic

disorders, pneumonitis, or retinochoroiditis (Remington et al., 2001). This last clinical manifestation is a common feature that can be observed whatever the time of maternal infection.

Although the vast majority of congenital infections result from primary acquired infection during pregnancy, parasite transmission can occur in rare instances in immunocompetent, previously immunized women who are re-infected with *Toxoplasma* during gestation. One such clinical case has been described due to an atypical strain of *T. gondii* (Elbez-Rubinstein et al., 2009).

Data from all over Europe, USA and South America (mainly from Brazil), identify *Toxoplasma gondii* as a risk for human health. It is known that the majority of the population at risk of complications derived from toxoplasmosis, are mainly people with concurrent health issues (organ transplant, patients with cancer, HIV, ecc..) and pregnant women who are at risk of abortion or grave congenital defects (Cenci-goga et al., 2011; Gangneux and Dardé, 2012; Campos et al., 2014). However, a high incidence and severity of ocular toxoplasmosis (chorioretinitis) in immunocompetent subjects and in congenitally infectioned babies, caused by atypical strains, have been reported from Africa, Brazil and Colombia (Gilbert et al., 2008).

Moreover, chronic toxoplasmosis has been shown to cause behavioural changes in mice, making them attracted rather than repelled by the scent of cat urine, due to inhibition of neuronal function and alteration of neurotransmitter levels (Gatkowska et al. 2012; Haroon et al. 2012). In humans, the condition has also been shown to correlate with schizophrenia (Yolken et al. 2001; Torrey et al. 2007) and bipolar disorder (Pearce et al. 2012; Hamdani et al. 2013).

This has highlighted the need to have more information about the diffusion of the coccidian and on the type of genotypes most diffused. Studying in depth the presence of atypical strains and their pathogenic potential is increasingly more important and necessary.

2.6 Foodborne toxoplasmosis: the main food products at risk

As said before, the consumption of contaminated food is the major route of infection with *T. gondii* for humans. The potential risk associated with the consumption of products derived from infected animals including raw or undercooked meat products, milk, cheese made with unpasteurized milk, has been shown in numerous studies. Meat consumption is certainly one of the main transmission routes for the infection with *Toxoplasma gondii*. Currently, there are no identification systems for

Toxoplasma gondii infection at slaughter, in any animal species, neither in USA nor in Europe. Even if the recorded data about infection in retail meat are limited, one study showed a prevalence of only 0.33% in over 2000 samples analyzed (Dubey et al., 2005). This may lead to think that the risk for the consumers related to meat consumption is very low. On the contrary, the consumption of sausages, typical meat products such as raw ham, or undercooked meat which has not been frozen before eating, is strongly associatedwith food-borne infection in humans (Vitale et al., 2014; Bonametti et al., 1996; Choi et al., 1997).

Pork, poultry, beef and sheep (in lower percentage) are the main meats consumed in Italy, Europe and the USA (www.assocarni.it/archivio3_comunicati-ed-eventi_0_310_173.html;Daniel et al., 2011). All these species have been identified as infected with *Toxoplasma gondii* and parasite DNA or antibodies against a specific antigen have been recorded all over the world. The presence of DNA or antibodies against *T. gondii* indicate a previous contact with the parasite and, in the former case, certainly the presence of tissue cysts.

The importance of *Toxoplasma* as a risk for human health was recently highlighted by European Food Safety Authority's (EFSA) opinion on modernisation of meat inspection, where *Toxoplasma gondii* was identified as a relevant hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer. (Call for proposals-GP/EFSA/BIOHAZ/2013/01: Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat)

Toxoplasma was reported by the European Member States (MSs) from pigs, sheep, goats, hunted wild boar and hunted deer, in 2011 and 2012. In addition, positive findings were detected in cats (the natural hosts), cattle and dogs as well as several other animal species, indicating the wide distribution of the parasite among different animal and wildlife species (EFSA, 2014).

Species	Origin	N. of bioassayed and tissues	techniques used	Positive samples (percentage)	References
PIGS	Finishing pigs, 10 different farms. Central Italy	960 sera	IFAT (Indirect Fluorescent Antibody Test) (pos≥1:16)	16.4% (at the heard the percentage ranged from 8.3% to 25%)	Veronesi et al., 2011
PIGS	Finishing pigs, slaughterhouse. Brazil	190 sera	IFAT (pos≥64)	19.5%	Feitosa et al., 2014
WILD BOARS	hunted during three seasons (2009-2011). Central Italy	400 sera	IFAT (pos≥1:40)	14 %	Ranucci et al., 2013
SHEEP	Ewes, Sardinia. Italy	422 Sera	IFAT (IgM pos ≥1:40, IgG pos ≥ 1:200)	About 30%	Zedda et al., 2010
		DNA extracted from feed	PCR (ITS1 region)	35%	
SHEEP	Serum submitted to APHA(animal plant health agency) (2005-2012). UK	4354 sera	LAT (latex agglutination test) pos ≥1:64	54.2 %	Hutchinson and Smith, 2015
GOATS	Lactating goats, 2012. Central Italy	127 sera	MAT(modified agglutination test) (cut-off 1:20)	60.6%	Mancianti et al., 2013
		DNA from blood	n-PCR	13% blood positive.	
		DNA from milk	n-PCR	All goats serologically positive excreted *T. gondii* in the milk (100%)	
GOATS	Hearts of goats from grocery stores. USA	234 bioassayed hearts, meat juice or blood clot	MAT (cut-off 1:20)	53.4%	Dubey et al., 2011
		112 hearts	BIOASSAY	25.9% of the bioassayed hearts	
CHICKENS	Free-range chickens. Southern-Italy	80 sera	MAT (cut-off 1:25)	(13.75%)	Dubey et al. 2008
		11 brains and hearts from positive animals	BIOASSAY	(27.3%)	
CHICKENS	Free-range chickens, slaughterhouse. Brazil	510 sera	IHAT (indirect haemagglutination test) (pos ≥1:16) and MAT (pos≥1:25)	40.4% (IHAT) and 38.8% (MAT)	Beltrame et al., 2015
CATTLE	Cows, 2013-	4487 sera	IHAT (pos≥1:64)	10.5% at	Sun et al.,

	2014. China	(blood collected from the tail vessel)		animal level and 27.% at herd level	2015
DONKEYS	Healthy lactating donkeys. Italy	Blood and milk from 44 lacting jennies	IFAT (pos ≥1:20)	25%	Mancianti et al., 2014
			PCR on blood and on milk from positive animals	13.6% (blood) and 6.8% (milk)	
DONKEYS	Donkeys. Brazil	88 sera	IFI (indirect immunofloresence) (pos 1≥64)	43.2%	De Oliveira et al., 2013

Table n. 2*: Toxoplasmosis infection in different animal species recorded in Italy, Europe, USA, China, Brazil using different techniques.*

The growing consumer demand for biological, free-range products in order to guarantee animal welfare and to have healthy food derived from them is another point to consider. Europe, together with North-America, is the main economic market for biological products, with an annual growth rate of 10-15% (IFOAM, 2007). Organic program standards (Italian regulation DM n. 18354/09, European Regulation CE 834/2007 and American legislation, USDA) require that all organically-raised animals must have access to the outdoors, including access to pasture for ruminants. Access to grass, soil, feed, or water contaminated with cat faeces, or to rodents and wildlife infected with *T. gondii*, during outdoor pasturage substantially increases the risk of exposure of pigs, sheep, cows and chickens to the parasite. Kijlstra and colleagues (2004) found that none of the 621 conventionally raised pigs studied were seropositive for *T. gondii*, while 38 out of 1295 (2.9%) pigs raised in "animal friendly" management systems were seropositive for *T. gondii*, with a farm positive rate of 39.0%.

All that have access to the outdoors, including livestock and wild animals, have a potentially higher risk of coming into contact with the coccidian. This has already been demonstrated in several studies in the USA (Dubey, 2010b, Dubey et al., 2012).

Free-range pigs raised in two different farms in the USA were selected to study the infection rate with *Toxoplasma gondii*. Out of 33 hearts analyzed serologically (MAT and ELISA test), 31 were positive and, after the bioassay in mice, *T. gondii* was isolated from 17 pigs (included one serologically negative animal). The high prevalence recorded in this study highlighted the potential health risk related to consumption of meat coming from these kind of farms (Dubey et al., 2012).
Free-range chickens are a good indicator of soil contamination with oocysts because they feed from the ground and they rarely become ill from *Toxoplasma gondii* (Ruiz and Frenkel, 1980; Dubey,

2010b). Infected chickens are also an important source of infection for cats that in turn shed oocysts after eating tissues of this intermediate host. The ingestion of undercooked poultry may be a source of infection for humans. Freezing and cooking by conventional methods will inactivate tissue stages in meat. In one study of retail meats, antibodies to *T. gondii* were found in 27/2094 (1.3%) of chickens from retail meat case, but viable parasite stages were not recovered from any samples by bioassay (Dubey et al.,2002b). While meat is considered to be a major source of human exposure to *T. gondii* (FAO/WHO, 2014), little risk is attributed to chicken because it is typically well cooked (Kijlstra and Jongert, 2008). Pork, on the other hand, is considered a major source of human exposure (Dubey and Jones, 2008a). Results of a recent study indicate that, while the density of *T. gondii* in poultry muscle is low, the ingestion of undercooked poultry muscle remains a potential source of infection for humans. The results showed that hearts of serologically positive chickens were used to infect cats and they all shed oocysts. Isolation of viable *T. gondii* from these free-range chickens is definitive evidence of the public health importance of the parasite (Dubey et al., 2015).

2.7 Techniques commonly used for the isolation and identification of T. gondii from different samples

Identification of *Toxoplasma gondii* infection may be done by different techniques, depending on what one is evaluating.

The diagnosis of toxoplasmosis may be confirmed by:

•Observation of parasites in patient specimens, such as broncho-alveolar lavage material from immunocompromised patients, or lymph node biopsy.

•Isolation of parasites from blood or other body fluids, by intraperitoneal inoculation into mice or tissue culture. The mice should be tested for the presence of *Toxoplasma* organisms in the peritoneal fluid 6 to 10 days post inoculation; if no organisms are found, serology can be performed on the animals 4 to 6 weeks post inoculation.

•Detection of parasite genetic material by PCR, especially in detecting congenital infections in utero.

•Serologic testing is the routine method of diagnosis. (CDC, www.cdc.gov/parasites/toxoplasmosis).

2.7.1 Isolation of *T. gondii* oocysts from cat faeces

The isolation of oocyst is done by collecting faeces of infected cats from day 3 to day 21 after the administration of infectious material. The method is based on flotation and foresees several steps, using sucrose and other solutions to create different gradient.

Faeces are mixed with a solution of sucrose and centrifuged. A drop of faecal float from the top of the meniscus is examined microscopically for *T. gondii* oocysts between a coverslip and a slide. The subsequent passages are described in detail in material and methods chapter.

2.7.2 Bioassay

Bioassay is the gold standard for isolation and identification of *Toxoplasma gondii*. The cat is the best choice but mice are also used with excellent results. Cats are infected through ingestion of suspected tissue. The faeces are collected from 3 to 21 days after feeding tissue cysts and the presence of oocysts of *T. gondii* was checked using the flotation method.

For mice, infection is through intraperitoneal inoculation a solution of suspected tissue following digestion, 1.5 ml maximum because tachyzoites grow in the peritoneal cavity. The inoculation of a virulent strain will induce death after approximately 2 days. Oral inoculation may be hazardous because enteritis and mesenteric lymphadenitis may occur, causing death. The presence of brain cysts is checked two months later squashing a piece of mouse brain between a slide and a coverslip. It is also possible to test body fluid, such as heparinized blood or cerebrospinal fluid, but in this case the samples are centrifuged, the sediment resuspended in saline and injected into mice. Heparinized blood may be inoculated directly (Dubey, 2010a).

2.7.3 In vitro cultivation

Toxoplasma gondii is not able to grow in cell-free media. The parasite can be cultivated in vitro on a cell monolayer to test the efficacy of new molecules or to allow the parasite to proliferate for use in infection of mice. Almost all cell types (e.g. Vero cells, HFF, Macrofages, Lymphocytes, etc..) and different culture medium with or without the addition of serum (Diab et al., 2008) may be used for tachyzioite maintenance. The yield of tachyzoites will vary with the cell line and the strain of *T. gondii*. Virulent strains, as RH (Type I), may destroy the entire monolayer within a few days, while avirulent or intermediate virulent strains grow slowly, causing minimal cell damage.

Cell monolayers can be be infected with tachyzoites, which penetrate into the cells in 2-4 hours, or with bradyzoites after acid peptic digestion of tissue cysts. The best results however are obtained with the cultivation of tachyzoites.

2.7.4 Serology in humans and animals

Serology reveals the immunologic response induced in the host.

The detection of *Toxoplasma*-specific antibodies is the primary diagnostic method to determine infection. Antibody detection tests are performed by a large number of laboratories with commercially available kits.

The IFA (indirect fluorescence antibody) and EIA (enzyme immunoassay) tests for IgG and IgM antibodies are the tests most commonly used today in human patients. Individuals should be initially tested for the presence of *Toxoplasma*-specific IgG antibodies to determine their immune status. A positive IgG titer indicates previous infection with the organism at some time. If more precise knowledge of the time of infection is necessary, then an IgG positive person should have an IgM test performed by a procedure with minimal nonspecific reactions, such as IgM-capture EIA. A negative IgM test essentially excludes recent infection, but a positive IgM test is difficult to interpret because *Toxoplasma*-specific IgM antibodies may be detected by EIA for as long as 18 months after acute acquired infection.

A major problem with *Toxoplasma*-specific IgM testing is lack of specificity. Two situations occur frequently: i) persons with a positive IgM but negative IgG, and ii) individuals with positive IgG and IgM results. In the first situation, a positive IgM result with a negative IgG test in the same specimen should be viewed with great suspicion; the patient's blood should be redrawn two weeks after the first and tested together with the first specimen. If the first specimen was drawn very early after infection, the patient should have highly positive IgG and IgM antibodies in the second sample. If the IgG is negative and the IgM is positive in both specimens, the IgM result should be considered to be a false positive and the patient should be considered to be not infected. In the second situation, a second specimen should be drawn and both specimens submitted together to a reference lab which employs a different IgM testing system for confirmation.

If the patient is pregnant, and IgG/IgM test resulted positive, an IgG avidity test should be performed. A high avidity result in the first 12 to 16 weeks of pregnancy (time dependent upon the commercial test kit) essentially rules out an infection acquired during gestation. A low IgG avidity

result should not be interpreted as indicating recent infection, because some individuals have persistent low IgG avidity for many months after infection. If the patient has clinicalsigns compatible with toxoplasmosis but the IgG titer is low, a follow-up titer two to three weeks later should show an increase in antibody titer if the illness is due to acute toxoplasmosis, assuming the host is not severely immunocompromised. Newborn infants suspected of congenital toxoplasmosis should be tested by both an IgM- and an IgA-capture EIA. Detection of *Toxoplasma*-specific IgA antibodies is more sensitive than IgM detection in congenitally infected babies. None of the current commercial assays offered in the United States have been cleared by the Food and Drug Administration for in vitro diagnostic use for infants; consequently, all specimens from neonates suspected of having congenital toxoplasmosis should be sent to the *Toxoplasma* Serology Laboratory, Palo Alto, CA which has the most experience with infant testing (http://www.cdc.gov/parasite/toxoplasmosis).

Serological determination of active central nervous system toxoplasmosis in immunocompromised patients is not possible at this time. *Toxoplasma*-specific IgG antibody levels in AIDS patients often are low to moderate, but occasionally no specific IgG antibodies can be detected. Tests for IgM antibodies are generally negative (http:// /www.cdc.gov/parasite/toxoplasmosis).

Several other commercial kits for *Toxoplasma* serologic testing are available, for example indirect haemoagglutination (IHA), indirect fluorescence antibody (IFA), modified agglutination test (MAT), latex agglutination (LA), enzyme-linked immunoabsorbent assay (ELISA) are commonly used, also in animals. However, the sensitivity and specificity of these kits may vary widely from one commercial brand to another.

IHA: soluble antigen of tachyzoites is coated on tanned red blood cells that are agglutinated by immune serum. With IHA titers remain elevated for long periods, so acute infections are likely to be missed by this test. It is also frequently negative in congenital infections. In animals titers lower than 1:128 may be nonspecific.

IFAT: in the conventional test, whole, killed tachyzoites are incubated with serum and antibody detection is enhanced by adding fluorescent-labeled antispecies IgG and viewing with a fluorescent microscope. Its disadvantages are the need of a fluorescent microscope and the cross-reaction with rheumatois factor and antinuclear antibodies.

MAT: no particular equipment is required for this test. The antigen is formalin-fixed parasites prepared in a mixture with 2-mercaptoethanol, dilution buffer and blue evans and the reaction takes place in U-bottom 96-well microtiter plates. Sera are treated with 2-mercaptoethanol to remove nonspecific IgM or IgM-like substances It is used extensively for the diagnosis of toxoplasmosis in animals.

ELISA: soluble antigen is absorbed on a plastic surface and the antigen-antibody complex is highlighted by the addition of a secondary antibody linked with an enzyme and the addition of a specific substrate allow the identification of a positive reaction by colour develop quantification. (Dubey, 2010a).

2.7.5 Molecular identification

T. gondii is frequently identified by PCR (both classic and quantitative one). Tested material can be of various nature. In cases of suspected human toxoplasmosis, PCR can be done on serum samples, mainly in pregnant woman or in immunocompromised subjects with suspicion of ocular toxoplasmosis, for example.

Regarding animals, the best organs to choose for the identification of the parasite are certainly the heart and the brain, but also all other muscle tissue can be used with a reduced sensitivity rate. DNA extraction is commonly done using a commercial kit. From a public health point of view, it is necessary to distinguish *T. gondii* oocysts from the oocysts of other related coccidium (as *Hammondia hammondii*, also present in cat faeces). For this, oocysts must be isolated, washed and broken with several steps of freezing and thawing, treatment with saline buffers, proteinase K and cety-trymethil ammonium bromide.

There are numerous PCR protocols validated for the identification of *T. gondii.* One of the genetic markers commonly used is the B1 gene that is a 35-fold repetitive sequence in the genome of the parasite. Burg and colleagues described the nested-PCR protocol in 1989.

The 300-fold repetitive element of 529 bp weight is another common genetic marker used. It is very sensitive, 10 to 100 times more sensitive than B1 (Su et al., 2010).

The ITS1 region is an internal transcribe spacer sequence with a sensibility rate similar to the B1 (Su et al., 2010; Yan et al., 2010).

Recently, a set of primers for PCR of *T. gondii* 18S rDNA, was found to be more sensitive than the B1 gene and this marker is of particular interest, as it can distinguish several protozoan parasites which are closely related to *T. gondii* (Su et al., 2010).

2.7.6 Immunohistochemical staining

Formalin-fixed, paraffin embebbed tissues can be used for this technique. Although *T. gondii* antigen can be detected as long as 1 year after fixation in 10% formalin, fixation for a short period (24h) is recommended. Polyclonal rabbit anti-*T. gondii* antibodies are better than monoclonal antibodies. Although rabbits can be infected with tachyzoites, bradyzoites and oocysts, the latter are preferred because they can be cleaned of faecal matter and treated with 5.25% sodium hypoclorite solution to remove any host material. This technique may also be used to distinguish tachyzoites from bradyzoites (Dubey et al., 2010a).

2.7.7 New biomolecular techniques

Sequencing is certainly one of the best and most common methods used for the analysis of the genotypes of *T. gondii*. Next generation sequencing (NGS) allows to identify the three clonal types of *T. gondii* and eventually atypical ones. All NGS technologies are capable of producing massive numbers of sequence reads per run, ranging from one million to several billion, at the cost of reduced read length and in some examples individual base accuracy (Blake, 2015). The Illumina approach attaches single template DNA molecules to a solid surface prior to polymerase-based amplification and the inclusion of base-specific fluorescently labelled reversible terminator bases, images of which translate into sequence detection. SOLiD sequencing operates by competing octamer ligation to a primer annealed to the DNA template, where the optimal octamer match is indicated by a fluorescent label prior to cleavage and ligation of the next octamer. Ion Torrent sequencing follows a similar process to pyrosequencing but detects hydrogen ions released during nucleotide addition in place of fluorescence. Recently, third generation sequencing from single molecules has become available using Pacific Biosciences (PacBio) technology. PacBio offers long reads of 20,000 bases or more, albeit with an error rate greater than the NGS technologies. Several other methods have been described in this fast developing field and are at various stages of development (Metzker, 2010).

The most common marker amplified and used for the sequencing analysis are the ITS1 region (Gjerde and Josefsen, 2015), the SAG2 locus (Fazaeli et al., 2007), the GRA6 marker (Biradar et al., 2014) and the 18S rDNA also useful for phylogenetic studies (Ellis et al., 1995).

The presence of snips in the region amplified by these markers is essential for the subsequent analysis and for the evaluation of the genotypes most commonly diffused in the area taken into account.

The subpopulation structure of *T. gondii* differs according to geographic location, exhibiting a largely clonal structure in Europe and North America although isolates from South America are more diverse and genetically distinct (Howe and Sibley 1995; Ajzenberg et al. 2004; Dubey et al. 2008b). Many South American isolates exhibit low linkage disequilibrium, indicating that these parasites have undergone frequent sexual recombination unlike the European and North American isolates (Ajzenberg et al. 2004). The life cycle of *T. gondii* is typical of the heteroxenous coccidian with sexual reproduction that occurs only in the definitive host. The life cycle of *T. gondii* has two features that may support the persistence of a largely clonal population structure in much of the world. Firstly, sexual development and self-fertilization can take place only in the cat to yield infectious oocysts. Simultaneous infection with different *T. gondii* strains occurs relatively infrequently in cats; thus, the opportunities for genetic recombination events are limited (Dubey et al. 2004). Secondly, *T. gondii* oocysts can infect intermediate hosts directly via the oral route, which is unusual among the Apicomplexa as it facilitates transmission without a sexual stage (Cornelissen and Overdulve 1985).

The first cross reported used the PLK and CEP strains to establish a genetic linkage map for *T. gondii* (Sibley et al. 1992). The map was based upon the inheritance patterns of 64 Restriction Fragment Length Polymorphism (RFLP) DNA markers that defined 11 different chromosomes (linkage groups). Preliminary linkage assignments were provided for genetic loci associated with resistance to sinefungin and adenine arabinoside on chromosomes IX and V, respectively (Sibley et al. 1992). However, the resolution of the original map was low and limited by fragmented sequence information and no corresponding physical map (Khan et al. 2005). The first high-resolution genetic map for *T. gondii* was developed by Khan et al. (2005) to facilitate forward genetic analysis. A total of 14 linkage groups, representing the 14 chromosomes, were identified comprising a total genetic size of approximately 592 cm (Khan et al. 2005). Several unusual features of the *T. gondii* genome were revealed during the construction of the high resolution genetic map. Firstly, high frequencies

of closely adjacent apparent double crossover events were observed, possibly representing gene conversions (Khan et al. 2005). Such a high frequency of closely spaced double crossovers is important for mapping phenotypes by linkage analysis; however, the mechanisms by which these occurr has not been determined (i.e. true double crossovers versus gene conversions). Crossovers, but not non-reciprocal conversions, can be used to rigorously delimitate intervals spanning quantitative trait loci (QTLs) (Khan et al. 2005). The current density of markers is therefore likely to underestimate the number of conversions if single marker events do represent gene conversions. Increasing the density of markers on the genetic map will enhance our ability to map complex traits that differ between the three clonal lineages. Secondly, large regions of genetic homogeneity were found among archetypal clonal lineages, indicating that relatively few outbreeding events had occurred since their recent origin (Khan et al. 2005). Another unusual feature of the *T. gondii* genome is that strain-specific SNPs were distributed asymmetrically, with several chromosomes exhibiting surprisingly homogenous haplotypes. On some chromosomes strain-specific SNPs occurred within restricted regions. The underlying cause behind the differing patterns of SNPs is unclear, but they indicate that little recombination has occurred in large regions of the genome when compared with the ancestral *T. gondii* SNP pattern (Su et al. 2003). Future investigation of *T. gondii* should aim to resolve these SNP patterns, especially as a global investigation of the distribution of SNPs across the genome detected a similar pattern (Khan et al. 2005). Linkage analyses have proved effective in identifying loci associated with several drug resistances (Khan et al. 2005). Genetic mapping has also been used with *T. gondii* to identify QTLs for complex phenotypes such as virulence (Su et al. 2002). In mice, for example, type I *T. gondii* is frequently lethal, while types II and III are less virulent (Sibley and Boothroyd 1992; Su et al. 2002). Following a genetic cross between a highly virulent type I strain (GT-1) and a type III strain with a much lower virulence level (CTG), a panel of recombinant progeny were produced and analyzed, identifying several QTLs associated with acute virulence.

3. Aims of the thesis

As said previously, the importance of *Toxoplasma gondii* as a risk for human health was recently highlighted by EFSA's opinions on modernisation of meat inspection, where *Toxoplasma gondii* was identified as a relevant hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer. (Call for proposals -GP/EFSA/BIOHAZ/2013/01: Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat)

Toxoplasma was reported by the MSs from pigs, sheep, goats, hunted wild boar and hunted deer, in 2011 and 2012. In addition, positive findings were detected in cats (the natural hosts), cattle and dogs as well as several other animal species, indicating the wide distribution of the parasite among different animal and wildlife species (EFSA, 2014).

There is a growing demand by the consumer of biological products, coming from free-range livestock animals, in respect of animal-welfare, and the desire for best quality of derived products, all leading to an increase in the farmoing of free-range animals. The risk of *Toxoplasma gondii* infectionincreases when animals have access to environment.

The absence of data in Italy, together with need for indepth study of both the prevalence and genotypes of *Toxoplasma gondii* present in Italy were the main reasons for the development of this thesis work.

Several species that represent intermediate hosts of *T. gondii* and that have been identified as important sources for human infection, were evaluated in the present project. The aims of the study included:

1. to determine meat juice seroprevalence and genotype of *T. gondii* in organically raised pigs reared for the production of fresh and cured pork products;

2. to verify the viability of *T. gondii* in cured meat (Parma ham) following experimental infection of pigs;

3. to evaluate the seroprevalence and genotype of *T. gondii* in an indigenous sheep breed reared for the production of dairy products;

4. to carry out a preliminary study of infection risk for *T. gondii* in free-range chickens;

5. to evaluate the prevalence genotypes of *T. gondii* in muscle tissue isolated from wild animals.

4. Materials and methods

Introduction

Several species that represent intermediate hosts of *T. gondii* and that have been identified as important sources for human infection, were evaluated in the present project. The aims of the study included:

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2. to verify the viability of *T. gondii* in cured meat (Parma ham) following experimental infection of pigs;

3. to evaluate the seroprevalence and genotype of *T. gondii* in an indigenous sheep breed reared for the production of dairy products;

4. to carry out a preliminary study of infection risk for *T. gondii* in free-range chickens;

5. to evaluate the prevalence and genotypes of *T. gondii* in muscle tissue isolated from wild animals.

4.1 Materials and methods for the study of *Toxoplasma gondii* in pigs.

4.1.1 Organically raised pigs

The "Suino Nero" of Parma is an ancient breed, indigenous to the province of Parma. The breed has been reared in the area since the 1400s. The current number of animals has been estimated at approximately 1000 units. They are animals easily adapted to adverse weather conditions, very rustic and for these reasons suitable for grazing outdoors and in the mountains. They have an important muscular mass and for this reason, they are commonly raised organically and are used for the production of meat, of "Prosciutto Crudo di Parma" and other fresh and cured pork products. According to national regulations, organic pig farming normally foresees that farrowing and weaning take place outdoors and that fatteners are kept either outdoors or indoors with an outdoor run. Pigs are sent to slaughterhouse when they reach 180 kg body weight (approximately 12-14 months of age). They may be slaughtered only in special slaughterhouses registered and approved by the "Consorzio of Suino Nero Parma" and at different times than other pigs (www.suinoneroparma.it).

For the present study, twenty-one hearts of the "Suino Nero" breed of black pig were collected at slaughter between March and April 2014. Pigs came from three different organic farms situated in the Parma province. All three farms gave outdoor access to fatteners for 8 months a year and for the remaining four months they were kept indoors on straw bedding.

Figure n. 4 shows an outline of the experimental design.

4.1.1a Meat juice serology

A piece of myocardial tissue was cut from the apex of collected hearts. Tissue was frozen at -20°C for 18-24 h and defrosted to obtain the meat juice, used subsequently for the serological test.

The ELISA test was carried out following the manufacturer's instructions (ID SCREEN® TOXOPLASMOSIS INDIRECT MULTI-SPECIES, IDvet). The micro-well are coated with the specific p30 antigen of *Toxoplasma gondii*; the immunodominant surface antigen of invasive tachyzoites (Flori et al., 2006). Briefly, in each well, 50 µL of meat juice and 50 µL of dilution buffer were distributed and incubated at room temperature for 45 minutes. After a washing with a specific buffer, 100 µL of the conjugate (final concentration 1X) were added and left to react with the immunocomplex. Before the addition of the substrate, each well was washed three times as

described above. After an incubation of 15 minutes in the dark, the reaction was stopped with a solution of H_2SO_4. Optical densities (OD) were read at 450 nm with a spectrophotometer.

Samples were considered positive with a S/P % value (S/P% = OD sample-OD negative controls/OD positive controls-OD negative control × 100) greater than 50%. Samples were considered positive with a S/P percentage calculated from the optical densities measured at 450 greater than 50%. Samples with S/P% ≤ 40% were considered negative, between 40% and 50% were considered doubtful. Positive and negative controls were included in each plate (see below).

$$\%\frac{S}{P} = \left[\frac{ODsample - ODcn}{(ODcp - ODcn)}\right] x\ 100$$

OD: optical densities read at 450 nm

CN: medium value of negative controls' optical densities

CP: medium value of positive controls' optical densities

RESULT

S/P ≥50%	**POSITIVE**
S/P ≤40%	**NEGATIVE**
40%< S/P <50%	**IN DOUBT**

4.1.1b DNA extraction and *T. gondii* identification with Polymerase Chain Reaction

DNA was extracted from all collected hearts. Two-hundred µl of tissue suspended in PBS were used for the DNA extraction made using a commercial kit (Qiagen).

T. gondii infection was confirmed by a PCR targeting the 529 bp region, using the primers TOX4 and TOX5, as described by Homan et al. (2000). The target 529bp is a 200- to 300- fold repetitive, non-coding fragment in the genome of *Toxoplasma gondii*; and has been shown to be more sensitive than the 35-copy B1 gene, with a detection limit of ≥ 1/50 of a genome equivalent and it is able to discriminate *T. gondii* from that of other parasites (Kasper et al., 2009). Amplification was performed by 7 min incubation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final 10 min incubation at 72°C. The products were fractionated on a 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

4.1.1c Genotyping

All the samples identified as *Toxoplasma gondii* with PCR were genotyped, according to Su and colleagues (2010) with a PCR-RFLP reaction, amplifying ten genetic markers (SAG1, SAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358, PK1, APICO). The PCR-RFLP is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), digest PCR products and subsequently display distinct DNA banding patterns on agarose gels by Electrophoresis (Sibley and Boothroyd, 1992).

A first PCR amplifying the external locus of each genetic marker was carried out; the pre-amplified PCR products are used as templates for a second nested-pcr. PCR-amplified marker regions were digested with restriction endonucleases (Thermo, USA) and analyzed as described (Su et al., 2006). The sensitivity of this method has been estimated at ten or more *T. gondii* genome equivalents, based on previous studies (Su et al., 2010). Positive controls of DNA from *T. gondii* Types I, II and III (RH, GG, VEG) were kindly provided by E. Pozio from the Istituto Superiore di Sanità, Rome, Italy.

4.1.1d Gene sequencing

The samples showing good bands on the gels after the amplification of the genetic markers or after the genotyping were purified (QIAquick PCR purification kit protocol, Qiagen) and eluted in 50 µL of water. The DNA was quantified and outsourced to be sequenced.

The sequences were aligned using the program CLC main workbench (version 6.0.2) and each contig was blasted on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi to check the presence of one or more significant alignment among each single sequence and those present in the database.

If the aligned sequence resulted to be a gene of Toxoplasma gondii, the next step was to evaluate if it belonged to the clonal type I, II or III. Based on the number and position of snips present within the query sequence and comparing it with the reference ones (recorded with a specific gene bank number on the site http://www.ncbi.nlm.nih.gov/nuccore) it is possible to define if the isolate is a type I, II, or III for a specific genetic marker.

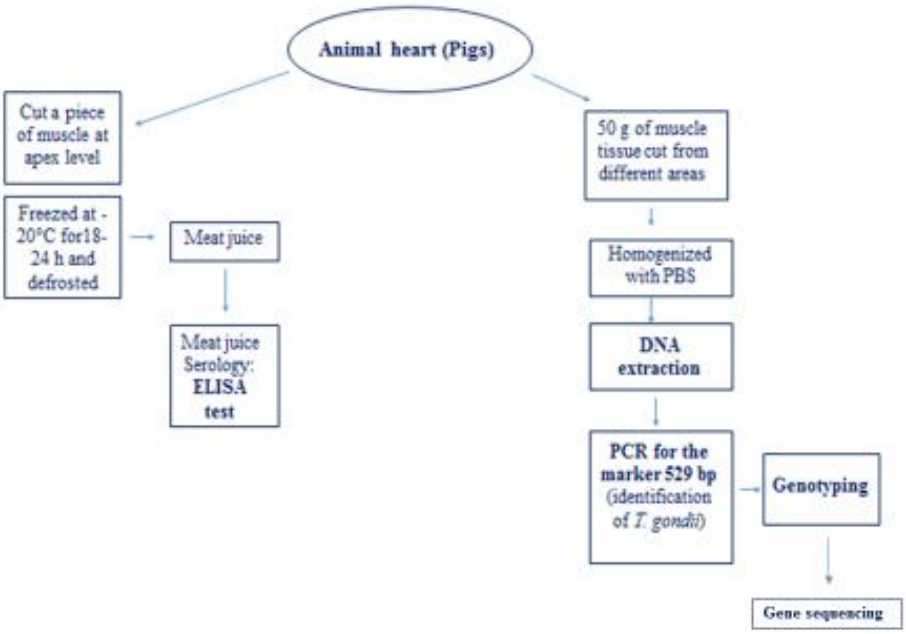


Figure 4: Outline of the experimental design for Suino Nero pigs.

4.1.2 Experimentally infected pigs

This part of the study was carried out in collaboration with the Istituto Zooprofilattico of Pavia and Brescia within the context of a larger project aimed at evaluating if the curing and aging process of Parma ham ("Prosciutto di Parma") is able to inactivate *Toxoplasma gondii* tissue cysts. A further aim of the project was to develop of an alternative technique for the isolation of viable parasites, thus avoiding the use of laboratory animals. In particular, in vitro isolation of the parasite was attempted, after the acid peptic digestion of the muscle tissue and seeding of the neutralized homogenate onto Vero cells.

The same aim was pursued also by the Istituto Zooprofilattico of Brescia, who infected 18 pigs with *T. gondii*. One of the objectives was to test the presence of viable parasite in different commercial cuts of pork meat, usually cooked by grill and consumed undercooked.

4.1.2a Isolation of *T. gondii* oocysts

Isolation of *T. gondii* oocysts was carried out at the University of Veterinary Medicine, Wien, thanks to the generous collaboration of Professor Anja Joachim, Head of the Institute of Parasitology at UVM.

Oocysts of a field strain of *T. gondii* isolated in Austria (Type II) were collected from faeces of cats fed with infected tissues (brains from experimentally infected mice). Cats usually shed oocysts from day 3 to day 10 after the ingestion of tissue cysts. The protocol foresees that all faeces for each cat are collected daily from day 3 to day 21 after feeding. For the screening, approximately 10 g of faeces, from each cat, for each day, are mixed thoroughly with 5 volumes of sucrose solution (specific gravity 1.15 or higher), poured into a 50-ml tube, and centrifuged at 2000 rpm for 10 min. A drop of fecal float from the top of the meniscus is examined microscopically for the presence of *T. gondii* oocysts between a coverslip and a glass slide. For the collection of a large number of oocysts, those fecal samples, containing a sufficient number of oocysts, are mixed with water, with few drops of detergent (household soap) and shaken in a shaker until faeces are completely broken. The mixture is then filtered through gauze and centrifuged in 250-ml bottles at low speed (1000 rpm, 15 min) without brake. The supernatant is discarded and the sediment mixed with water. The bottles are centrifuged two or three times, with the supernatant decanted, and replaced with clean water each time. After this, the final supernatant is decanted, and the sediment mixed with 5 volumes of sucrose solution of 1.15 specific gravity and centrifuged for 10 min at 2000 rpm in a 50-

ml tube, with a conical bottom. Most oocysts float to the top of the tube and can be aspirated. However, not all oocysts rise to the top. Therefore the entire supernatant (40-45 ml) can be poured into a 250-ml bottle, mixed thoroughly (but not vigorously), and centrifuged at 1500 rpm for 15 min. The sediment containing the oocysts should be washed one more time, mixed with 2% H_2SO_4 and aerated on a shaker for 7 days at room temperature (20-22°C).

Sporulated oocysts have a lighter density than the unsporulated oocysts. Oocysts can be further cleaned using a cesium chloride gradient. The procedure used by Dubey was as follows:

1. After floating oocysts in sucrose solution, add 10 ml of fecal solution (in 2% sulphuric acid) into a new 50-ml falcon tube. Neutralize the acid with 6 ml of 1N NaOH and bring volume to 50 ml with water. Vortex to mix well.
2. Centrifuge fecal sample for 20 min at 1200 *x g* at room temperature with no brakes.
3. Discard supernatant and resuspend pellet in Tris Buffer (TE, 50mM Tris, 10 mM EDTA, pH 7.2).
4. Prepare stock solution of CsCl (specific gravity=1.15) by mixing 21.75 g of CsCl with 103.25 ml of TE buffer and prepare gradient as shown below:

Solution	**Specificity gravity**	**TE**	**CsCl (1.150 specific gravity)**
A	1.050	30 ml	20 ml
B	1.110	20 ml	30 ml
C	1.125	10 ml	40 ml

5. Pour discontinuos gradient in Nalgene Oak Ridge. Gradient is formed by carefully underlaying each solution using syringe/needle with a two-way stopcock as illustrated in **Figure 5.**
6. Slowly add the following in the order listed below into the bottom of the Nalgene Oak Ridge tube.
   i. 10 ml TE/oocysts suspension sample
   ii. 8 ml of solution A
   iii. 8 ml of solution B
   iv. 8 ml of solution C

7. Centrifuge at 12000 *x g* 60 min at 4°C with no brakes. For best separation, use a high-speed swinging bucket rotor, but a fixed-angle rotor is OK.
8. Collect oocysts from the opaque-to-with interphase layer (between 1.05 and 1.11) by using a 10-ml pipet. Be mindful and minimize disturbing the gradient and debris when transferring interphase into new 50-ml falcon tube.
9. Wash oocysts by adding water up to 50 ml. Centrifuge at 2000 *x g* for 10 min, at room temperature with no brakes. Carefully aspirate supernatant. Repeat to wash two times.
10. Resuspend oocysts in 2% sulphuric acid for long-term storage.

Sporulated oocysts can be stored for one year with minimal loss of infectivity. Usually, 10-fold dilution are made in 2% H_2SO_4 at one time, stored at 4°C and used at the desired concentration for inoculations into animals (Dubey, 2010).

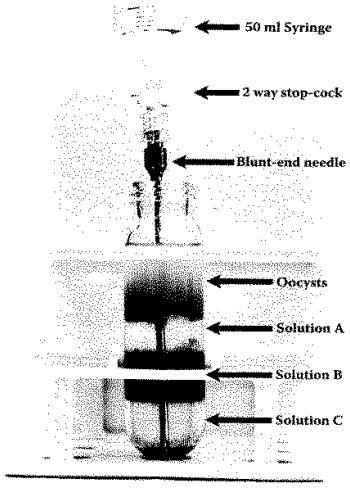


Figure n.5: *Pour discontinuos gradient in Nalgene Oak Ridge. (image taken from "Toxoplasmosis of animals and humans" J.P.Dubey).*

4.1.2b Experimental infection of pigs

In January 2014, a total of 30 Large White pigs, of 10 months of age and 160 kg weight approximately, were used in the study. All were seronegative for *T. gondii* and had no other parasitic infections (all the animals were subjected to stool test and visual ears examination). Pigs were infected with *Toxoplasma gondii* oocysts obtained in Vienna. Meatballs made of "panettone" (a sweet food of which pigs are greedy) were spiked with 1000 sporulated oocysts after fasting for 24 hours. All animals were housed indoors, in an independent stable, eight pigs in each stall, separated from the other infected groups with an empty pen in the middle.

During the period between infection and slaughter the health of infected animals was continuously monitored (body temperature, weight, blood exams to identify the presence of antibodies against *T. gondii*, etc.). Several animals showed clinical signs of infection such as fever (>39°C) accompanied by inappetence and poor general conditions.

All pigs were tested periodically by ELISA (Istituto Pavia) for anti-*T. gondii* antibodies and those that did not seroconvert were excluded from the experiment.

4.1.2c Slaughter and tissue collection

Pigs were slaughtered at three months post-infection. One thigh was sent to the curing house for the production of "Prosciutto di Parma" according to the regulations dictated by Consorzio of Parma Ham (see appendix 1)

The other thigh was sampled immediately. It was cut into three pieces, one third was used for molecular analysis (as described in point 4.1.1c), one third was digested and inoculated into mice, and one third was used for meat juice serology and in vitro seeding onto Vero cells to check for the presence of viable and infective parasites.

4.1.2d. Bioassay in mice

Bioassays were carried out at the Operative Unit of Istituto Zooprofilattico of Pavia, under the supervision of Doctor Marco Genchi, project head.

Swiss White mice were infected with eight millilitres (1 ml/day) of digested tissue obtained from 400 g of muscle, treated in different days, following the procedures described by Dubey (1998), for a total of 10-12 mice for each thigh. The bioassay was carried out on fresh thigh (three months after the infection) and on ham for each pig (12 months after the sacrifice of infected animals).

Briefly, from 50 g of muscle tissue, digested with an acid-pepsin solution (1h at 37°C), 1 ml was inoculated intra-peritoneally in each mouse. The same operation was repeated the following days. For each ham three different areas were analyzed: the rind, the most central part (very close to the bone) and an intermediate part located between the rind and the bone. In this way it could be possible to show if the presence of lower salt concentrations present near the bone for example, were able to kill the parasite.

All the mice were sacrificed six to eight weeks after inoculation and a cerebral squash was prepared to verify the presence of brain cysts of *T. gondii*. Briefly, an about 10-mm^3 portion of the cerebrum

of each mouse was placed on a microscope slide, squashed under a coverslip and examined for *T. gondii* tissue cysts by direct microscope.

4.1.2e. Meat juice serology

A sample of thigh muscle from experimentally infected pigs slaughtered at 3 months p.i. was used for meat juice serology, according to methods described in point **4.1.1b.**

4.1.2f. Acid-peptic digestion and seeding onto Vero cells

As said earlier, one of the aims of the project on Parma ham was to develop an alternative model for evaluating parasite viability that does not depend on bioassay. To this aim, samples from cured hams were digested and seeded onto Vero cells.

Approximately 50 grams of tissue were cut from three areas of the ham as done for the bioassay.

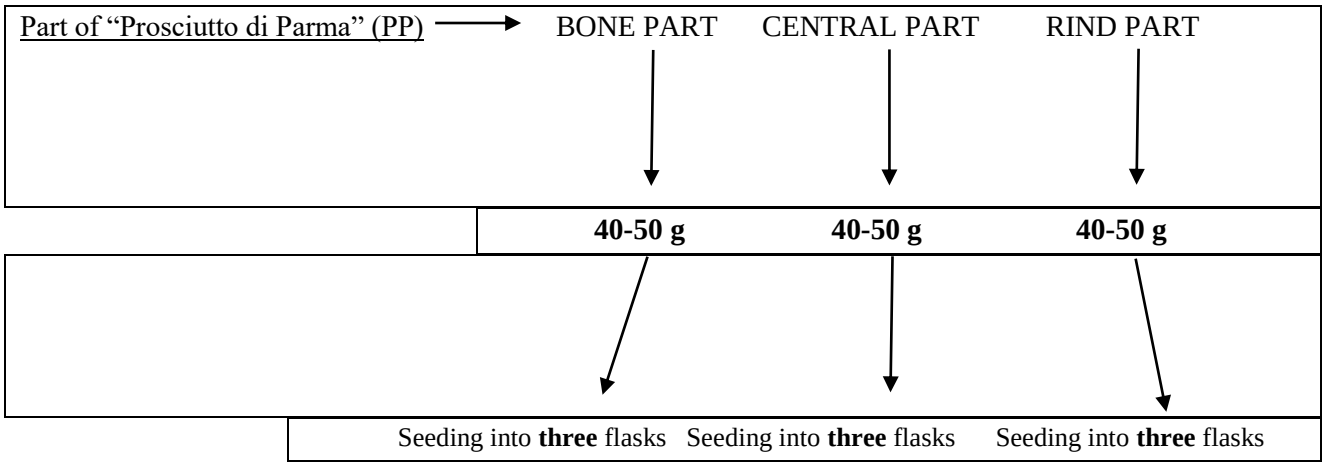
Due to the high concentration of salt in the final, cured product (4.4 to 6.0%), a technique was developed to remove all the NaCl present in each sample before acid-peptic digestion and seeding onto Vero cells. Indeed, an excess of NaCl is harmful to cells and may cause suffering in inculated mice.

Thus, 40-50 grams of each sample were weighed, cut and blended. Then, 1 L of bi-distilled water heated at 40°C was added and the solution maintained in a shaking water bath for half an hour at room temperature. Salted water was poured off and the same operation was repeated for another half an hour. After making up to volume with bidistelled water, the solution was filtered with a filter paper. Ten ml of filtrate were diluted with a same volume of bidistilled water and the titration of chlorides was done with $AgNO_3$.

Acid peptic digestion was then carried out. Briefly, blended tissue was added with 125 ml of PBS, kept warm at 37°C, and then 250 ml of a pepsin solution (pH 1.1-1.2) was added, as suggested by Dubey (1998). This treatment was able to break the cyst wall of *Toxoplasma gondii* leaving free the bradizoites. This solution was maintained a 37°C in a shaking water bath for one hour. Two-hundred-fifty ml of the homogenate were filtered with two gauzes and centrifuged at 1200 x *g* for 10 minutes. The supernatant was poured off and the pellet resuspended with 20 ml of sterile PBS (pH 7.4). The homogenate was centrifuged again as done before, the pellet resuspended with 20 ml of PBS and pH was neutralized with around 10 ml of Sodium Bicarbonate (1.2% w/v pH 8.3 8.4). Another centrifugation was done and now the pellet was resuspended with 10 ml of a saline solution containing penicillin, streptomycin, and Fungizone® (final concentration was 3%) (Euroclone).

One millilitre of digested tissue was put inside 75 cm^2 T-flasks seeded with VERO cells with a confluence around 80% cultivated in RPMI 1640 supplemented with 10% FBS.

For each of the three digested areas of the ham, three different flasks were seeded (see the scheme below). This operation was made because the odds of contamination are very high when working with material of animal origin; in this way there was a greater chance of success in maintaining cells culture for 21 days. After three hours incubation at 37°C, 5% CO_2, the homogenate was poured off, the monolayer was washed three times with sterile PBS and new fresh medium, with a lower percentage of FBS (2%), added. The medium was changed after 24 hours and then after 3-5 days for the following three weeks as suggested by Zintl and collegues (2009). All the flasks were controlled every day to verify any sign of infection.



4.1.2g Real-time PCR for determination of parasite growth in vitro

A Real-time PCR protocol was applied to verify if, during the 21 days of culture, the parasite was able to proliferate and in this way determine an increase in the amount of DNA. Extracted DNA was used as template for the qPCR, using the SsoAdvanced SYBR Green Supermix (Bio-Rad) in a CFX96 Real Time machine (Bio-Rad). Primers used amplify a small sequence in the 529 bp region. The forward and the reverse sequences were 5'-CACAGAAGGGACAGAAGT-3' and 5'-TCGCCTTCATCTACAGTC-3', respectively. The amplification protocol was characterized by a denaturation step (95°C for 5 minutes) and 45 repeated cycles (95°C – 15 sec; 58,5°C – 30 sec). Fluorescence signals were collected in every cycle and the presence of aspecific products was

avoided through melting curve analysis. To determine the copy number of the sequences a standard curve was made. Sample of DNA of *T. gondii* (extracted from tachyzoites) were amplified for the 529 bp marker (the same used in real-time PCR) and run on agarose gel. DNA was extracted directly from the gel using a commercial kit (JETQUICK Gel Extraction Spin Kit, Genomed) and quantified through spectrophotometry (Eppendorf). The number of copy per ng of DNA was then calculated. The molecular weight of each copy was determined using the sequence registered in the NCBI database (accession number: AF146527) and a web tool (http://www.bioinformatics.org/).The standard curve was fitted within 4 points, starting from a 1:50 dilution of the standard sample and subsequent 1:10 dilutions (detected range 1.6 ng – 1.6 pg).
In all experiments, parasite quantification was expressed as DNA vs. T0, where time zero is the DNA extracted from the digested product seeded onto Vero cells.

4.2 Materials and methods for the study of *Toxoplasma gondii* in sheep.

Animals

Twenty four sheep of the Cornigliese breed were analysed for the presence, prevalence and genotype of *T. gondii* infection. The Cornigliese sheep breed is indigenous to North Western Italy. It is an ancient sheep breed descended from Vissana, with some Merino and Bergamasca blood. It is an indigenous breed and was historically reared in an area near the village of Corniglio (hence the name), 1500 meters above the sea level (Emilia-Romagna) and the breed is known for its adaptation to the local marginal conditions. Morphologically, they appear white with dark spots on face and legs. The flocks are usually made up of a hundred animals each, sometimes mixed with goats. The breeding is traditionally conducted in transhumant flocks, moving in the highest areas of the Apennines in the summer and in pastures more accessible and repaired in winter www.agraria.org /ovini/cornigliese). It is known for the excellent dye uptake of its wool, for the quality of its milk for cheese production and for its meat (Ceccobelli et al., 2015). It is currently considered at risk of extinction and counts approximately 1600 head (Ceccobelli et al., 2015).
Hearts from 24 animals slaughtered from March 2014 to May 2015 were sampled and analysed by meat juice serology, PCR and in vitro isolation of *T. gondii*.

A seroprevalence study was also carried out on two sheep farms in the Parma province. Breeds included Cornigliese, Lacaune and Bergamasca. Sera from 587 adult sheep were tested for the presence of IgG antibodies against *T. gondii.* A total of 170 samples were from the first farm (A) and 417 samples from the second one (farm B). The consistence of the two farms was 170 sheep (5 male and 165 female) for the first one (A) and 747 sheep comprising 30 males and 717 females for farm B. Sheep were bred in extensive or semi-extensive farms. Animals were reared inside during winter and grazed outside in summer.

4.2.1 Serology

Serum samples from the two farms and meat juice obtained from sampled hearts were analyzed with the same kit and according to the same methods described for pigs.

4.2.2 PCR

DNA extracted from 50 g of hearts, cut and blended, was used for the identification of *Toxoplasma gondii* infection in sheep using the marker 529 bp, as done for pigs.

4.2.3 Genotyping

The same protocol described for genotyping *T. gondii* isolated from pigs was applied for sheep.

4.2.4 In vitro isolation

An attempt to isolate *T. gondii* in vitro was carried out on four heart samples. Following the same protocol applied for swine, but without pre-treatment for salt removal, heart muscle tissue was treated with acid-peptic digestion and then one milliliter of the digested material was seeded onto Vero cells.

Two weeks after the inoculum, the monolayer was scraped, centrifuged at 1800 rpm, for 10 minutes at 4°C, and the pellet resuspended with sterile PBS. A part (500 µL) was used for DNA extraction and the other one was seeded again onto a new flask. The same operation was repeated four weeks post-inoculum. The DNA was used to amplify the 529 bp region (for the identification of *T. gondii*). The sample resulted positive for the presence of *T. gondii* DNA were tested in RT-PCR to quantify the amount of DNA two and four weeks after inoculum, and in this way to determine if the parasite was alive and able to proliferate.

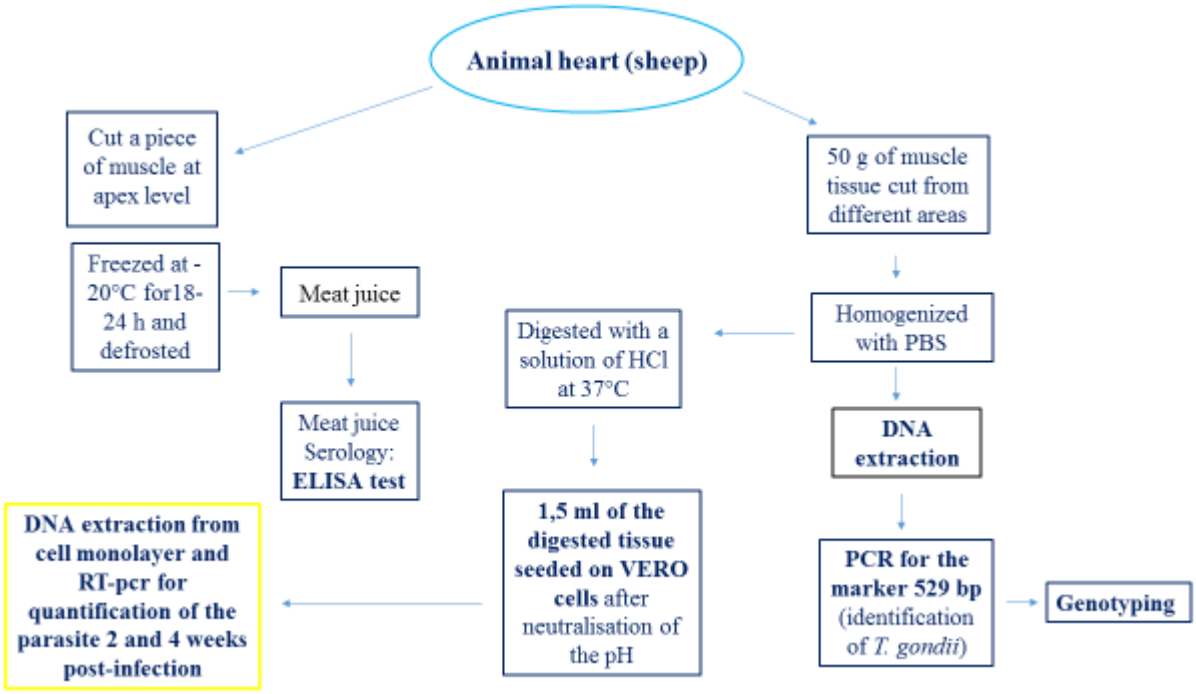


Figure n. 6: *Outline of the experimental design for Cornigliese sheep.*

4.3 Materials and methods for study of *Toxoplasma gondii* in chickens.

Animals

From April to July 2015 we analysed 77 free-range organic chickens two farms, one in northern Italy (Piacenza; 40 chickens) and one from central Italy (Teramo province; 37 chickens).

Those coming from Piacenza province were chickens of the "pollo passerino" and "pollo perniciato" breeds. They are free-range chickens, biologically certified ICEA (Istituto Certificazione Etica ed Ambientale- http://www.icea.info/it/), and fed with grains. All the animals were slaughtered at 52 days of age in small slaughterhouses located near the poultry farm.

Those reared in central Italy were from a large poultry farm belong to an international company. The animals are reared outdoors with strict biohazard regulations to prevent any possible infection with pathogens.

4.3.1 Meat Juice serology for chickens

There is no available information on the use of meat juice serology for *T. gondii* in chickens. Therefore it was necessary to validate the commercial ELISA that was used throughout the study (ID SCREEN® TOXOPLASMOSIS INDIRECT MULTI-SPECIES, IDvet) for sheep and pigs, by changing the secondary antibody.

All the animals' hearts were frozen at -20°C for 18-24 hours and the meat juice obtained used for the serology. The total amount of meat juice was poor, considering the limited dimensions of a chicken heart (medium weight around 4-5 g). For this reason all the hearts were put singly in small bags and immediately frozen after the slaughter.

Another problem with poultry is that there are no commercial kits validated for the research of antibodies against *T. gondii* in poultry. For this reason, we attempted to validate a method using the same commercial ELISA kit used before for other species.

Briefly, we tested the secondary antibody, an anti-chicken linked with a peroxidase (SigmaAldrich), at different concentrations against serum of experimentally infected poultry (kindly provided by Dr. B. Bangoura, University of Leipsig, Germany). Firstly, the positive serum were distributed in each well-plate in toto and diluted 1:40, 1:80, 1:160, 1:320, while the negative controls were tested in toto. In this first experiment, the secondary antibody was used at a final concentration of 1:30000.

After the first attempt we tested the positive serum with the lowest OD value (obtained in the first experiment) (in toto and diluted 1:40) and the negative control serum with the highest OD value

(only in toto). In this second test, we wanted to verify which was the best concentration of the secondary antibody to use. Three different dilutions (1:15000, 1:20000 1:30000) were tested. We also changed the incubation temperature: usually room temperature and here 37°C for the first two steps of the protocol (1.reaction between meat juice and p30 antigen and 2. reaction between the immunocomplex and the secondary antibody). The incubation time was extended (1 hour for the first point and 45 minutes for the second one).
The best results were obtained with the secondary antibody at a dilution 1:15000.

N° **34** in toto	N° **36** in toto	N° **52** in toto
N° **34** 1:40	N° **36** 1:40	N° **52** 1:40
N° **34** 1:80	N° **36** 1:80	N° **52** 1:80
N° **34** 1:160	N° **36** 1:160	N° **52** 1:160
N° **34** 1:320	N° **36** 1:320	N° **52** 1:320
N° **53** in toto	N° **53** 1:40	N° **53** 1:80
N° **53** 1:160	N° **53** 1:320	N° **19** in toto (C-)
N° **21** in toto (C-)	N° **22** in toto (C-)	N° **26** in toto (C-)

Table n. 3: *scheme representing the ELISA plate in the first experiment. Different dilutions of the positive controls (n° 34-36-52-53) were tested. The final concentration of the secondary antibody used was 1:30000. The negative controls (n° 19-21-22-26) were used in toto.*

N° 34 in toto + 1:15000 secondary Ab
N° 34 1:40 + 1:15000 secondary Ab
N° 34 in toto + 1:20000 secondary Ab
N° 34 1:40 + 1:20000 secondary Ab
N° 34 in toto + 1:30000 secondary Ab
N° 34 1:40 + 1:30000 secondary Ab
N° 22 in toto + 1:15000 secondary Ab
N° 22 in toto + 1:30000 secondary Ab

Table n.4: *scheme representing the ELISA plate in the second experiment. Sample n° 34 in toto and diluted 1:40 was tested with different concentrations of the secondary antibody (1:15000; 1:20000; 1:30000). The negative control n° 22 were used in toto and allowed to react with the dilution rate 1:15000 and 1:30000 of the conjugate. The incubation temperature was 37°C. Incubation time was one hour for the reaction between serum and p30 antigen and 45 minutes for the reaction between the immunocomplex and the secondary antibody linked with a peroxidase.*

4.3.2 PCR (529 bp marker and B1 nested-PCR)

As described above for swine and sheep, DNA was extracted from hearts collected at slaughter the previous day. Each heart was frozen at -20º C for 18-24 hours, defrosted and used for DNA extraction. Connective tissue was removed and the cardiac muscle tissue (about 10-20 g weight) blended with 30 ml of PBS. The DNA was extracted from 350 µL of this suspension following the manufacturer's instructions (Qiagen). For the identification of *Toxoplasma gondii* infection in chickens, the 529 bp region was amplified as done for the other samples.

The genetic marker was not present in any of the 77 samples analyzed. However, there was a band at around 600 bp in all the samples. The presence of B1 gene, a marker commonly used for the diagnosis of toxoplasmosis, was used to evaluate the nature of the band in order to confirm (or not) the presence of *T. gondii* DNA. The B1 gene is a 35-fold repetitive gene sequence with unknown functions. The amplification reaction was a nested-PCR managed following the protocols described by Burg (1989) and Jones (2000). The oligonucleotides sequences are described in **table n.5** and the protocol provided a first amplification with external primers, followed by a second amplification with internal primers made on the template obtained from the first PCR reaction.

PCRs mix were prepared with a 2x reaction mixture (*Mytaq mix*-Bioline), 0.1 µM of each primer and 1 µL of DNA water to bring to a final volume of 25 µL. Reactions, after a denaturation at 94º C for 1 minute, were cycled 40 times with a denaturation step at 94º C for 10 sec, followed by annealing at 57º C for 10 sec and finally an extension at 72º C for 15 sec. Negative and positive controls were insert in each PCR reaction.

The nested mix reactions contained 1 µL obtained from the first-round product, a 2X reaction mixture (*Mytaq mix*-Bioline), 0.5 µM each primer and water to bring to volume. The amplification protocol was characterized by a denaturation step (94°C for 1 minute) and 40 repeated cycles (94°C – 10 sec; 62.5°C – 10 sec; 72°C - 15 sec). Negative control samples, from first-round amplification, an additional second-round negative control of sterile water were included in all nested reactions together with a positive one.

The products (10 µL), coloured with a dye to facilitate the loading (*5X loading die* –Bioline-), were fractionated on a 1% agarose gel, stained with Sybr-safe gel stain (Life Technologies) and visualized by UV transilluminator.

Oligonucleotide primer	**Genetic sequence**	**Sequence position**	**Band molecular weight**
Tox4 (F)	5'CGCTGCAGGGAGGAAGACGAAAGTTG3'	Repetitive fragment	**529 bp**
Tox5 (R)	5'CGCTGCAGACACAGTGCATCTGGATT3'		
B1 (F) outer primer	5′-GGAACTGCATCCGTTCATGAG-3′	694-714	**193 bp**
B1 (R) outer primer	5′-TCTTTAAAGCGTTCGTGGTC-3′	887-868	
B1 (F) inner primer	5′-TGCATAGGTTGCAGTCACTG-3′	757-776	**96 bp**
B1 (R) inner primer	5′-GGCGACCAATCTGCGAATACACC-3′	853-831	

Table n.5: *primer sequences used for the identification of Toxoplasma gondii DNA*

4.3.3 Gene sequencing

Three of the ampliflied samples with the 529bp marker (13PC, 23PC, 31PAM) presenting a strong band at 600bp were purified and sequenced.

The purification was done using the QIAquick PCR purification kit protocol (Qiagen) eluting the products in a final volume of 50 µL of water (better than EB buffer for sequencing). The purified DNA was then quantified with the Nanodrop (Thermoscientific) and appropriate dilutions were made to have an equal amount of DNA in each sample (final volume 20 µL).

The products, together with the two primers used (TOX4-TOX5) at a final concentration of 10 pmol/ µL, were outsourced to be sequenced with an Illumina.

The sequencing results were analyzed and the sequences aligned with the program CLC main workbench version 6.0.2, blasted on the website http://blast.ncbi.nlm.nih.gov/Blast.cgi to check the presence of homologies with the data recorded in the database. Another check was also made to verify the presence of some sequence omology with the chicken genome, blasting the aligned sequences on the website http://www.ensembl.org/Gallus_gallus/Info/Index.

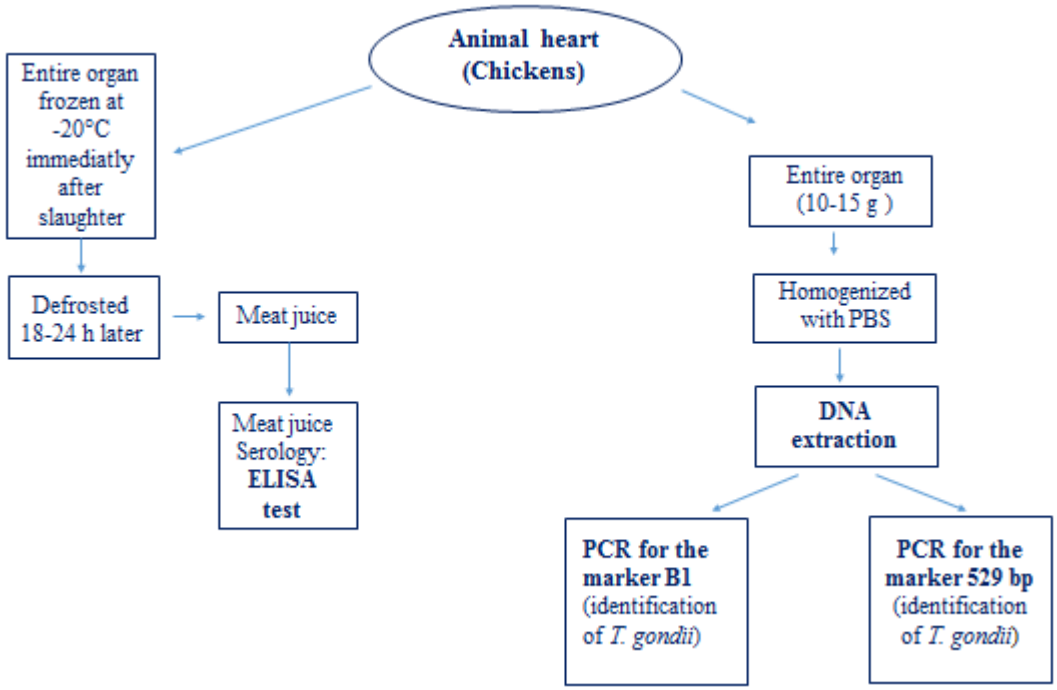


Figure n. 7: *Outline of the experimental design for free-range chickens.*

4.4 Materials and methods for study of *Toxoplasma gondii* in wild animals.

Animals

Thirty wild animals were also studied. The presence of *T. gondii* DNA and seroprevalence in both wild herbivores and carnivores could provide information about the contamination level of the environment and on the diffusion of the parasite.

The sample collection includes 2 jackdaws, 1 coypu, 1 crow, 4 foxes and 22 magpies.

4.4.1 DNA extraction and PCR for the 529 bp marker

DNA was extracted from 30 samples of hearts and diaphragms following the same protocol described before.

The PCR for the 529 bp marker was applied to identify the presence of *T. gondii* DNA.

4.4.2 Genotyping

A nested-PCR protocol for 12 genetic markers was also applied on wild animal samples positive for the 529 bp marker. The samples showing good bands after the amplification were genotyped using specific restriction enzymes as described before (Su et al., 2010). Only some markers were used to genetically characterise the samples. They were chosen based on their ability to discriminate among the three clonal types of *T. gondii* (identifying specific snips) and on the results recorded before, evaluating the number of the samples and the specificity of the bands obtained on the gel after the nested-PCR.

4.4.3 Gene sequencing

The samples showing good bands on the gels after the amplification of the genetic markers or after the genotyping were purified (QIAquick PCR purification kit protocol, Qiagen) The DNA was quantified and outsourced to be sequenced. The sequences were aligned and each contig blasted as done for pigs' samples.

If the aligned sequence resulted to be a gene of *Toxoplasma gondii*, the next step was to evaluate if it belonged to the clonal type I, II or III. Based on the number and position of snips present within the query sequence and comparing it with the reference ones (recorded with a specific gene bank

number on the site http://www.ncbi.nlm.nih.gov/nuccore) it is possible to define if the isolate is a type I, II, or III for a specific genetic marker.

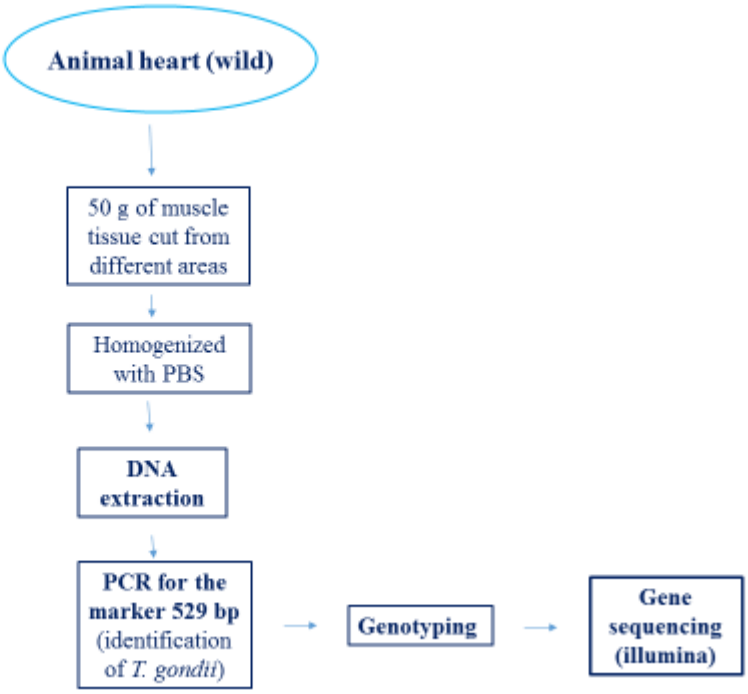


Figure n. 8: *Outline of the experimental design for wild animals.*

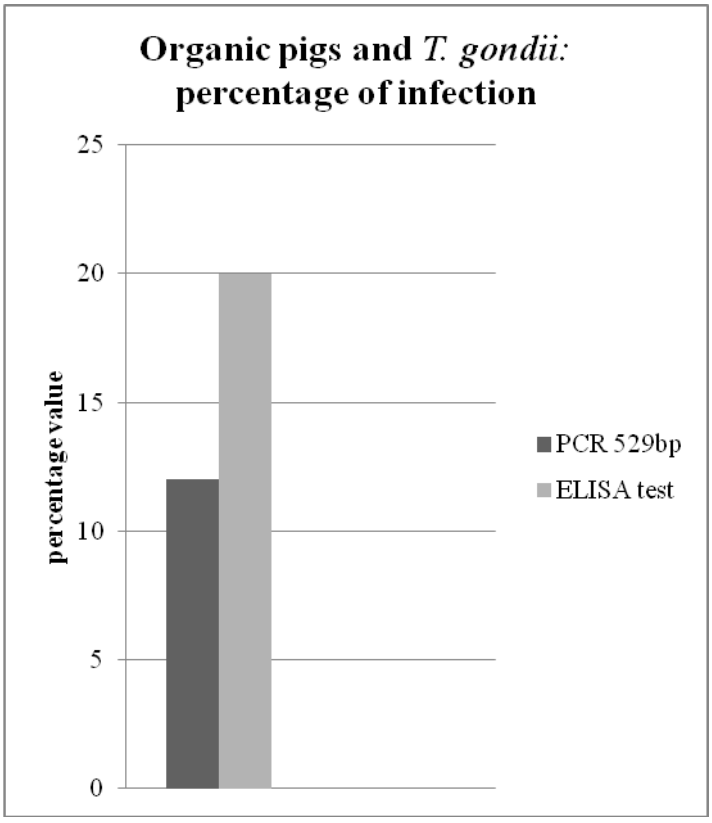
5. Results

5.1 *Toxoplasma gondii* in swine

5.1.1 Organic pigs

PCR and ELISA test

The results of PCR and ELISA from the organic, free-range pigs analysed are shown in the graph below. Of the 21 animals tested, 57.1% (12/21) were positive for the 529 bp marker and almost all (20/21) showed IgG antibodies against *T. gondii* (95.2%).



Graph n. 1: *graphic representation of the percentage of positive samples using two different techniques.*

Genotyping

Two of the 12 TOX4/TOX5 positive samples (8, 21) were fully genotyped, showing Type II alleles at all markers except SAG1 (SAG1 Type III variant). One sample (12) that was genotyped at 7 loci had a predominant Type II-pattern, while an additional five samples, genotyped at 4-6 loci were of a

Type-I/II pattern (2, 10, 11, 17, 20). Finally, three samples (7, 18, 19) genotyped at 5-7 loci showed a predominant Type-I pattern (**Table n. 6**).

Several of the samples were sequenced in 2015 to confirm the genotype. The amount of *T. gondii* DNA was low and for this reason some markers could not be amplified or they showed weak bands on the agarose gel. In some cases (for example, GRA6) the bands visualized on the gel were acceptable, but sequencing revealed that the amplified DNA belonged to swine and not to *T. gondii*. The genetic pattern was fully confirmed only for samples n. 8 and 21.

The table below (**Table n.7**) shows the results obtained after a second attempt at genotyping and sequencing (in red).The majority of the samples showed, for the analysed markers, a Type-II profile.

	Sample number										
MARKER	**2**	**7**	**8**	**10**	**11**	**12**	**17**	**18**	**19**	**20**	**21**
SAG1	n.d.*	II or III	II or III	II or III	n.d.	n.d.	n.d.	I	n.d.	I	II or III
5'SAG2	II	I/II	I/II	I/II	I/II	I/II	I/II	I/II	I/II	I/II	I/II
3'SAG2	II	I/III	II	II	II	II	II	I/III	I/III	II	II
altSAG2	II	n.d.	II	II	n.d.	II	II	II	n.d.	n.d.	II
SAG3	III	III	II	III	III	III	III	III	III	III	II
BTUB	n.d.	n.d.	II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	II
GRA6	I	II	II	I	I	I	I	I	I	I	II
C22-8	n.d.	n.d.	II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	II
C29-2	I	I	II	I	I	I	I	I	n.d.	I	II
L358	n.d.	n.d.	II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	II
PK1	n.d.	n.d.	II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	II
APICO	I/III	I/III	II	n.d.	II	II	n.d.	n.d.	II	n.d.	II
Type	I/II	I	II	I/II	I/II	II	I/II	I	I	I/II	II

n.d. = not determined

Table n. 6: *T. gondii genotype patterns in organically raised pigs.*

	Sample number										
MARKER	**2**	**7**	**8**	**10**	**11**	**12**	**17**	**18**	**19**	**20**	**21**
SAG1	n.d*	**II or III**	**II or III**	**II or III**	n.d	n.d	n.d	n.d	n.d	n.d	II or III
5'SAG2	**II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**	**I/II**
3'SAG2	**II**	**I/III**	**II**	**II**	**II**	**II**	**II**	**I/III**	**I/III**	**II**	**II**
altSAG2	**II**	n.d	**II**	**II**	n.d	**II**	**II**	**II**	n.d	n.d	**II**
SAG3	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
BTUB	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
GRA6	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
C22-8	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
C29-2	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
L358	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
PK1	n.d	n.d	**II**	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**
APICO	**I/III**	**I/III**	**II**	n.d	n.d	**II**	n.d	n.d	**II**	n.d	II
Type	II	II/III	II	II	II	II	II	II	II	II	II

n.d. = not determined

Table n. 7: *T. gondii genotype patterns in organically raised pigs after revision and sequencing.*

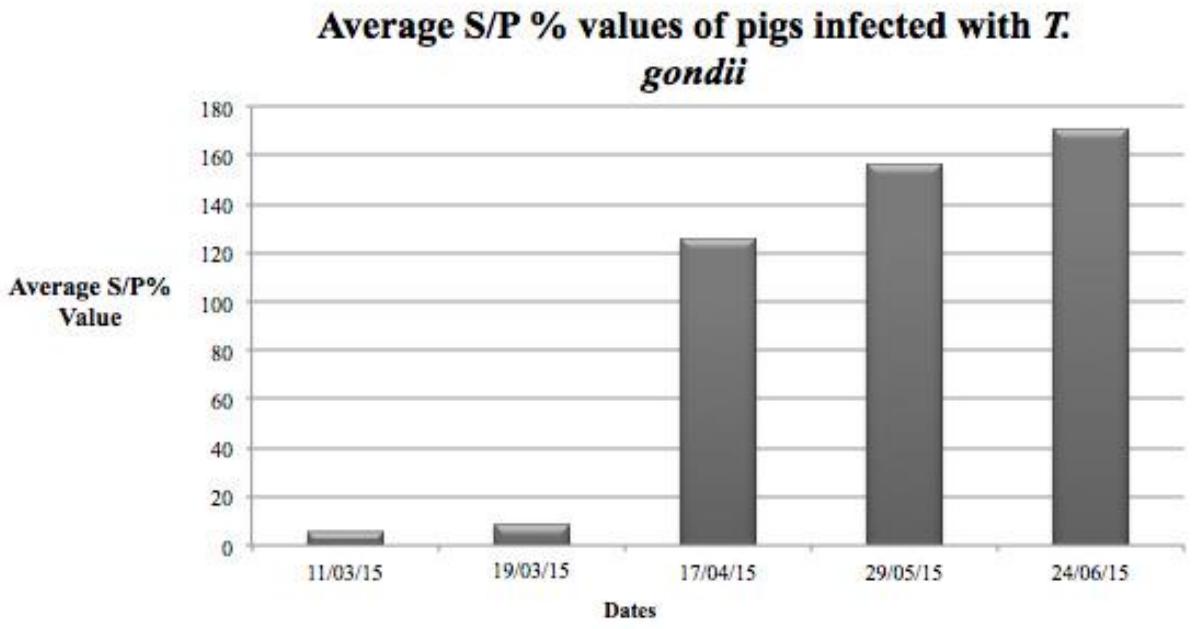
5.1.2 Experimentally infected pigs

Serology

All 12 pigs infected in March 2014 with 1000 oocysts/each, were positive for anti-*T. gondii* IgG in ELISA. Sera were analyzed at five different time points. From day 42 post-infection (p.i.), all animals resulted positive (**Table n. 8**). The average value of S/P % (calculated on the positive samples) was 2.7 times higher (138.17) than the cut-off (50). The OD values increased with a positive trend in almost all the samples. **Graph n. 2** shows the increase of S/P% values (average calculated on the 12 samples) related to time.

Sample	Dates				
1	11/03/2014	19/03/2014	17/04/2014	29/05/2015	24/06/2014
Od value	0.111	0.208	2.192	2.089	2.396
S/P %	8.09	9.88	132.80	126.42	145.44
3					
Od value	0.126	0.157	2.793	2.431	3.112
S/P %	4.80	6.72	170.0	147.61	189.80
4					
Od value	0.137	0.137	2.398	2.435	3.027
S/P %	5.48	5.48	145.57	147.86	184.54
5					
Od value	0.162	0.199	1.233	2.080	2.489
S/P %	7.032	9.32	73.38	125.86	151.20
6					
Od value	0.108	0.225	1.953	3.108	3.246
S/P %	3.68	10.93	117.99	189.56	198.11
8					
Od value	0.212	0.206	2.518	2.913	2.962
S/P %	10.13	9.75	153.0	177.47	180.51
9					
Od value	0.164	0.222	2.458	2.878	3.150
S/P %	7.15	10.74	149.28	175.30	192.16
10					
Od value	0.153	0.254	2.329	2.920	3.299
S/P %	6.47	12.73	141.29	177.91	201.39
12					
Od value	0.086	0.209	2.227	2.837	2.980
S/P %	2.32	9.94	134.97	172.77	181.62
13					
Od value	0.102	0.266	1.779	2.980	2.663
S/P %	3.31	13.48	107.21	181.62	161.98
15					
Od value	0.129	0.098	1.221	1.260	1.509
S/P %	4.98	3.06	72.64	75.06	90.48
16					
Od value	0.083	0.108	1.798	2.947	3.037
S/P %	2.13	3.68	108.4	179.6	185.16

***Table n.8**: OD values and S/P% of pigs measured at five different time points obtained with a commercial ELISA test on serum.*



***Graph n. 2**: Histogram representing the increase in the mean value of S/P% related to time. The presence of specific IgG Ab is confirmed when the S/P% value is greater than 50%.*

Meat juice serology

All the pigs (except one doubtful) slaughtered 6 months p.i. and used for the production of Parma ham, were positive on meat juice ELISA, with particularly values of Optical Density. The average value of S/P% was 154.32, i. e. three times the positive cut-off. Meat juice obtained from thighs showed a lower value of OD compared to other tissue samples. In fact, if only heart samples (or diaphragm in the absence of heart) were considered, the average S/P% was 174.99. If instead, only OD values obtained from thigh meat juice were considered, the mean S/P% value was lower, 134.83.

It was, however, not possible to have a heart sample and a thigh sample from all pigs, due to problems at slaughter.

Samples		
C+	**4** heart	**10** heart
C+	**4** thigh	**10** thigh
C-	**5** diaphragm	**12** heart
C-	**5** thigh	**13** heart
1 diaphragm	**6** heart	**13** thigh
1 thigh	**8** heart	**15** heart
3 heart	**9** heart	**16** heart
3 thigh	**9** thigh	**16** thigh

A

OD values		
15,651	31,073	32,065
15,862	18,415	17,180
0,0455	10,600	36,087
0,0454	0,8094	31,037
26,849	32,070	20,263
11,976	29,358	15,989
36,082	32,444	28,448
22,784	19,198	22,009

B

S/P % value		
Average value C+ 1.575	199.7	206.2
	117.4	109.5
Average value C- 0.045	66.3	232.8
	49.3	199.7
172.5	206.2	129.1
74.8	188.6	101.5
232.4	208.8	182.9
145.4	122.5	140.8

C

Table n. 9 A, B. C: *Schematic representation (A), OD values (B) and S/P% (C). Meat juice was collected from different muscle samples infected with oocysts of T. gondii after slaughter 6 months p.i*

Bioassay in mice

Results from bioassay in mice are partial. All mice infected intraperitoneally with fresh thigh samples obtained at slaughter were positive for *T. gondii* showing cerebral cysts with numerous bradyzoites (Fig. 9). At the time of writing, bioassay has been completed for 6 hams of 12 and 16 months of aging (n. 4, 9, 13, 16,, 8, 10) and none of the infected mice were positive.

For each sample, on five mice, at least two presented brain cysts, highlighting the presence of viable parasites.

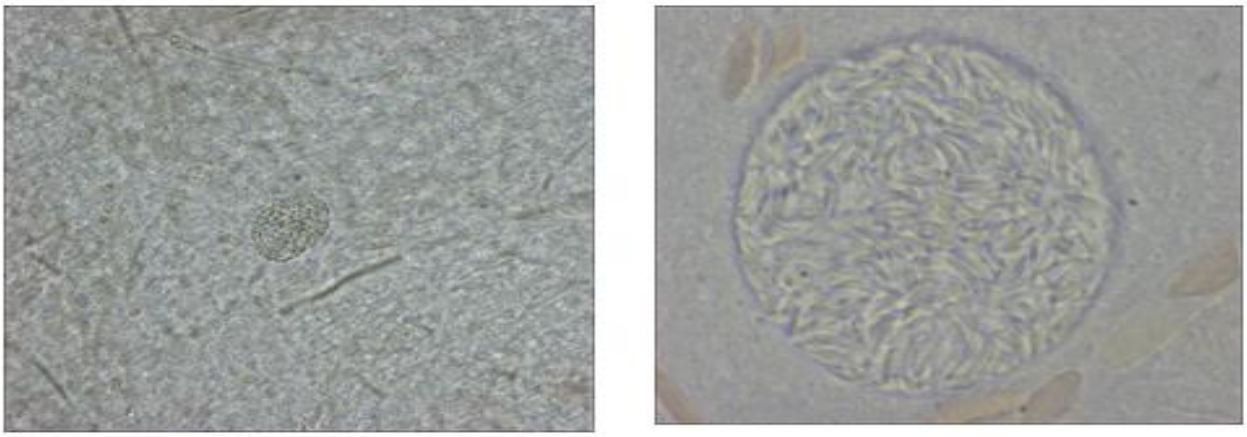


Figure n.9*: Tissue cysts full of bradizoites (seen at microscope 20X and 100X) in a mouse brain killed two months after the inoculation seen at the microscope after having squashed it on a slide.*

Figure n. 10: *Parma ham after 12 months of aging. The mean concentration of salt was 5.7%.*

Real-time PCR for determination of parasite growth in vitro

The table below (**n. 10**) reports the Ct values of the 12 thighs analysed after 12 and 16 months of aging. No increase in the amount of DNA was recorded for any of the samples. The graphs of all RT-PCR analyses are reported in appendix n.3. For one sample, Ct values appeared to decrease at T1 and T2 compared to T0, apparently indicating an increase in the amount of *T. gondii* DNA, but the data was not statistically significant.

It was not possible to analyse all ham samples due to contamination of the flasks (bacteria and mold).

Sample number	Months of aging	RT-PCR T0 (Ct value)	RT-PCR T1 (Ct value)	RT-PCR T2 (Ct value)
n. 4 bone	12	32.77	34.14	32.30
n. 4 rind	12	33.11	32.68	32.16
n. 4 middle	12	32.66	35.63	31.51
n. 9 bone	12	32.72	32.74	32.25

n. 9 rind	12	32.57	33.40	32.25
n. 9 middle	12	32.78	34.36	31.64
n.13 bone	12	31.71	32.40	31.84
n.13 rind	12	31.25	32.53	31.32
n.13 middle	12	31.31	31.74	33.10
n.16 bone	12	30.72	28.73	32.91
n.16 rind	12	33.78	31.90	33.51
n.16 middle	12	23.39	22.10	23.85
n.3 bone	16	31.63	32.20	32.50
n. 3 rind	16	27.78	32.43	n.d*
n. 3 middle	16	28.69	35.01	n.d
n. 8 bone	16	33.31	33.30	33.67
n. 8 rind	16	32.93	33.28	33.21
n. 8 middle	16	33.41	34.06	35.03
n.15 bone	16	31.36	32.21	33.44
n.15 rind	16	n.d	n.d	n.d
n.15 middle	16	30.98	30.12	32.27
n.1 bone	16	31.19	33.53	32.84
n. 1 rind	16	23.02	23.97	23.08
n. 1 middle	16	31.41	33.03	33.30
n. 5 bone	16	33.86	34.53	n.d
n. 5 rind	16	n.d	n.d	n.d
n. 5 middle	16	n.d	n.d	n.d
n.12 bone	16	n.d	n.d	n.d
n. 12 rind	16	33.96	35.25	38.02
n.12 middle	16	23.21	24.20	21.86
n. 10 rind	16	34.29	34.72	35.41
n.10 middle	16	34.34	36.76	36.61

n.10 bone	16	32.58	35.31	34.61
n. 6 rind	16	33.95	36.29	35.59
n. 6 middle	16	34.61	38.42	35.80
n. 6 bone	16	33.43	36.31	36.17

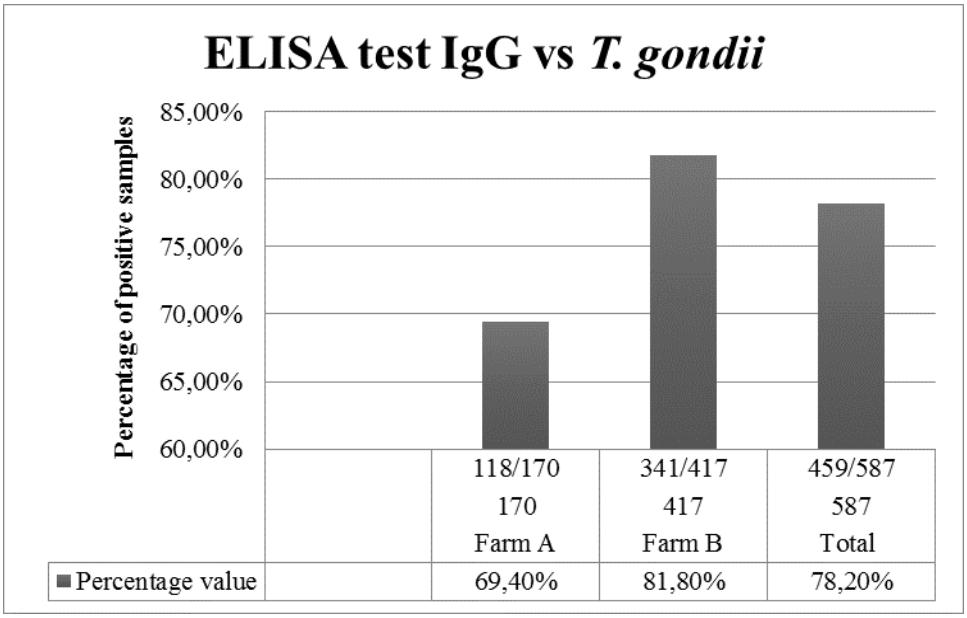
n.d: not determined due to flask contamination*

Table n. 10: *Ct values of the samples seeded on Vero cells and analyzed in RT-PCR amplifying the 529 bp marker. The DNA extraction was made at three different time points to evaluate a potential increase in the amount of DNA, proving the viability of the parasite.*

5.2 *Toxoplasma gondii* in sheep

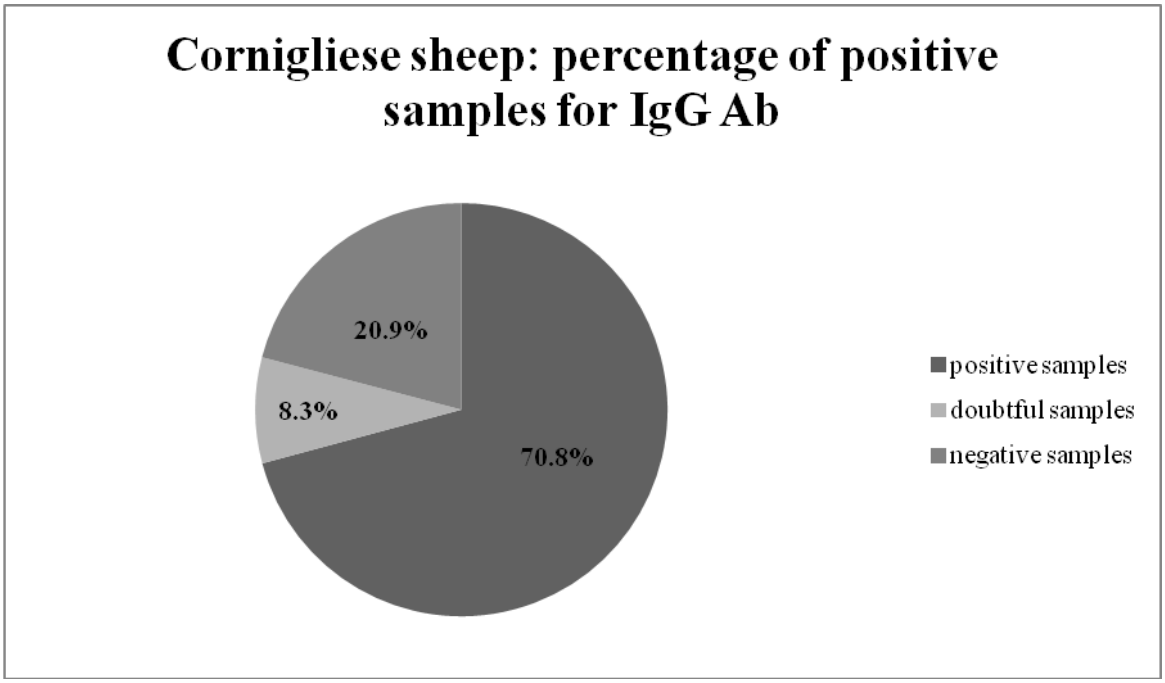
Serology

The serum of 587 sheep reared in two different farms in Parma province was tested for the presence of antibodies direct against the p30 protein of *T. gondii*. Animals from farm A showed a percentage of positive of 69.4%, while those of farm B of 81.8%. The final percentage of positive sheep in the analysed population was 78.2% (**Graph n.3**).



Graph n. 3: *percentage of animals that resulted positive in ELISA test for the presence of specific IgG antibodies against T. gondii in two different farms situated in the North of Italy.*

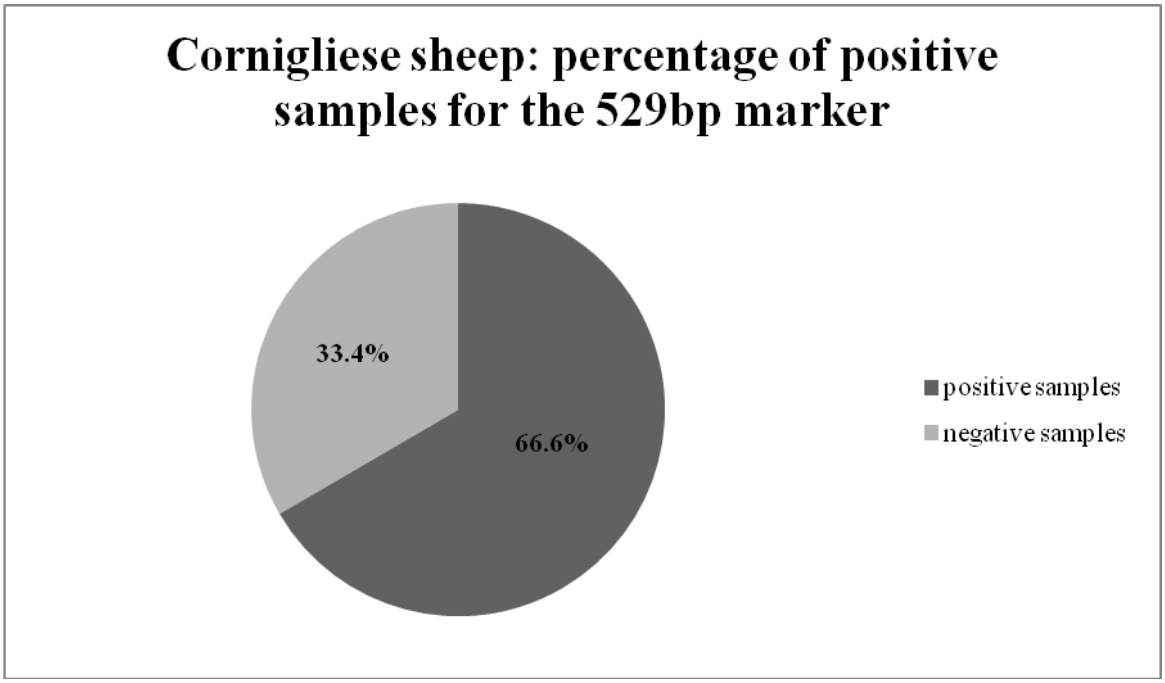
In **graph n.4** the results of serology of the 24 Cornigliese sheep studied are reported. Seroprevalence indicated a positive rate of 70.8% (17/24), while two samples were interpreted as doubtful (with an S/P % value between 40% and 50%).



Graph n. 4: *percentage of positive Cornigliese sheep to the serological test.*

PCR

The molecular analysis of the DNA extracted from myocardial tissue of sheep showed that *T. gondii* was present in sixteen (66.6%) of the samples analysed.



Graphn.5: *percentage of positive sheep for T. gondii DNA extracted from myocardial tissue.*

Genotyping

Nine samples were partially genotyped as reported in **table n. 11**. One sample (n.18) was genotyped at 7 loci, showing Type-II/III pattern. Samples n. 5, 6, 12 and sample n. 15 were genotyped at four loci showing a Type-II profile for the first one and a Type-II/III pattern for the other ones. Only three genetic markers characterized samples n. 7 and n. 8 highlighting a Type-I/III profile for the first one and a Type-II/III for the second one. For the other two samples (n. 16 and n. 22) only two genetic markers have been amplified, likely for the small amount or low quality of *T. gondii* DNA extracted from muscle tissue.

Sample number									
Marker	**5**	**6**	**7**	**8**	**12**	**15**	**16**	**18**	**22**
SAG1	n.d*	n.d	n.d	**II/III**	**II/III**	n.d	**I**	**II/III**	n.d
3'SAG2	**II**	**II**	n.d	n.d	**II**	**II**	n.d	**II**	n.d
5'SAG2	**I/II**	**III**	**I/II**	n.d	**I/II**	**I/II**	n.d	**I/II**	n.d
SAG3	**II**	n.d	**I**	**II**	n.d	n.d	n.d	**II**	**I**
C22-8	n.d	**III**	n.d	n.d	n.d	**III**	**III**	**III**	n.d
APICO	**III**	**III**	**III**	**III**	**III**	**III**	n.d	**III**	**III**
GRA6	n.d	n.d	n.d	n.d	n.d	n.d	n.d	**II**	n.d
Type	II	II/III	I/III	II/III	II/III	II/III		II/III	

n.d: not determined*

Table n. 11: *T. gondii genotype patterns in Cornigliese sheep.*

In vitro isolation and molecular identification

Samples number 21, 22, 23, 24 were digested and seeded onto Vero cells, as described in the materials and methods section DNA was extracted from each flask two and four weeks post inoculum to verify the infection with *T. gondii*. Only sample n. 22 was positive after both two and four weeks post inoculum. The other samples were negative at both time points. DNA quantification with RT-PCR for sample n. 22 showed a negative trend (**Fig. 13**). No increase in the amount of the DNA was observed among the three time points considered (T0; T1, i.e. two weeks post inoculum; T2, i.e. four weeks post inoculum). Results are reported below.

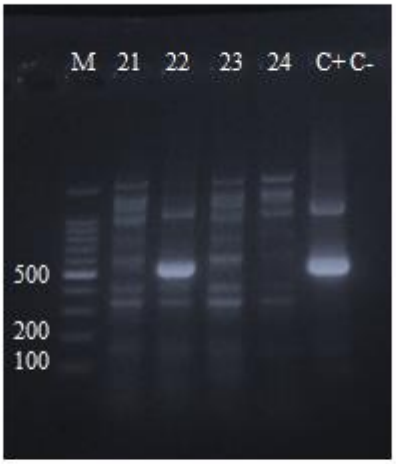


Figure n. 11: *PCR for the identification of T. gondii (marker 529 bp) done on the extracted DNA two weeks post inoculum. Sample number 22 resulted positive.*

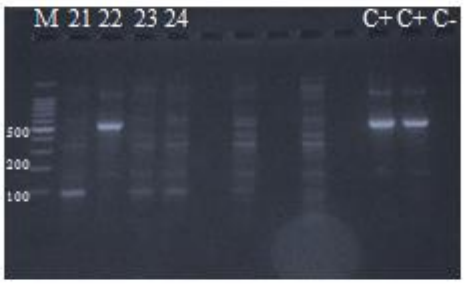


Figure n. 12: *PCR for the identification of T. gondii (marker 529 bp) done on the extracted DNA four weeks post inoculum. Sample number 22 resulted positive again.*

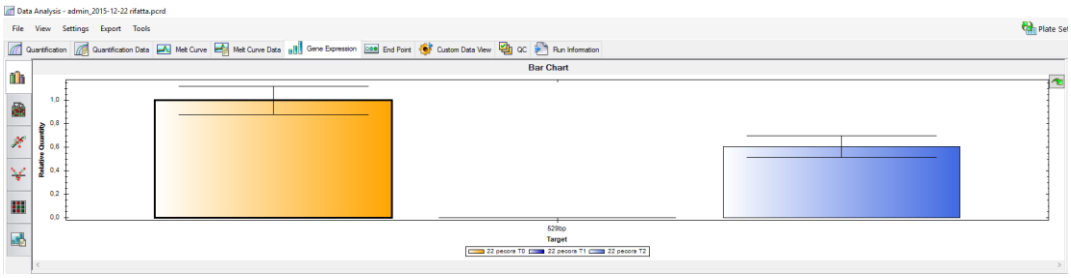


Figure n.13*: quantification of sheep's DNA in sample n. 22 at three different moments. DNA used as time zero (T0) was extracted from the digested tissue and time one (T0) and time 2(T2) two and four weeks post inoculum, respectively.*

5.3 *Toxoplasma gondii* in chickens

Meat juice serology

The OD values from the meat juice tested for the presence of IgG antibodies against *T. gondii* are reported in **table n. 12**.

In this first attempt of validation an ELISA test using meat juice for chickens, only the samples with an OD value higher than 5*SD calculated on the mean value of four negative controls have been considered as positive. **Table n. 13** shows the OD values obtained with chicken meat juice coming from two different farms, with two different systems of biosecurity. Of the 77 hearts collected, only 66 gave sufficient meat juice for use in ELISA. None of the meat juice samples (0 %) were positive when considering S/P % values, while 24 (36.4%) were positive if OD values were expressed as 5*SD.

Farm A located in Emilia-Romagna region had a percentage of positive of 62.8% (22/35 samples analyzed), while farm B, located in Central Italy, with stronger biohazard measure, showed a lower percentage value of positive animals (6.45%, 2/31).

CTRL	**Average value OD**	**Standard deviation**	**5*ST.DEV.**	**Threshold** (average value OD+5*ST.DEV)
Positive controls	0.4312	0.076533582	0.38266791	
Negative controls	0.0470	0.003075711	0.015378556	**0.0624**

***Table n.* 12**: *ELISA test cut-off used to discriminate positive samples from negative samples was calculated as the threshold of four negative controls. The addition of the average value of OD of the negative controls and 5*ST.DEV gives the threshold value. All the samples with a value of Optical density higher than 0.0624 was considered positive for the presence of Ab against Toxoplasma gondii.*

PCR for the identification of *T. gondii* and sequencing results

DNA extracts from all samples analyzed were negative for both 529bp and B1 markers, indicating that *T. gondii* was not present in muscle tissue of the chickens analyzed. A band of approximately 600 bp was observed in all the samples amplified for the 529bp marker. This was subsequently sequenced and, after the alignment of the sequences (made on CLC main workbench program 6.0 version) and blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), was compared with the genome of chickens (www.ensembl.org/Gallus_gallus/Tools/Blast/Results). A region with a high rate of

homology with the sequence amplified with the 529bp marker was present in the chicken genome. In particular, on chromosome 3, the gene RIMS (coding for an uncharacterised protein) of chickens there is a long sequence with a percentage of identity of approximately 97.0%, with a score E value 0.0, compared to the 529 bp sequence. This would indicate in all likelihood that the band seen on the gel was a highly conserved chicken gene that was amplified during the PCR reaction for the 529 bp marker.

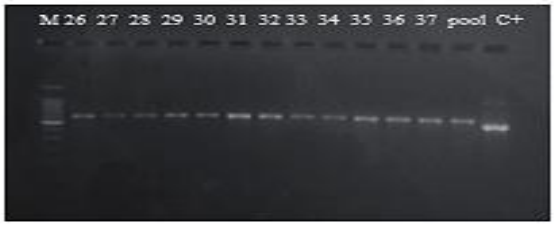


Fig. n.14: *Bands amplified with the 529bp marker in chicken samples (n. 12 PAM to n.37 PAM).*

Sample Farm Piacenza (PC)	PCR	ELISA		OD Value		Sample Farm Teramo (PAM)	PCR	ELISA		OD Value
		S/P%	5*SD					S/P%	5*SD	
1	-	-	+	0.1387		2	-	-	-	0.0489
2	-	-	+	0.0710		4	-	-	-	0.0474
3	-	-	+	0.0720		5	-	-	-	0.0513
5	-	-	+	0.0772		6	-	-	-	0.0498
6	-	-	+	0.1107		8	-	-	-	0.0515
8	-	-	+	0.1886		9	-	-	+	0.0740
11	-	-	+	0.0838		10	-	-	-	0.0529
12	-	-	+	0.0854		11	-	-	-	0.0484
13	-	-	+	0.0668		12	-	-	-	0.0487
14	-	-	+	0.0625		13	-	-	-	0.0469
15	-	-	+	0.1113		14	-	-	-	0.0519
17	-	-	+	0.0643		15	-	-	-	0,0495
18	-	-	-	0.0577		17	-	-	-	0.0503
19	-	-	+	0.0769		19	-	-	-	0.0522
20	-	-	+	0.0881		20	-	-	-	0.0513
21	-	-	+	0.0636		21	-	-	-	0.0483
22	-	-	-	0.0580		23	-	-	-	0.0500
23	-	-	-	0.0586		24	-	-	-	0.0563
24	-	-	-	0.0576		25	-	-	-	0.0542
25	-	-	+	0.1024		26	-	-	-	0.0518
26	-	-	+	0.1203		27	-	-	-	0.0570
27	-	-	+	0.0671		28	-	-	-	0.0520
28	-	-	+	0.0632		29	-	-	-	0.0500
29	-	-	-	0.0585		30	-	-	-	0.0500
30	-	-	-	0.0588		31	-	-	+	0.0631
31	-	-	-	0.0556		32	-	-	-	0.0496
32	-	-	-	0.0562		33	-	-	-	0.0500
33	-	-	-	0.0573		34	-	-	-	0.0519
34	-	-	-	0.0554		35	-	-	-	0.0497
35	-	-	+	0.0742		36	-	-	-	0.0524
36	-	-	+	0.0850		37	-	-	-	0.0597
37	-	-	+	0.1370						
38	-	-	-	0.0556						
39	-	-	-	0.0517						
40	-	-	-	0.0574						

Table n. 13: *results of PCR and Elisa test of the 77 chickens tested from April and July 2015***.**

Results for Job 1: BLASTN against Chicken Galgal4 (Genomic sequence)

Job details

Job name	Job 1: BLASTN against Chicken Galgal4 (Genomic sequence)
Species	Chicken (Gallus gallus)
Assembly	Galgal4
Search type	BLASTN (NCBI Blast)

Download results file

Results table

Show All entries | Show/hide columns (2 hidden) | Filter

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Length	Score	E-val	%ID
3:81477136-81477717 [Sequence]	RIMS1	Forward	21	602	582 [Sequence]	1098	0.0	98.80 [Alignment]

HSP Location of selected alignment

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81477676 CAAAGAACGCACTTCTGGCGTGCCGTGGAAATCCAGATGCACCAGGTCTGCAGCTGTTAC 81477735
81477736 TTATGATGAGAAGCAGACAGTCTATTGATAAGATCCAGTCAGCCCACATTAAGCACTTAA 81477795
81477796 TTGAGCCTAACATTCAGCTGCTAATTAATAAGGCAGTTCTAAGCACATCCCTAAGAAACT 81477855
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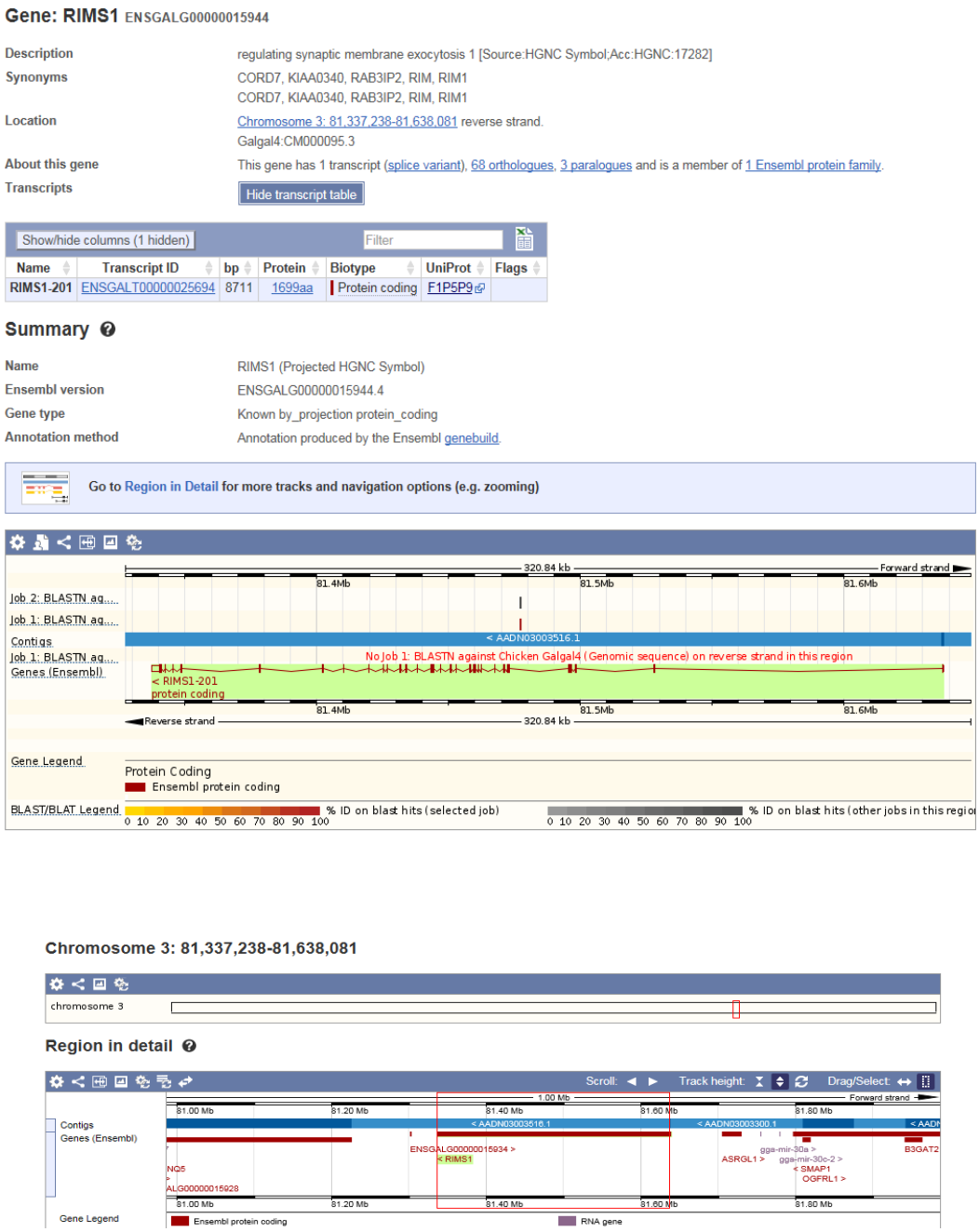

Figure. n.15: *Blastn results obtained with the sequence of sample 31 PAM on ensemble.org. In the first picture the percentage of identity with the 529bp marker (98.8%) is highlighted, together with the E value. In the second one it is possible to see the location of the alignment followed by, in the third, the region that overlaps the gene RIMS1(the same for the fourth one but in more detail).*

Results for Job 2: BLASTN against Chicken Galgal4 (Genomic sequence)

Job details

Job name	Job 2: BLASTN against Chicken Galgal4 (Genomic sequence)
Species	Chicken (Gallus gallus)
Assembly	Galgal4
Search type	BLASTN (NCBI Blast)

Download results file

Results table

Show All entries | Show/hide columns (2 hidden) | Filter

Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Length	Score	E-val	%ID
3:81477136-81477717 [Sequence]	RIMS1	Forward	16	597	582 [Sequence]	1051	0.0	97.77 [Alignment]

HSP Location of selected alignment

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>chromosome:Galgal4:3:81476836:81478017:1
81476836 CAAAATTCTACTGGGGAAATCCTCAATTTAGCGCAGCTTCAACCACTTTCAATTGCTTGT 81476895
81476896 TAAACAACGTTCTAAATTTAACAACATGAAAAATGTAAAAATAAGAAGCCTCTAAGACAT 81476955
81476956 GATTTGCACTGTATCATTTACTTCTGAACATTACTAATACATTCCTATAAAAAATACACA 81477015
81477016 GACATATTTTAAAATCGAGCCTATGTTAAAAAATGTATTTGCAATTATAATTTAATTCAG 81477075
81477076 ACTAATGGAGTGAAAGAAGTATATCTTTATAAAATCCTTCTACCTGAGGGAGTAATTCAA 81477135
81477136 AAAGTTGTATTCTGTCAGCAATAAAATACTTTTCCTATTAACTGCTCAATTACTATTCTG 81477195
81477196 TTGGACTTTAACTTGCCACTGTCATAAGTTACTTAACATGACACAAATTGGCTTAAGTCT 81477255
81477256 TTTCTTGAGAAGTCCTTTGCAGTCCTCAATCAAGTTTCAAGGTGAAGAGTAAAACTCTAA 81477315
81477316 TACGCCGCTTTGATTTTATGTCTGCAACATGCATGCCTTCCAAATAAAGGGAAAGCAAGC 81477375
81477376 TTCAGAACACATTTTCTCTGAAATACTTCATCCTGAGCAATGCTACTCATTTGAAAAGCT 81477435
81477436 GATGTTGCAATGCTGTGATTTCAAGTGAAATACTACGAAACTGGTAAGCATTAGAAATGA 81477495
81477496 CAGCCTTGTCTTTGCTGCTAAGCTGAAACACGGCTGTGTCGAAACAGGCACAAAATACAA 81477555
81477556 GAAAGCCTGTTATCCCATATGACCAGCCATGCTGCTTCAATAAGTCCTGAACAGTGGAGA 81477615
81477616 TAAAACACCCCAAGATGCTTCCCCAAATCCAGACTGTGTATGGAGAATGGTGATATTCAG 81477675
81477676 CAAAGAACGCACTTCTGGCGTGCCGTGGAAATCCAGATGCACCAGGTCTGCAGCTGTTAC 81477735
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81477796 TTGAGCCTAACATTCAGCTGCTAATTAATAAGGCAGTTCTAAGCACATCCCTAAGAAACT 81477855
81477856 CAGACAATTATTTTCAGGGGCTGCATCTATAATTAAAGCACGAACTGGCTTCCTATACAG 81477915
81477916 ACAGACTAGATCTATCTAATTAACAGTCACAGCAGCCATCTCTGTATTTGTCTTTGTTAA 81477975
81477976 CTTTTAGGCTCAGATACAATGTGCGCTAATGAGGAAAAAAAA                   81478017
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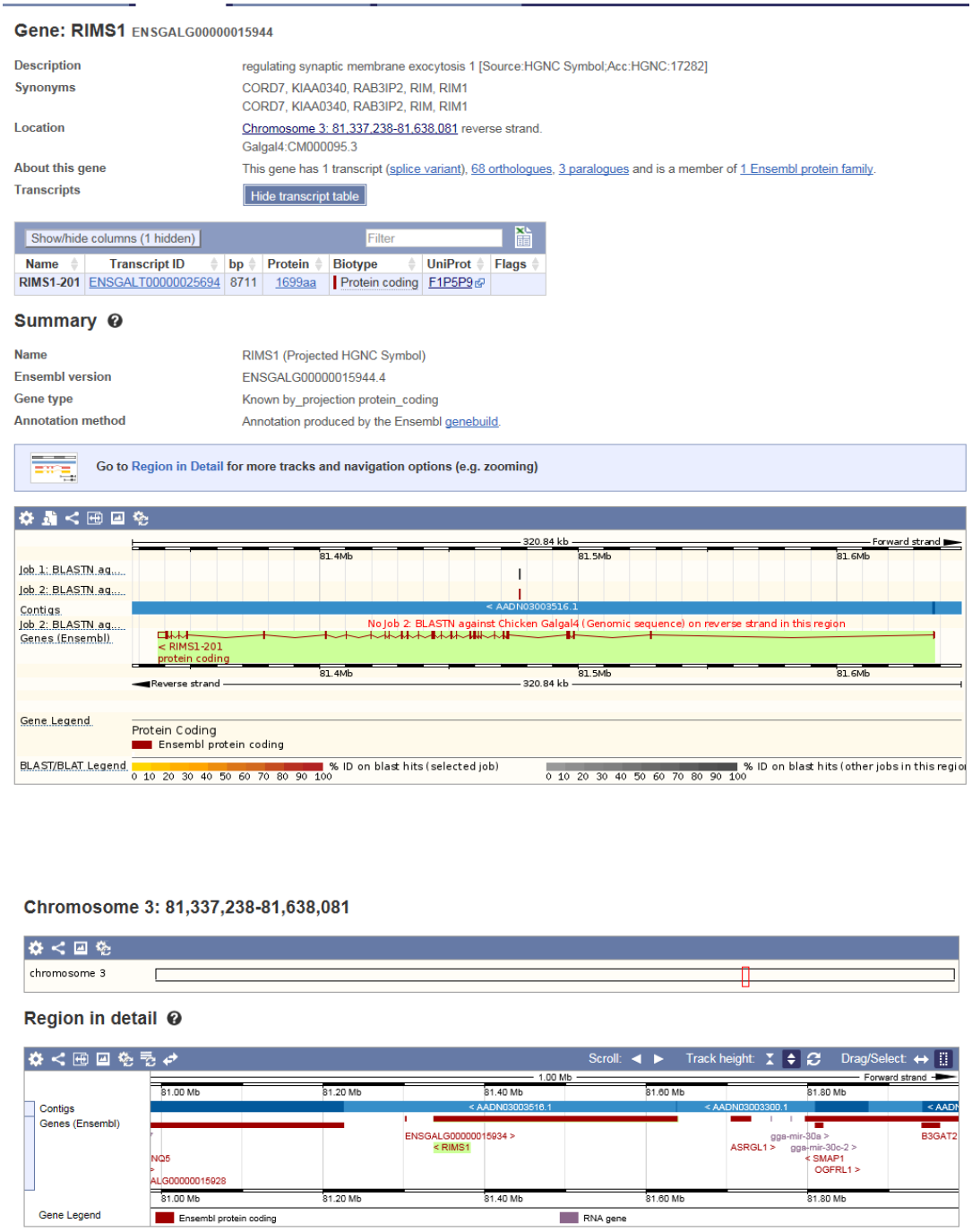


Figure. n.16: *Blastn results obtained with the sequence of sample 13PC on ensemble.org. In the first picture the percentage of identity with the 529bp marker (98.8%) is highlighted, together with the E value. In the second one it is possible to see the location of the alignment followed by, in the third, the region that overlaps the gene RIMS1(the same for the fourth one but in more detail).*

5.4 Toxoplasma gondii in wild animals

PCR

A PCR reaction for the 529 bp marker was performed to identify *T. gondii* in DNA samples extracted from muscle samples from different wild animal species. All the four foxes analyzed and 42.8% of magpies resulted positive. The table below (**n. 14**) resumed the data.

Sample number	PCR 529 bp
Jackdaws n.2	0/2
Coypu n. 1	0/1
Fox n. 4	4/4
Crow n. 1	0/1
Magpies n.21	9/21

Table n.14: *molecular identification of T. gondii (529bp marker) in wild animals.*

Genotyping

Based on previous data obtained with other samples, a selected number of genetic markers (giving the best results) were chosen to genotype muscle samples.

One sample from a magpie (n. 8) was genotyped at seven loci, showing Type II/III profile. Magpie n. 21 showed a Type–III pattern with genotyping by four markers. All other samples were only partially genotyped: samples n. 10 and 15 had a predominant Type-III pattern, considering the studied alleles. Only the C22-8 marker was characterized in foxes and the genotype for that allele was Type-II/III.

	Sample number							
Marker	1 fox	3 fox	4 fox	6 fox	8 magpie	10 magpie	15 magpie	21 magpie
C22-8	**II/III**	**II/III**	**II/III**	**II/III**	**II**	n.d*	n.d	n.d
SAG3	n.d	n.d	n.d	n.d	**III**	**III**	n.d	**III**
APICO	n.d	n.d	n.d	n.d	**I/III**	n.d	n.d	**I/III**
GRA6	n.d	n.d	n.d	n.d	**II**	n.d	n.d	n.d
5' SAG2	n.d	**III**	n.d	n.d	**III**	**III**	**III**	**III**
3'SAG2	n.d	n.d	n.d	n.d	**II**	n.d	n.d	n.d
C29-2	n.d	n.d	n.d	n.d	**I/III**	**I/III**	**I/III**	n.d
PK1	n.d	n.d	n.d	n.d	n.d	n.d	n.d	III
Type		III			II/III	III	III	III

n.d*: not determined

Table n. 15: *T. gondii genotypes studied using a PCR-RFLP protocol.*

Gene sequencing results

A large portion of the samples genotyped through PCR-RFLP were sent for sequencing after having purified the PCR products (only those with a concentration of at least 10 ng/µL). It was possible, however, to obtain contig (after having assembled the forward and reverse sequence with CLC program) for only three samples, blasted on http://blast.ncbi.nlm.nih.gov/Blast.cgi.

The alignment made using the program "Seqman" (Lasergene) showed that samples n. 8 magpie and n.21 magpie had the same genomic sequence of isolate W-N4 of *T. gondii* for the APICO marker (GenBank accession number: **KM583876.1**). For the sample n.3 fox, the alignment was good only for the forward sequence and for this reason the comparison with the recorded sequences and the definition of the genetic strain was not possible. Blast showed a high concordance with the sequence coding for the p22 antigen (**Fig. 18**).

	Sample number		
Marker	3 fox	8 magpie	21 magpie
5'sag2	III		
Apico		I/III	I/III
GenBank Accession number	LN14498;DQ000461	KM583876.1	KM583876.1

Table n. 16: *T. gondii genotypes obtained with Illumina gene sequencing.*

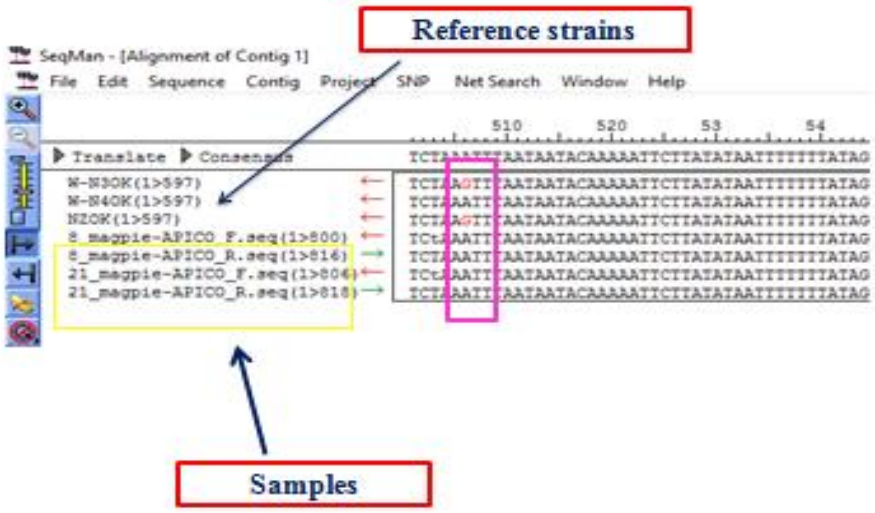
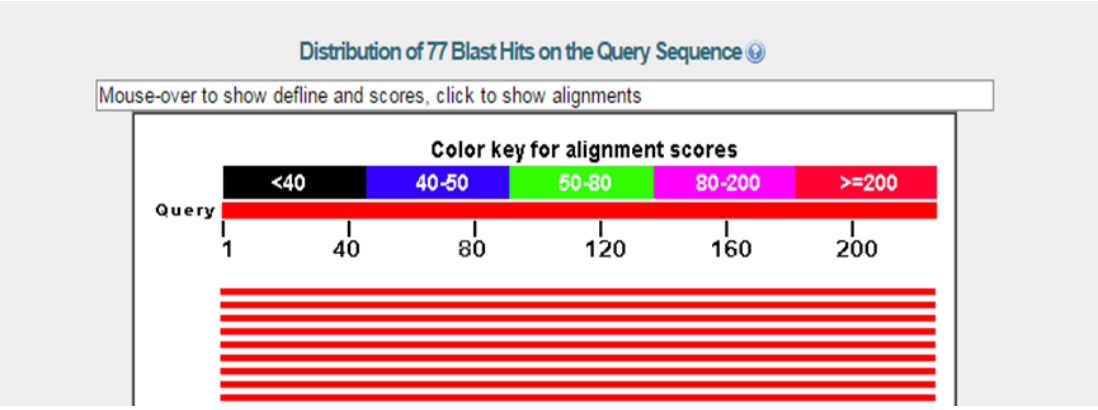


Figure n.17: *Screenshot of the alignment made with the bioinformatic program "Seqman" (Lasergene). Samples n. 8 and n.21 magpie have been aligned with the three reference genotypes recorded on ncbi. Both samples have the same genomic sequence of isolate W-N4.*



Sequences producing significant alignments:

Select: All None Selected:0

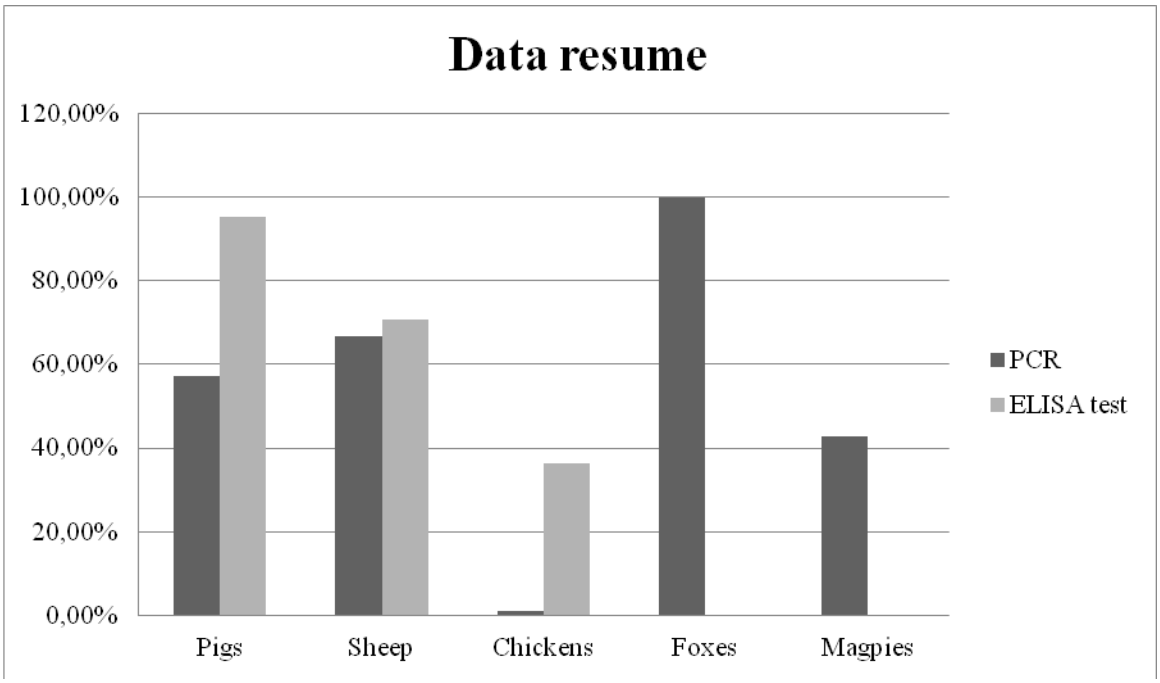
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
TPA: Toxoplasma gondii VEG, chromosome chrVIII, complete genome	403	403	100%	7e-109	99%	LN714498.1
Toxoplasma gondii from skunk surface antigen 2 (SAG2) gene, partial sequence	403	403	100%	7e-109	99%	DQ000461.1
Toxoplasma gondii strain C56 SAG2 (SAG2) gene, partial cds	399	399	100%	9e-108	99%	KJ524441.1
Toxoplasma gondii P22 gene for surface antigen, partial cds, strain: Tehran	399	399	100%	9e-108	99%	AB667974.1
Toxoplasma gondii P22 gene for surface antigen, partial cds, isolate: S4	399	399	100%	9e-108	99%	AB667973.1
Toxoplasma gondii P22 gene for surface antigen, partial cds, isolate: S7	399	399	100%	9e-108	99%	AB667972.1
Toxoplasma gondii cDNA, clone: XTG05125.2, full cDNA, XTG Sugano cDNA library	399	399	100%	9e-108	99%	AK317818.1
Toxoplasma gondii ME49 surface antigen P22, mRNA	399	399	100%	9e-108	99%	XM_002365730.1
T.gondii surface antigen P22 mRNA, complete cds, clones:c(86,88, 120,122)	399	399	100%	9e-108	99%	M33572.1

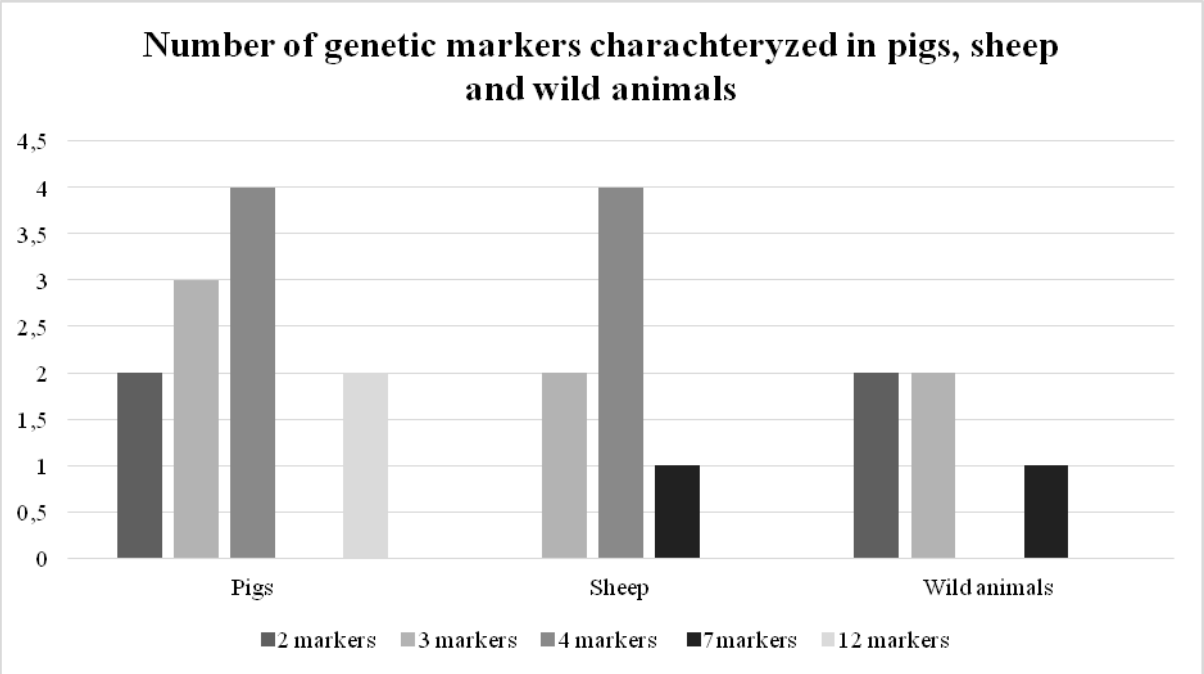
Figure n. 18: *screenshot of results obtained blasting the contig of sample n. 3 fox for 5'sag2 marker.*

5.5 Summary of results

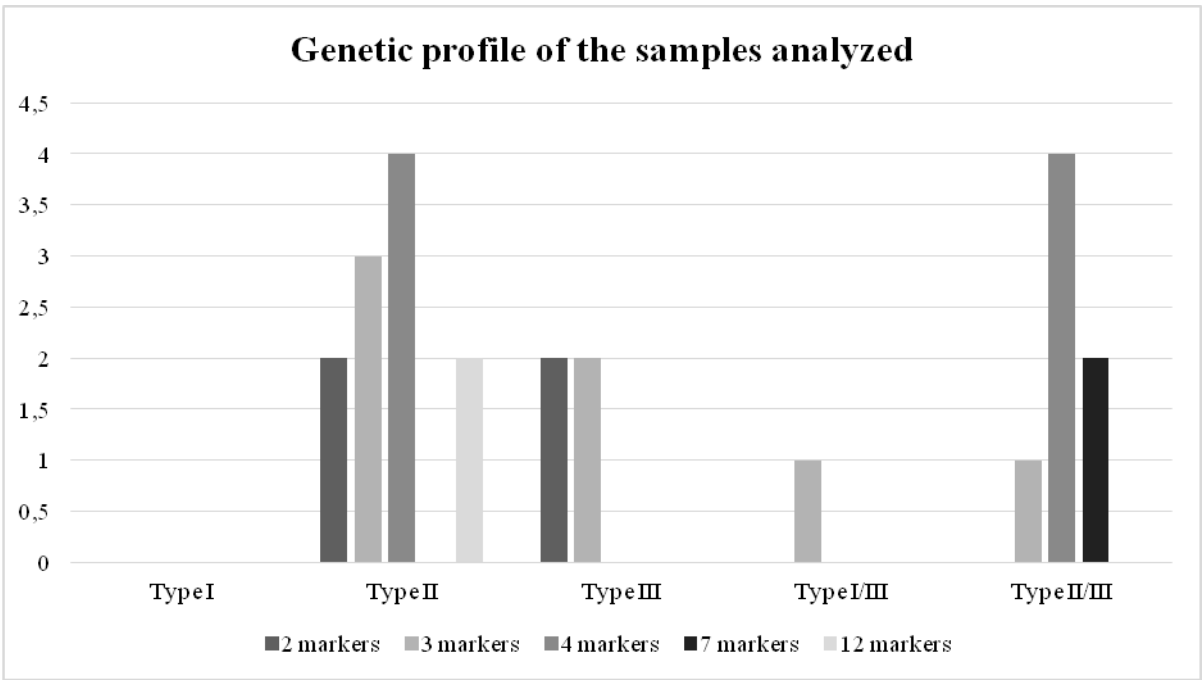
The figures below shows a summary of the results obtained for the prevalence of *T. gondii* infection and genotypes in the different species analyzed.



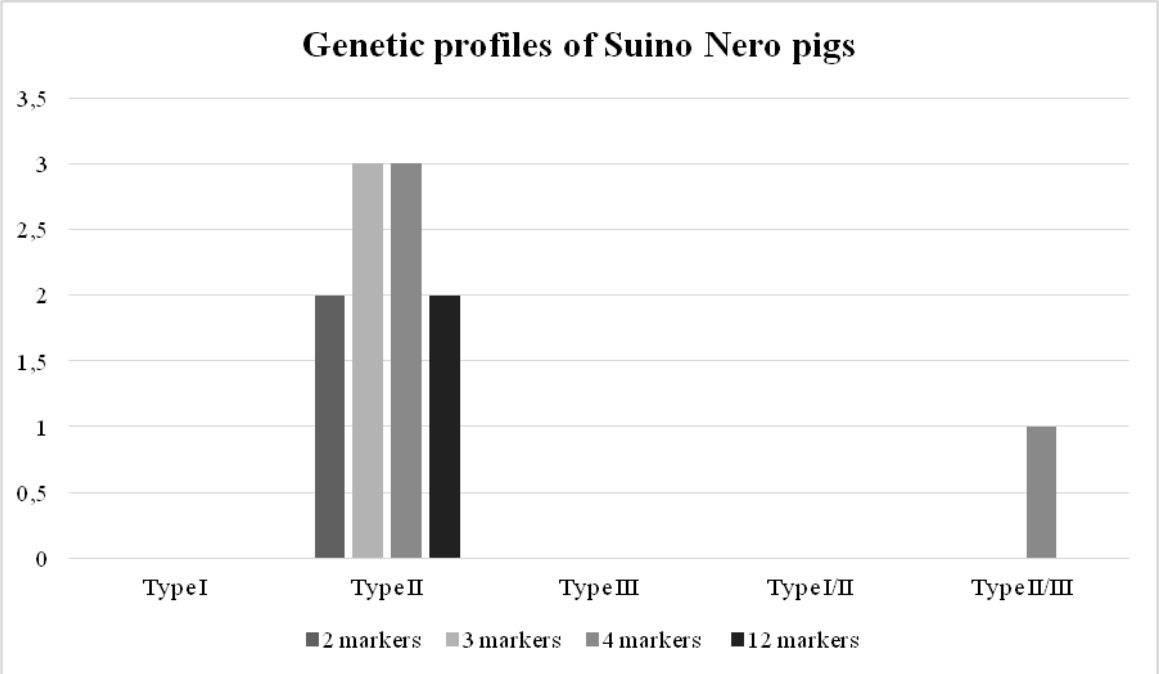
Graph n. 6: *histogram resuming the results for the infection of T. gondii in different animal species.*



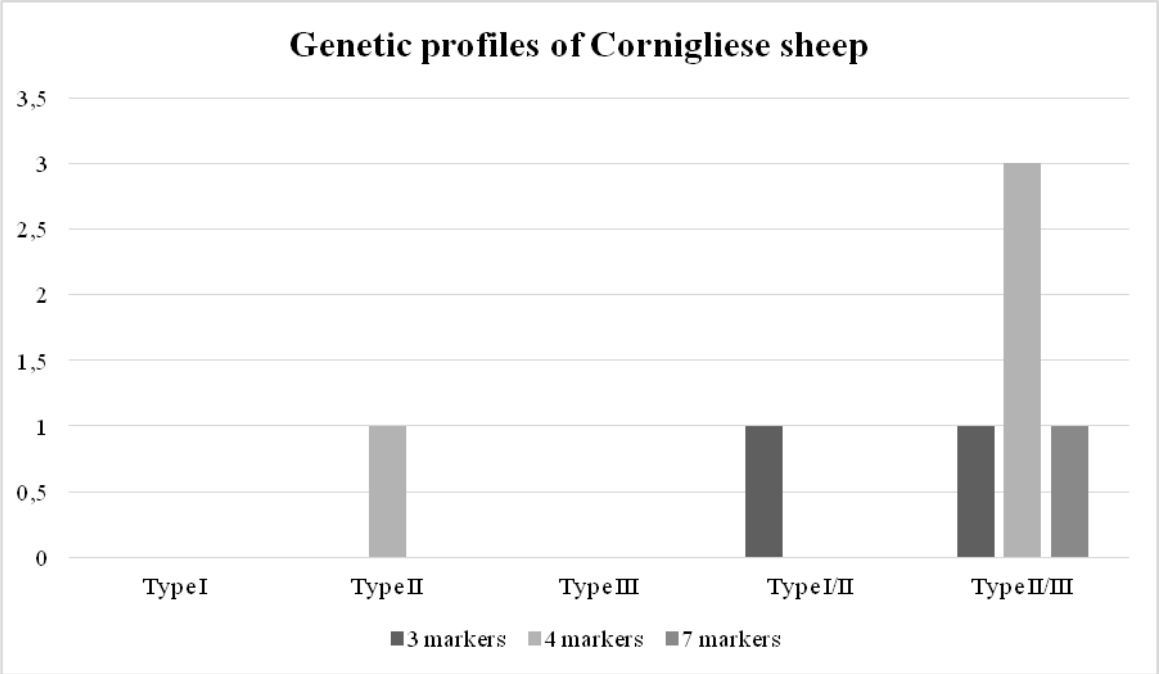
Graph n. 7: *Number of genetic markers characterized in different animal species*



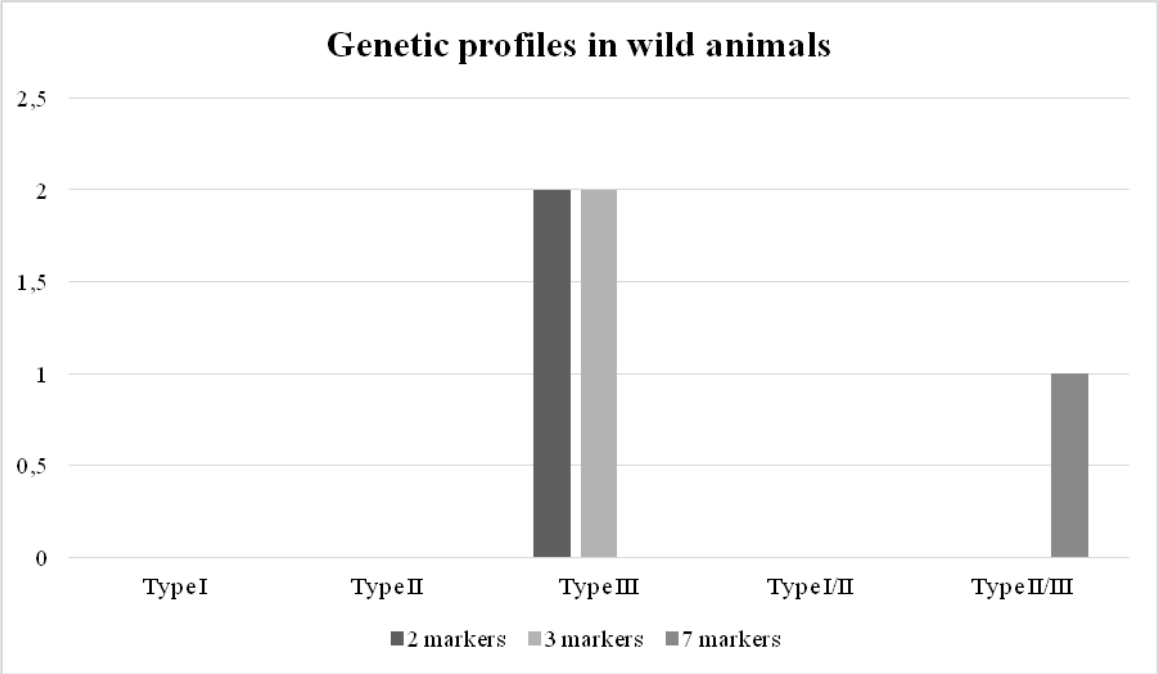
Graph n. 8: *genetic profiles of the samples analyzed. All the samples are divided basing on the number of the genetic markers amplified and on the genetic profile that have been characterized.*



Graph n. 9: *genetic profiles of Suino Nero pigs. Type-II pattern was highlighted in the majority of the samples.*



Graph n. 10: *genetic patterns of Cornigliese sheep. Mixed profiles are predominant.*



Graph n. 11: *genetic patterns in wild animals. A predominant Type-III profile was highlighted.*

5. Discussion

Toxoplasma gondii is a coccidian parasite considered the second leading cause of foodborne disease–related deaths and the fourth leading cause of foodborne disease–related hospitalizations in the United States (Scallan et al., 2011). The importance of this parasite as a risk for human health was recently highlighted by EFSA's opinion on modernization of meat inspection, where *Toxoplasma gondii* was identified as a relevant hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer (Call for proposals - GP/EFSA/BIOHAZ/2013/01). This, together with a growing demand by the consumer for organic products, coming from free-range livestock, in respect of animal-welfare, and the desire for the best quality of derived products, have all led to an increase in the farming of free-range animals. The risk of *Toxoplasma gondii* infection increases when animals have access to environment and the absence of data in Italy, together with need for in depth study of both the prevalence and genotypes of *Toxoplasma gondii* present in Italy were the main reasons for the development of this thesis project.

The first part of the study took into consideration the prevalence of infection by *T. gondii* in free-range pigs reared in the North of Italy. All but one of the pigs tested (95.2%) were positive for *T. gondii* antibodies. This prevalence was higher if compared to the few studies carried out in confinement-raised pigs in Italy, that report seroprevalence values ranging from 16 to 36%; (Ranucci et al., 2013; Veronesi et al., 2011; Villari et al., 2009). Results of the present study confirm that outdoor access dramatically increases the risk for *T. gondii* infection.

In the present study, direct PCR for the 529bp marker on tissue samples was significantly less sensitive than serology, with a prevalence of 57.1%. This lower sensitivity has been reported by others (Hill et al., 2006a; Hamilton et al., 2015).

Hill et al. (2006a), when comparing diagnostic methods in both experimentally and naturally infected pigs and retail pork products, reported values of sensitivity of 100% for serum ELISA, of 76.9% for tissue fluid ELISA and lower values for PCR methods. In particular, the real-time PCR showed a sensitivity of 20.51%, 12.82% was the value of the semi-nested PCR (12.82%) and 0% for direct PCR (0%). The authors cited different reasons for this lack of sensitivity, including limited sample size and random distribution of tissue cysts. The same limitations were likely present in this study.

Hamilton et al. (2015) reported values of positive serology in pigs, sheep and goats of 48%, 26% and 34%, respectively. On the same samples *T. gondii* DNA was detected in 21.0% of pigs'

samples, in 16.0% and 23.0%% of sheep and goats respectively. The data reported demonstrated one more time that serology is more sensitive than molecular biology methods for identification of *T. gondii* infection. The authors suggested extracting DNA after the acid peptic digestion as a way of improving the quality of extracted DNA and the problem of interaction with host DNA. Indeed, digestion causes destruction of muscle fibers and the cysts' wall, with subsequent release of parasites, thus allowing a greater amount of DNA to be obtained from the total amount of tissue (50-100 g) used for extraction.

Meat juice (MJ) serology has been reported as an excellent method for testing animals at slaughter to eventually exclude the commercialization of meat from *Toxoplasma gondii* positive-animals. In fact, in the absence of serum, a piece of muscle tissue, diaphragm for example (already used for the research of *Trichinella* spp.), could be frozen for 18-24 hours and after thawing, the "juice" obtained, used for the detection of IgG antibodies with a validated commercial ELISA test. Results from the present study would confirm the performance of meat juice serology. Indeed, all experimentally infected pigs that seroconverted at 45 days post-infection when tested with serum were also positive with high OD values after slaughter when meat juice serology was performed. The same result was obtained by other authors confirming that meat juice serology has proven to be an excellent method for detection of *T. gondii* infection at slaughter in pigs and has been confirmed to correlate well with serum serology (Hamilton et al., 2015; Meemken et al., 2014; Basso et al., 2013;).

With the aim of satisfying the pressing demand from EFSA for rapid methods of *T. gondii* detection in different animal species, meat juice serology could be a valid screening method at slaughter.

In contrast to conventional systems, organic standards require that animals are raised with outdoor access. In Italy, organic pig farming regulations normally require that farrowing and weaning take place outdoors and that fatteners are kept either outdoors or indoors with an outdoor run. The examined animals had outdoor access for at least 8 months a year and for the remaining four months were kept indoors on straw bedding.

Parasitic pressure is definitely higher in free range pigs compared to confinement reared animals. As highlighted by Dubey et al. (2012): "access to organic material contaminated with cat faeces or to rodents or wildlife potentially infected with *T. gondii* during outdoor pasturage substantially increases the risk of exposure of pigs to *T. gondii"*. The authors reported that 30 out of 33 pigs had antibodies against the parasite and viable *T. gondii* was isolated from 17 of these.

A single *T. gondii* infected pig can be a source of infection for many consumers, since one market weight hog (100 kg or more) can yield over 600 individual servings of meat. Although seroprevalence of *T. gondii* in market hogs raised in confinement has decreased drastically in the last decade (Dubey and Jones, 2008; Hill et al., 2010; Kijlstra et al., 2004) high prevalence has been recorded in free-range pigs in poorly managed facilities (Dubey et al., 2002a, 2008b).

Results of gentyping from the present study represent one of the first reports on *T. gondii* genotypes circulating in Italy. Unfortunately, the genetic pattern was not completed for all the animals analyzed. After sequencing and repeated genotyping with several markers, the predominance of a Type-II profile was evident. For some loci a Type-I and a Type-III pattern was highlighted. As mentioned previously, the low quantity and poor quality of *T. gondii* DNA extracted from heart tissue is a major limitation of this study. The PCR used here amplified a marker (529bp) that is repeated approximately 300-times in the *T. gondii* genome (Su et al., 2010*).* On the other hand, the protocol used for the genetic characterization amplified single-nucleotide markers. This could be one explanation for the incompleteness of the genetic profiles obtained. Other Italian authors such as Mancianti et al. (2013, 2014) reported the presence of common genotypes II and III in naturally infected donkeys and genotypes II, III and the possible presence of atypical, Type I genotypes in goats. However, the genetic profiles were not complete.

Organically bred swine have access to potentially new strains of *T. gondii* that may circulate in wildlife. These farms are typically located in more isolated, rural areas that likely share the same habitat with wild boars, ungulates and birds. Indeed, Grigg and Sundar (2009) suggested that a large diversity of *T. gondii* strains infecting a variety of prey in the selvatic cycle can produce new strains that are capable of clonal expansion and may sweep the domestic cycle. Recombination events could potentially generate more *T. gondii* strains that may possess new biological properties, such as increased virulence for humans. It would be of interest to expand this research to a larger number of farms in different areas of Italy to make a map of the major genotypes of *T. gondii* diffused in our country.

Looking at the data reported here it could be affirmed that food products derived from pigs reared in organic and free-range farms represent a risk for health's consumers. As these animals are often bred for the production of typical sausages or salami that can be consumed either raw (during processing) or undercooked, the zoonotic risk of *T. gondii* infection should not be ignored.

T. gondii infection and subsequent evaluation of food-borne risk were also evaluated in experimentally infected pigs. A total of 30 pigs were infected with 1000 oocysts of *T. gondii* (field strain)/each. This is a relatively low dose, considering that as many as 1 million oocysts may be present in 1g of infected feline faeces (Dubey and Frenkel, 1972).

All animals showed clinical signs, including fever, weight loss, inappetence and poor general conditions, as also reported by Wingstrand et al., (1997). Serology carried out at five different time points showed the presence of specific IgG antibodies against *T. gondii* from the 45th day after the infection. Meat juice serology was performed on fluids obtained from a part of each thigh: all the samples resulted positive for IgG antibodies with high OD values, highlighting the sensitivity of the method. Wingstrand et al. (1997) reported similar serological data in 17 experimentally infected pigs in Denmark. Different serological tests (IFAT and Western blot) were evaluated by Basso and colleagues (2013) and both the ELISAs (PrioCHECK® Toxoplasma Ab porcine ELISA and TgSAG-1 ELISA) tested showed a higher specificity and sensitivity compared to other methods. This again highlights that ELISA is a rapid and sensitive method useful for identification of a specific immunological response.

Eighteen of the infected pigs were used by the group of "Istituto Zooprofilattico of Brescia" to evaluate the presence and viability of *T. gondii* in different commercial cuts of pork meat. The food risk associated with the evaluated cuts was high. The cuts of pork considered here are those usually cooked by grill and consumed undercooked. Our contribution included the digestion and seeding of homogenates on Vero cells and applying the Elisa test on meat juice. At the moment of writing, only preliminary results, obtained with bioassay and MJ serology, are available. They suggest that the distribution of the parasite is nearly uniform in all the cuts and at least two mice out of five infected for each cut had brain cysts. The most heavily infected organs, however, were the heart and brain, as previously reported (Dubey et al., 1986).

It can be confirmed that grilled pork meat infected with *T. gondii* represents a real and important risk for human health.

The viability of the parasite following the aging process was also evaluated in the present study. Experimentally infected pigs were used for the production of "Prosciutto di Parma" on *T. gondii*. Parma ham is a cured product whose production is limited in the area of Parma province and the regulation methods are dictated by Consorzio of Parma Ham. The legal recognition of the

"Consorzio del Prosciutto di Parma" means that the marking is a real mark of the State, as well as a guarantee of quality and sweetness, and the absolute respect of the traditional methodology, legally sanctioned (Law 26 of February 13, 1990 and DL 253 of 15 February 1993) (www.prosciuttodiparma.com/it_IT/prosciutto/segreti). The regulation foresees that only salt (NaCl) is be added to cured meat and the minimum period for curing is 12 months. The survival time of tissue cysts is highly dependent on the concentration of the salt solution and the temperature of storage. It is well known that isolated tissue cysts can survive for 56 days in a solution of 0.85% salt, 49 days in 2% salt and 21 days in 3.3 % salt (Dubey, 1997). Under laboratory conditions, Dubey found that tissue cysts were killed in 6% NaCl solution at all temperatures examined (4 to 20 at °C) (Dubey, 1997) and more recent data have indicated that injection of >2% NaCl and/or >1.4% lactate salt solutions into experimentally infected pig meat could kill the parasite (Hill et al., 2006b). The medium percentage of salt concentration contained in Parma ham is 5.7%. The preliminary results obtained with bioassay and RT-PCR on DNA extracted from cell cultures inoculated with homogenates of hams indicate that the production process is able to kill the parasite. No brain cysts in infected mice have been found for the first six hams analyzed and no increase in the amount of DNA of *T. gondii* have been highlighted in RT-PCR. Similar results were recorded by Bayarry and colleagues (2010) studying the viability of *T. gondii* in cured ham in Spain. Viable *T. gondii* was found, using bioassay, in hams at 7 months of curing (middle time of seasoning) but no viable parasites were found at the end of curing (14 months), where the salt concentration reaches 3.9%. The loss of viability of the bradyzoites and tachizoytes during the curing process may be due to the dehydratation suffered by the salting and subsequent curing process.

On the contrary, another study carried out in Spain on Serrano ham using magnetic capture and bioassay revealed the presence of *T. gondii* (Gomez-Samblas et al., 2015). They analyzed 475 ham samples from seven different companies, comprising vacuum-packed sliced hams and unpacked ham pieces bought at super-market. The global prevalence of *T. gondii* was 8.84% with a viability rate of 4.84%. The data also revealed that, on the samples tested, the 7.94% were positive when conserved in vacuum-packed and 9.58% when conserved without vacuum-packed process. In some companies the prevalence of the parasite was zero, whereas in other it reaches 32%. The differences in the viability of *T. gondii* were probably related to the time required for the curing process. However, possible post-processing contamination by oocysts was not considered in this study, suggesting that the infection by tissue cysts may have been overestimated.

The curing process employed in this study appears to produce hams that do not pose a serious concern to human health and therefore could be marketed and consumed without significant health risk. However, further studies are needed to complete the risk assessment and the analysis of other products cured using technological processes other than those investigated in the present study. For example, it could be interesting to analyze products such as salami, produced with pig meat all over the Italian country, with very different recipes, also in domestic and rural contexts, characterized by a very short period of curing (1 to 6 months).

The seeding process carried out on fresh thighs and hams derived from infected pigs has several limitations. This technique was attempted in order to find an alternative and sensitive method in substitution of bioassay. In a proposal call, EFSA clearly asked for new methods for meat inspection for the identification of *T. gondii* (Call for proposals -GP/EFSA/BIOHAZ/2013/01) and for this reason the method was evaluated on the experimentally infected pigs. From a theoretical point of view the protocol exactly reproduces what happens in nature. The acid-peptic digestion has the purpose to destroy the cyst's wall to release the bradyzoites; the contact with the cell monolayer for three hours should allow the penetration of the parasite into cells.

One of the limitations was the absence of a positive control to digest and seed together with the samples to be analyzed. A second weak point was the high level of cell culture contamination due to bacteria and mold, mainly recorded for fresh meat and fresh thighs. This has also been reported by Zintl et al. (2009), who previously described the use of cell culture and semi-quantitative PCR as a method for evaluation of *T. gondii* infection and viability of the parasite in substitution of bioassay. Other studies have suggested using a different method for the in vitro isolation of tachyzoites: suspected infectious material is fed to mice and different organs (lungs, brain or peritoneal fluids) of positive mice is seeded on cell cultures to obtain tachyzoites. In this way, contamination can be avoided (organs are sterile), they are sure to seed infected material and they also obtained purified tachyzoites avoiding the problem of hosts' tissue contamination during genotyping (Unzaga et al., 2014; Dubey et al., 2013).

EFSA in recent reports on zoonoses highlighted that an increasing numbers of animals resulted infected with *T. gondii* in EU (reported by the European Member States for pigs, sheep, goats, hunted wild boar and hunted deer, in 2011 and 2012). In addition, positive findings were detected in cats (the natural hosts), cattle and dogs as well as several other animal species, indicating the wide

distribution of the parasite among different animal and wildlife species (EFSA, 2014). Currently, there are no identification systems for *Toxoplasma gondii* infection at slaughter, in any animal species, neither in USA nor in Europe. Meat juice serology (using validated ELISA test) could be a sensitive, rapid and low cost method useful to identify *T. gondii* at slaughterhouse in different animal species. Pigs producing-meat and meat-products are certainly the most important host that should be controlled at slaughterhouse. All this in the prospect of a relevant economical value and for the parasitic pressure; pigs, in fact, resulted one of the animal species more sensitive to the infection with *T. gondii* (Dubey et al., 2005).

Results for Cornigliese sheep concerning sensitivity of serology and molecular methods for the identification of *T. gondii* infection are similar to those reported for organic pigs. MJ serology found that the number of positive animals with IgG antibodies was 70.8% while PCR revealed a positive rate of 66.6%.

The serology data related to Cornigliese sheep and to 587 adult sheep analyzed here showed an high level of infection (70.8% and 78.2%, respectively) in this geographical area. Similar values were reported in Belgium by Verhelst et al. (87.4%) (2014) and in Tunis City by Boughattas et al. (73.6%) (2014), while lower values have been recorded in Morocco (27.6%) (Sawadogo et al., 2005) and in UK (54.2%) (Hutchinson and Smith, 2015). Several studies carried out in recent years have reported prevalence values of *T. gondii* infection in sheep in Italy ranging from 66-97% (Zedda et al, 2010; Cenci-Goga et al, 2013; Chessa et al, 2014; Gazzonis et al, 2015) and important risk factors for infection include extensive grazing and transhumance.

T. gondii has been reported as the cause of over 25% of ovine abortions (Gutierrez et al, 2012). Even though we have no data concerning the prevalence of abortion from the herds studied here, it is likely that *T. gondii* plays an important role in reproductive failure in these animals as well.

Future studies should concentrate upon further evaluation of the reproductive and economic effects of these parasitic infections, in light of the necessary conservation of local, indigenous sheep breeds. It is thought that the Cornigliese sheep has its origins from cross breeding between local breeds and the Merino sheep breed, as early as the 1500's (Ceccobelli et al., 2015). It has been reported that Merino sheep are particularly resistant to gastro-intestinal helminth infections (Kelly et al, 2013). It would be of interest to evaluate if the Cornigliese sheep breed is naturally resistant to the pathogenic effects of infections by *T. gondii*.

The high seroprevalence observed in the present study was confirmed by molecular identification of the parasite. Obviously, the presence of parasite DNA is not indicative of viable parasites and this is important from the point of view of the zoonotic potential of *T. gondii*. In vitro isolation has been reported as a possible alternative to bioassay for determining the infectivity of *T. gondii* tissue cysts in sheep (Zintl et al, 2009; Vilares et al, 2014). Zintl et al. (2009) described an in vitro isolation method followed by real-time PCR to evaluate parasite growth in culture and viable parasites were identified in 89% of placentomes from 4 experimentally-infected ewes. It would be of interest to develop in vitro methods that are able to determine viability of parasites in infected meat at slaughter. In the present study, only one out of four cultured tissues resulted positive in PCR for the presence of *T. gondii* following seeding onto Vero cells. Further work is likely necessary to validate this technique for wide scale use in meat/dairy animals.

Genotyping of nine samples positive in PCR showed a predominance of Type II/III or Type II patterns, even though several loci were unable to be amplified. These results, which should be confirmed by further genotyping analysis, may suggest that there are different genotypes circulating in the area where these sheep are grazing, but would indicate the absence of the more virulent, Type I genotype. The presence of mixed genotypes could be explained by the presence of mixed infections, that appears also to be frequent in other countries (Boughattas et al., 2014; Lindstrom et al., 2008).

Even if the foodborne-risk of sheep-derived products is lower than for other animal species because sheep-meat is usually consumed well cooked, this part of the study gives important data relating to the diffusion of the parasite in our territory. Transhumant sheep have a higher risk of contracting *T. gondii*, circulating in wildlife, in particular with atypical strains. This should be studied further to better understand the role of these animals in the diffusion and genetic diversity of the parasite.

Several recent studies have evaluated sheep milk as a potential source of infection of *T. gondii*. In Campania region (Italy), of 117 milk samples examined (10 milk samples collected for each farm and pooled) the presence of *T. gondii* DNA was detected by PCR in 4 milk samples (3.4%) (Fusco et al., 2007). A Brazilian group studied the contamination of sheep milk with *T. gondii*. *T. gondii* DNA was detected in seven milk samples from five seropositive sheep (5 out of 20 sheep). Sequences of species shared 97-100% identity with *T. gondii*. The authors suggested that the peripartum period may lead to the resurgence of tissue *T. gondii* tachyzoites cysts which can

circulate again and be excreted in the milk (Camossi et al., 2011). Evaluation of foodborne risk associated with raw milk (and cheeses) of Cornigliese sheep is certainly an aspect to study in depth in the near future.

Chickens are important intermediate hosts of *T. gondii* (Dubey, 2010b). A recent review has reported that prevalence of *T. gondii* in free-range chickens can be as high as 100% (Dubey, 2010b), confirming their role in the epidemiology of infection and the related zoonotic risk through consumption of undercooked chicken meat. Chickens experimentally infected usually do not showed symptoms related to clinical toxoplasmosis (Biancifiori et al. 1986; Dubey et al. 1993) However, Dubey et al. (1993) described cases of death due to acute toxoplasmosis in chickens after inoculation of a high dose (100,000 oocysts) of the *T. gondii* GT1 strain. Differences in infectivity and pathogenicity in avian toxoplasmosis may be explained by the use or occurrence of *T. gondii* strains of variable virulence and infective doses. Nonetheless, as confirmed by Geuthner et al. (2014), gallinaceous birds have a high tolerance with respect to high infection doses of *T. gondii*.
Serum serology for *T. gondii* in chickens can be carried out with different methods, including MAT, IFAT and ELISA. Sensitivity and specificity vary and depend on different factors, including age, number of animals sampled, and test used. Currently there are no available ELISA tests for *T. gondii* serology in chickens. However, Casartelli-Alves et al. (2014) recently reported sensitivity and specificity of 85% and 56% respectively, in naturally exposed chickens, using a commercial ELISA adapted to chicken sera. Meat juice serology (MJS) is increasingly used to determine the prevalence of various pathogens in different animal species, including *T. gondii* infection in pigs and sheep (Lundèn et al., 2002; Berger-Schoch et al., 2011; Glor et al., 2013). There are currently no reports on the use of MJS for *T. gondii* infection in chickens at slaughter. Dubey et al. (2005) applied the ELISA technique to evaluate the presence of anti-*T. gondii* antibodies in tissue fluid obtained from retail breast meat, reporting a prevalence of 1.3%. In the present study, hearts were used for obtaining meat juice. A recent study has shown that comparison of *T. gondii*–specific antibody titers in meat juice and serum of pigs revealed a strong positive correlation for meat juice from heart tissue, making this the organ of choice for MJS (Wallander et al., 2015). The commercial kit used in the present survey has been validated for multiple species, but not for chickens, and the protocol was adapted by changing the species-specific conjugate and testing performance with serum from experimentally infected chickens. Serum from experimentally infected chickens was used both in toto for kit validation and at a 1:40 dilution when testing meat juice samples in order to

decrease antibody concentration and to better mimic OD values in meat juice, which are consistently lower compared to serum (Wallander et al., 2015). Positive titers in meat juice collected from the hearts were established in two different ways, according to manufactures' instructions and according to Dubey et al. (2005), resulting in a prevalence of approximately 0% and 36.4% respectively. The results obtained from the two evaluation methods were very different. If the positive control had a very high concentration of IgY, thus giving a high cut off OD value, samples from chickens with low antibody titres may have resulted negative with the S/P% calculation. On the other hand, 5 times the mean standard deviation of the mean of all samples tested may have resulted in a higher number of false positive samples. It would be necessary to further study the validation of this kit with experimentally infected birds.

Considering 5*SD values, prevalence values were notably different between the two farms (62.8% and 6.45%), probably due to different farm management. The large scale, industrial free-range chickens are kept under strict biosecurity measures, including barriers which inhibit access by cats, thus making soil contamination by oocysts nearly impossible. The backyard farm in northern Italy, on the contrary, is family run and the grounds are open to the surrounding countryside.

Parasite-specific PCR was negative in all the samples, thus it was not possible to verify positive serology with the presence of *T. gondii* in myocardial tissue of naturally-exposed, free-range chickens. Geuthner et al. (2014) also reported a very low prevalence of *T. gondii* DNA in muscle tissue from experimentally infected chickens (2.1%), suggesting that *T. gondii* does not persist for long periods in this species. Dubey et al. (2005) found no positive samples of retail breast meat when tissue was bioassayed in mice. The results of the present study would confirm these previous reports and would suggest that while positivity in MJS may be an indicator of infection risk, it likely does not correlate with detection in the meat. The limited amount of sample material that it was possible to use for DNA extraction and the following PCR is another point to consider for the absence of correlation between MJ serology and PCR results, as affirmed also by Geuthner et al. (2014).

In Italy, free-range farms have increased in recent years and approximately 3,500,000 chickens were raised in 2013 (www.istat.it), making Italy one of the most important producers in Europe. For its features, free-range livestock could be a source of infection of *T. gondii* and an indicator of contamination, even if studies on infection prevalence in Italian poultry are still limited.

Considering the data analyzed here, it can be concluded that the validation of a specific ELISA test for the identification of infection with *T. gondii* is an urgent need, in order to satisfy the demands of EFSA and for the large amount of gallinaceous meat consumed annually worldwide.

Another aspect that should be analyzed in depth is the seroprevalence of infection of *T. gondii* in turkeys. A recent study demonstrated the persistence of *T. gondii* in experimentally infected turkeys up to 16 weeks after infection, i.e. over a whole fattening cycle. Thigh and breast muscle were positive for *T. gondii* DNA showing that often several muscles are parasitized simultaneously. All these data indicate that an infection of turkeys with *T. gondii* possibly leads to *T. gondii*-positive edible tissues and a potential risk for infection of the consumer cannot be excluded (Geuthner et al. 2014). Poultry meat, in fact, is one of the most consumed in Italy; in 2014 there was an increase of 1.0% in the consumption and production.
The avian meat consumption in Italy is 13.92 Kg for chicken meat and 4.31 Kg for turkey (www. Istat.it). Overall, avian meat consumption per capita resulted 19.51 kg (+0.9%) in 2014.
In Italy, a total of 117.8% of turkey meat consumed is produced in our country, confirming that the sector of poultry meat production is completely self-sufficient (http://www.unaitalia.com). All this could support the interest to study in depth the possible food risk associated with turkey meat in Italian country.

A small number of wild animals were analyzed in the present study. The aim was to obtain preliminary data concerning the infection rate in wildlife in our geographical area, given the scarcity of current data at the time of writing. The majority of studies have been carried out in Piemonte region or in general in the Alps or Apennines areas. A recent study by Ferroglio et al., (2014) recorded a relatively high prevalence (p = 10.99%) of *T. gondii* and evidenced a widespread presence of the parasite in wildlife species. The parasite was absent or was found at very low prevalence in ruminants, while higher prevalence of infection was recorded in omnivores (wild boar p = 16.19%) and carnivores (red fox p = 20.21%) respectively. These findings reflect the higher probability of a carnivore or omnivore to consume tissues infected with *T. gondii* than the probability of an herbivore to ingest *T. gondii* oocysts from the environment. This is especially true in epidemiological contexts where there is only one species acting as definitive host (in the studied area it is the domestic cat) and contributing to oocyst dissemination (Smith et al., 1995).

Our results show a high infection rate by PCR for foxes (4/4) and magpies (9/21). Seroprevalence for red foxes have been reported as ranging from 14% in Germany (Hermann et al., 2014) to 16.1% in Central Italy (Mancianti et al., 2010), 18.8% in Belgium (De Craeye et al., 2011) and 68% in Hungary (Jakubek et al., 2007). To the authors' knowledge, there is no published data for magpies or in general for corvids. However, a recent study conducted in California by Straub et al. (2015) indicated that free-flying condors had a seroprevalence of 3% (3/92) for *T. gondii*, in golden eagles was 15% (4/26) and in turkey vultures 11% (7/66). Wild birds may represent an important source of infection for domestic animals and for the spread of new and recombinant genotypes (Wendte et al., 2011).

In this study, out of the 13 samples positive for *T. gondii* DNA, one sample (magpie n. 8) was genotyped at seven loci, showing Type II/III profile. Magpie n. 21 showed a Type–III pattern with genotyping by four markers. All other samples were only partially genotyped: samples n. 10 and 15 had a predominant Type-III pattern, considering the studied alleles. Only the C22-8 marker was characterized in foxes and the genotype for that allele was Type-II/III. As seen before the genotyping was only partial for the reasons cited before. The studied samples showed mainly a Type-III and Type-II/III profile. A research conducted in China typed three isolates from wild birds at 10 genetic markers with complete data for all loci, and two genotypes (Type I and Type II variant) were identified. The results suggested that the Type I and II variant strains are circulating in wild birds in China, and these birds are potential reservoirs for *T. gondii* transmission (Huang et al., 2012). As affirmed before by Wendte et al. (2011) in wild birds it is possible to isolate recombinant and atypical strains of *T. gondii* and the same data was recorded also in Mexico (Alvarado-Esquivel et al., 2011).

Wild animals can be a source of *T. gondii* infection in humans, cats, and other carnivores and can contribute to the spread of new and recombinant genotypes of *T. gondii*. They represent a sort of ring between domestic animals and wilderness and for this reason an in depth study to evaluate this interface should be carried out in the future. Beyond that, the idea that tissue cysts are important as oocysts in the transmission of *T. gondii* and that wild animals (especially wild boar and roe deer) could be an important source of foodborne toxoplasmosis in humans (Ferroglio et al., 2014; Smith et al., 1995) is another point to take into account for future evaluations.

Conclusion and Future Perspectives

This thesis project has highlighted widespread diffusion of *T. gondii* in the geographical area taken into account. Pigs, sheep, chickens and wild animals showed high prevalence of infection, indicating the spread of the parasite among numerous animal species.

Meat juice serology appears to be a very useful, rapid and sensitive method for screening carcasses at slaughterhouse. As indicated by EFSA, and due to the high prevalence of infection recorded in all the European member States, MJ serology could be a valid test for screening and for marketing "Toxo-free" meat. New techniques such as Loop Mediated Isothermal Amplification (LAMP) could represent an important future prospect for meat inspection at slaughterhouse. Recently, this method was found to be a powerful diagnostic tool, and LAMP assays targeting the SAG1 gene (SAG1-LAMP), 529-bp repetitive element, B1 gene, SAG2 gene, and TgOWP of *T. gondii* were developed (Sotiriadoua et al., 2008; Zhang et al., 2009; Kong et al., 2012;). The LAMP assay based on the 529-bp repetitive element was shown to be useful for detection of *T. gondii* DNA extracted from veterinary samples (Zhang et al., 2009;Lin et al., 2012). LAMP is an isothermal nucleic acid amplification technique. In contrast to the polymerase chain reaction (PCR) technology in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature, and does not require a thermal cycler. In LAMP, the target sequence is amplified at a constant temperature of 60 - 65 °C using either two or three sets of primers and a polymerase with high strand displacement activity in addition to a replication activity. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than PCR based amplification. It is a very sensitive, easy, and less time-consuming method. The advantage of this assay is that LAMP products can easily be detected by the naked eye due to the formation of magnesium pyrophosphate, a turbid white by-product of DNA amplification that accumulates as the reaction progresses (Mori et al., 2001; Mori et al., 2004). LAMP products can also be detected by direct fluorescence (Tomita et al., 2008). Fluorescent dyes, such as ethidium bromide, SYBR green, and Evagreen, can also be used for visualization of LAMP products under UV light (Qiao et al., 2007). Development of rapid, low-cost, easy-to-use methods are required for screening test to use at the slaughtehouse in the next future and validate a LAMP protocol, able to discriminate among viable and non-viable parasite should be a perspective.

The free-range farming put in evidence a marked risk for meat-producing animals and as a consequence also for the consumer.

Genotyping revealed the diffusion of Type-II and in a lower percentage of Type-III. There are also mixed genotypes (Type-II/III) that could be explained by the presence of mixed infections. Free-range farming and the contact with wildlife could facilitate the spread of the parasite and the generation of new and atypical strains, with unknown consequences on human health.

Little is known about the diffusion and genotypes of *T. gondii* in wild animals; further studies on the way in which new and mixed genotypes may be introduced into the domestic cycle should be very interesting, also with the use of NGS techniques, more rapid and sensitive than PCR-RFLP. Economic, social and bioclimatic changes are causing ever-increasing contact among wildlife, humans and domestic animals (Daszak et al., 2001) and the role of wildlife as a source of zoonotic diseases should be specially monitored (Daszak et al., 2000). Furthermore wildlife can become a valuable indicator of environmental contamination with *T. gondii* oocysts.

Other future perspectives regarding pigs include the expansion of the number of free-range animals and farms and for Cornigliese sheep the evaluation of other food products as raw milk and cheeses as a food-safety aspect.

It should be interesting to proceed with the validation of an ELISA test for infection in chickens, using both serum and meat juice on a larger number of animals and the same should be done also for wildlife (at the moment no ELISA tests are available and MAT is the reference method for them).

Toxoplasma gondii is one of the most diffuse food-borne parasites globally. Public health safety, improved animal production and protection of endangered livestock species are all important goals of research into reliable diagnostic tools for this infection. Future studies into the epidemiology, parasite survival and genotypes of *T. gondii* in meat producing animals should continue to be a research priority.

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8. Appendix

CURING AND AGING PROCESS FOR THE PRODUCTION OF PARMA HAM ("PROSCIUTTO DI PARMA")

The fresh leg rests for 24 hours in cooling cells until it reaches a temperature of 0 ° C, in this way the meat becomes firm with the cold and can be trimmed easily (**cooling phase**). During the cooling phase, the product undergoes a first weight loss of about 1%.

The second phase involves the **trimming**. It gives to "Prosciutto di Parma" the characteristic rounded shape. The trimming is performed by removing part of the fat and rind, and it promotes the subsequent salting. By trimming the thigh loses muscle and fat for 24% of its weight.

The third phase is **salting**. Salting is done in different ways: the parts of the rind are treated with damp salt, while the lean parts are sprinkled with dry salt. This operation is extremely delicate and must be performed on thighs that are at the right temperature and uniform. A too cold thigh absorbs little salt, while a thigh not cold enough may suffer deterioration phenomena. Then, each thigh is placed in refrigerator at a temperature between 1 and 4 ° C, with about 80% of humidity. After a week spent in this cell, called "primo sale", thighs are cleaned from the residual salt, subjected to a very light salt, and then put into a new cold room, called "secondo sale"; they'll remain there for 15-18 days, depending on their weight. At the end of this period of salting the weight loss it is of about 4%.

After having removed the residual salt, the thigh remains for periods varying from 60 to 80 days in a special cell called "rest cell" (**resting phase**), with a humidity rate of about 75% and a temperature between 1 and 5 ° C. During this phase the product must 'breathe' without moistening or drying out and for thiese reasons air exchange in the cells is very frequent. The absorbed salt deeply penetrates evenly distributed within the muscle mass, and the weight loss in this phase is around be 8/10%.

Thighs are then washed (**washing and drying phase**) with warm water to remove any salt crystals or impurities. Drying was done taking advantage of natural environmental conditions, in sunny and dry days, or in special drying rooms.

Hung on the traditional “scalere”, the hams dry naturally in large rooms with windows (**pre-seasoning**). The windows are opened depending on the indoor humidity, with respect to the outdoor moisture climate or to the product humidity. This operation should allow a gradual drying of the product. The weight loss at this stage is equal to 8/10%.

The bare part of the muscle was covered with “sugna”, a mixture of minced pork fat with a bit 'of salt and pepper and sometimes rice flour (“**sugnatura” phase**). “Sugnatura” performs the function of softening the muscular surface avoiding a more rapid drying compared to the internal ones, while allowing further loss of moisture. “Sugna” is a product of the slaughter, completely natural and without any preservatives.

At the 7th month, the ham is transferred in the "cellar" of the plant, where the particular environmental characteristics have an influence during maturation process (**seasoning phase).** Important biochemical and enzymatic processes occur in this phase and they determine the characteristic aroma and flavor of Prosciutto di Parma.

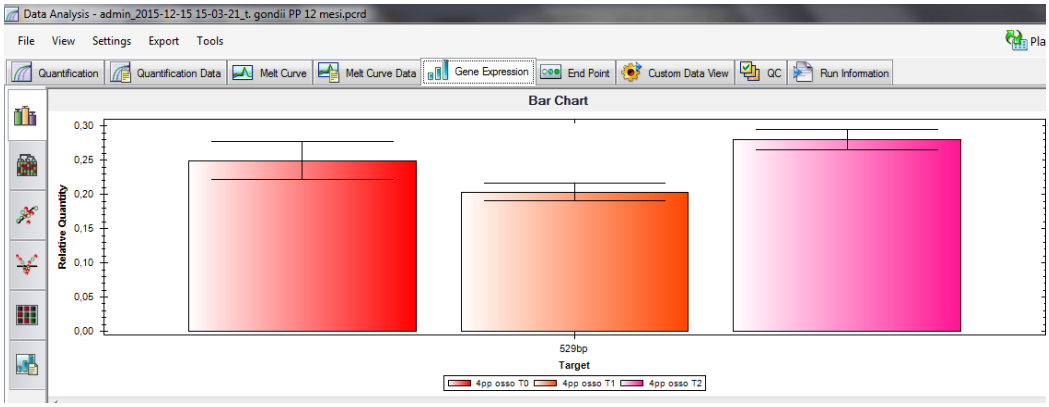
At the end, 12 months later the first salting process, after appropriate findings of the inspectors of Parma Quality Institute, it is affixed the branding of the '5-pointed crown'. The legal recognition of the “Consorzio del Prosciutto di Parma” means that the marking is a real mark of the State, as well as a guarantee of quality and sweetness, and the absolute respect of the traditional methodology, legally sanctioned (Law 26 of February 13, 1990 and DL 253 of 15 February 1993) (www.prosciuttodiparma.com/it_IT/prosciutto/segreti).

Real-time PCR results

Samples n. **4pp, 9pp, 13pp, 16pp** bone-rind-middle part T0-T1-T2

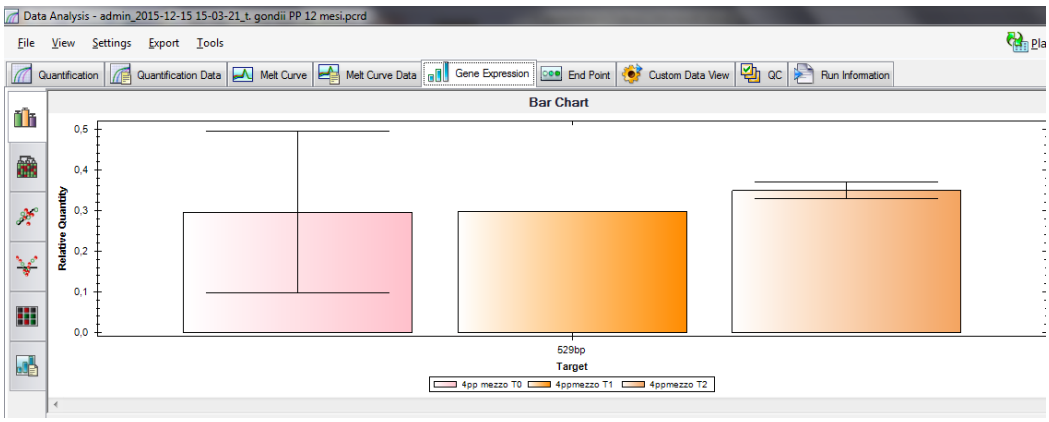
Parma ham **12 months**

4pp bone part

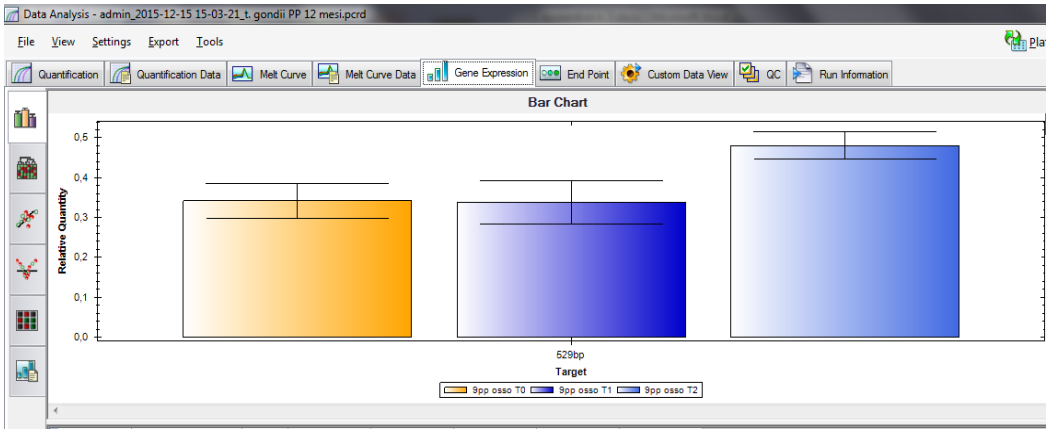


4pp rind part

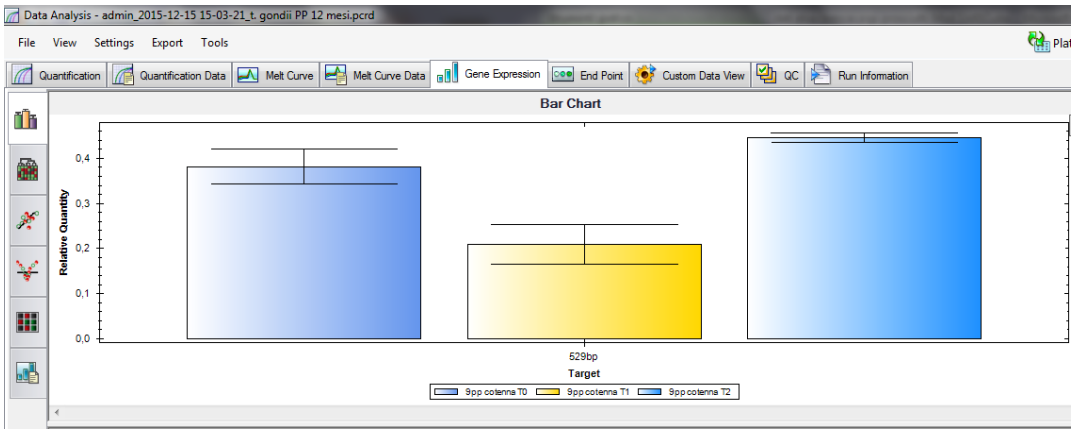
4pp middle part



9 bone part

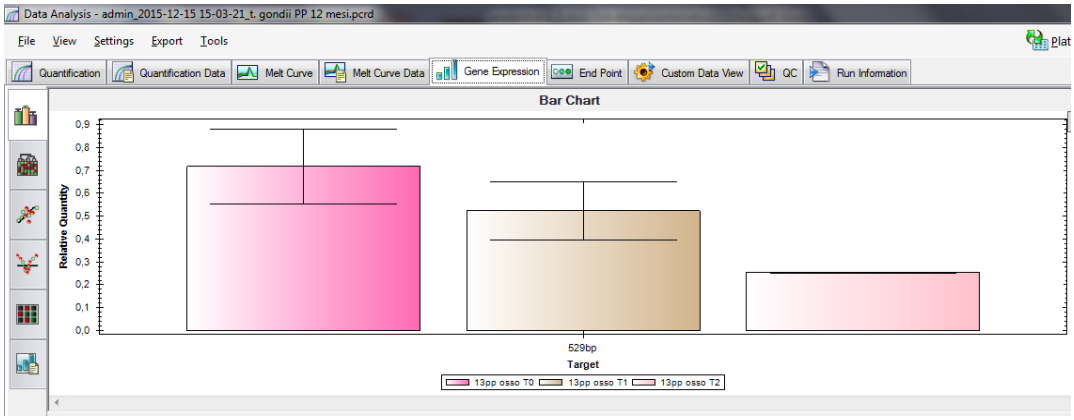


9pp rind part

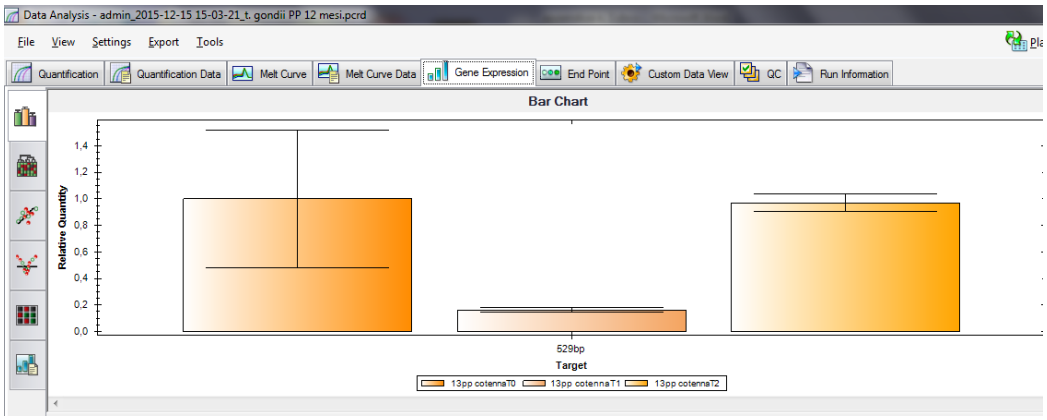


9pp middle part

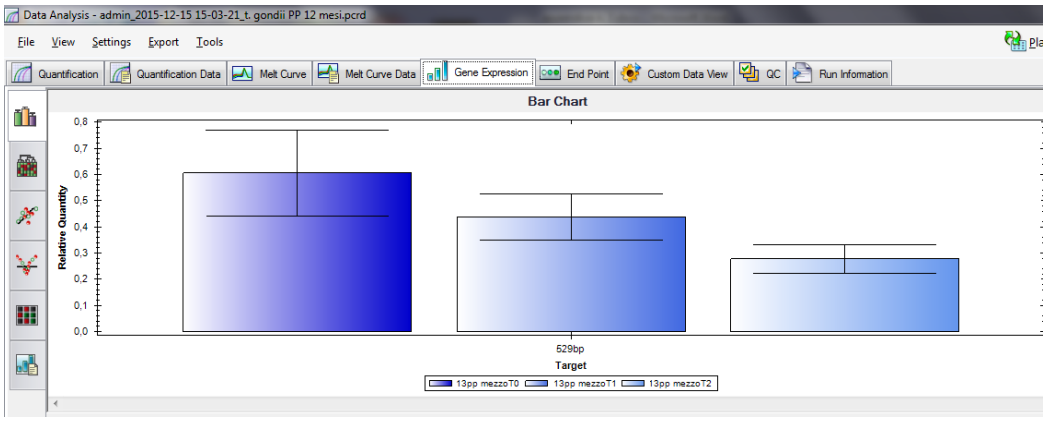
13pp bone part



13pp rind part

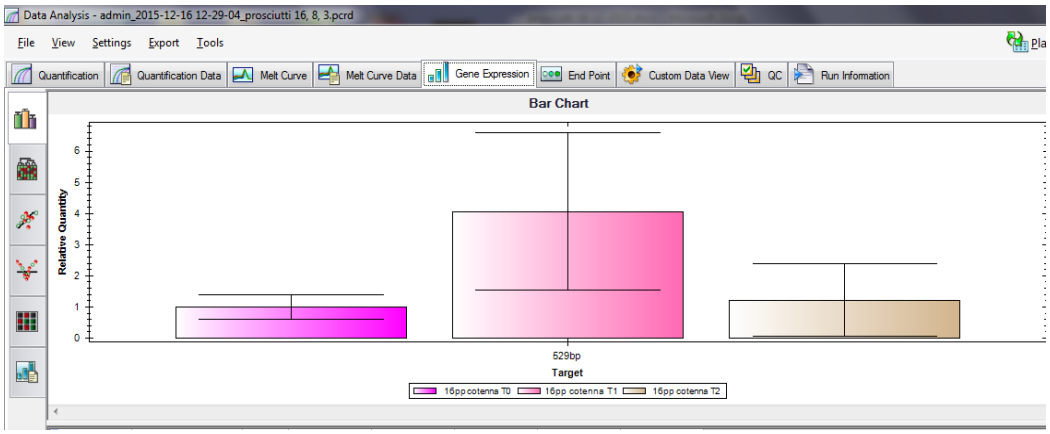


13pp middle part



16 bone part

16 rind part

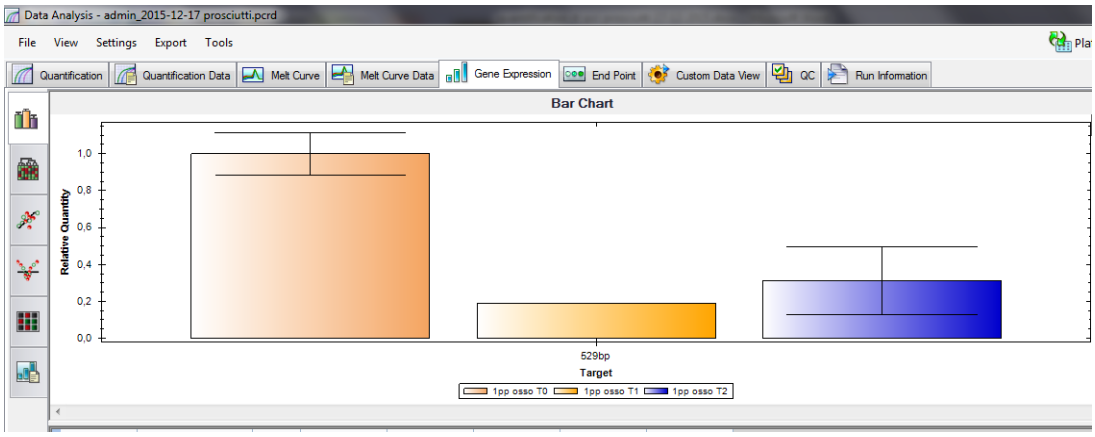


16 middle part

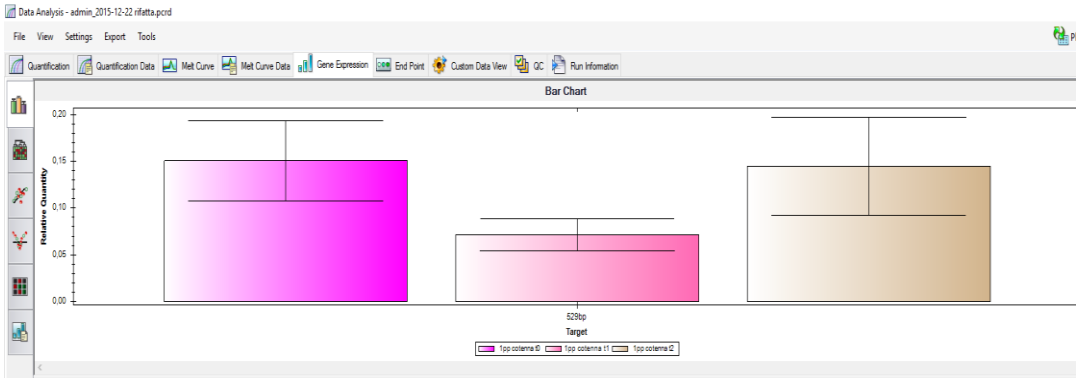
Samples n. 1, 3, 5, 6, 8, 10, 12, 15 bone-rind-inner part T0-T1-T2

Parma ham **16 months**

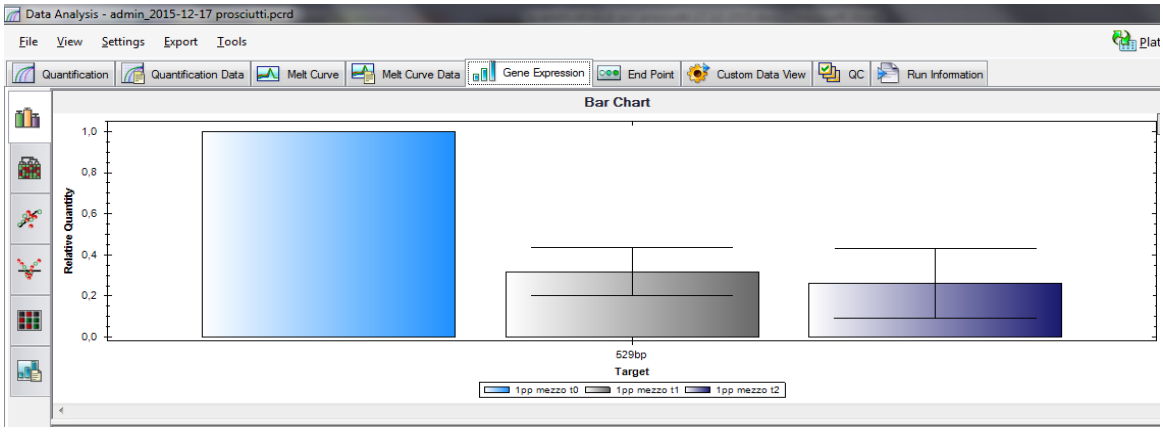
1pp bone part



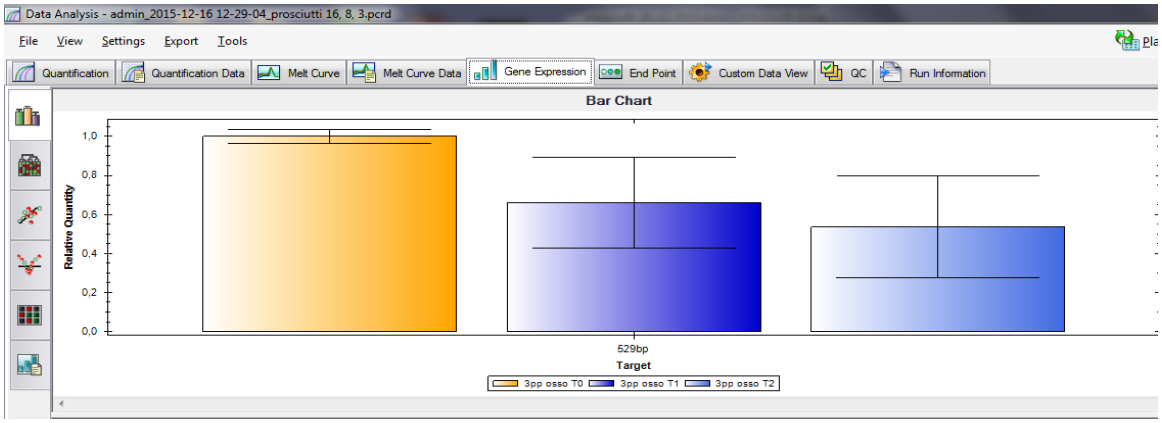
1pp rind part



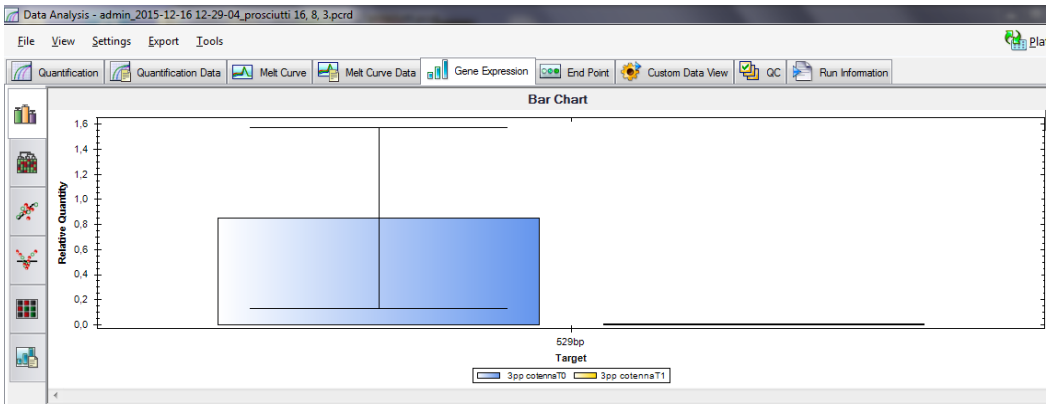
1pp middle part



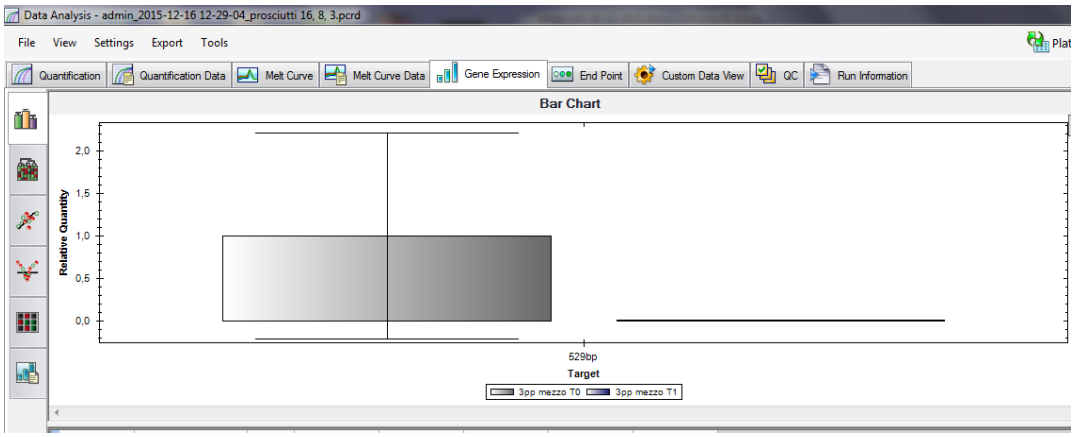
3pp bone part



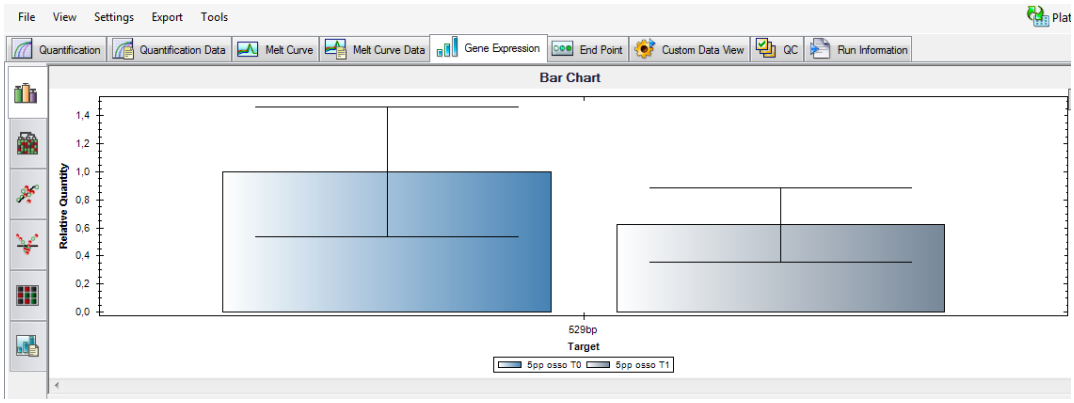
3pp rind part (T2 contaminated by bacteria)



3pp middle part (T2 contaminated by bacteria)

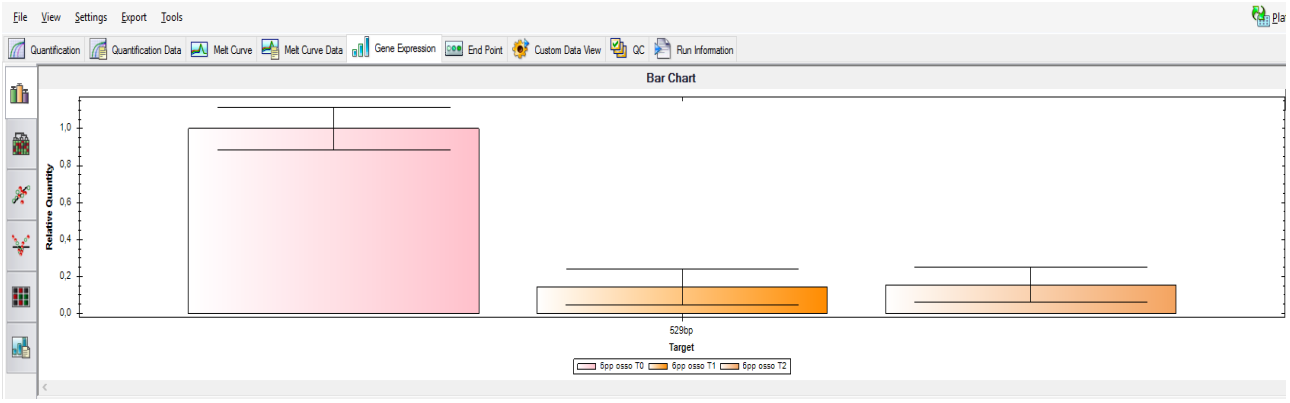


5pp bone part

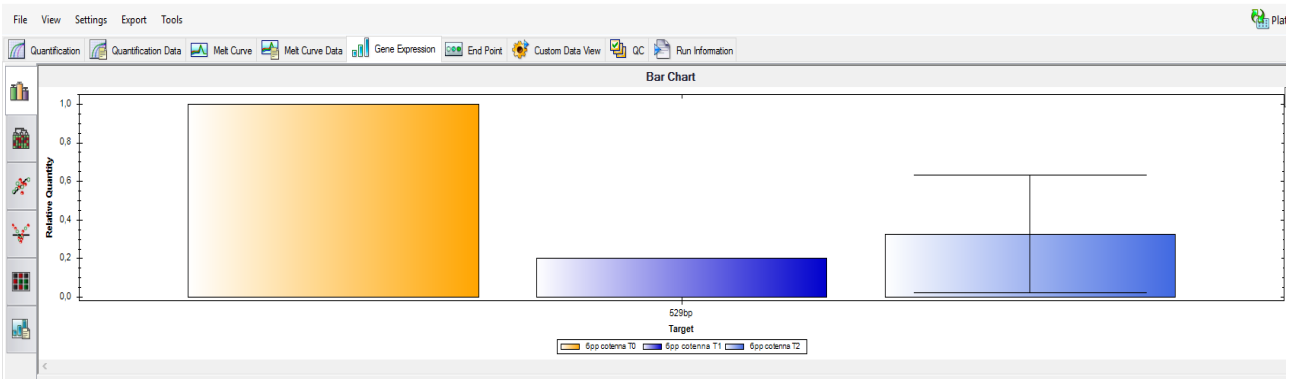


5pp rind part and 5pp middle part (data not available for contamination)

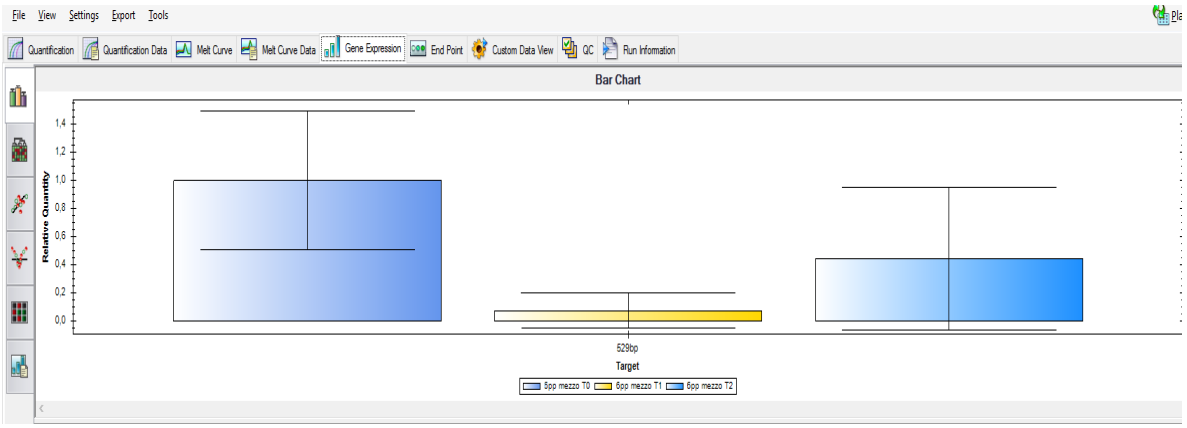
6pp bone part



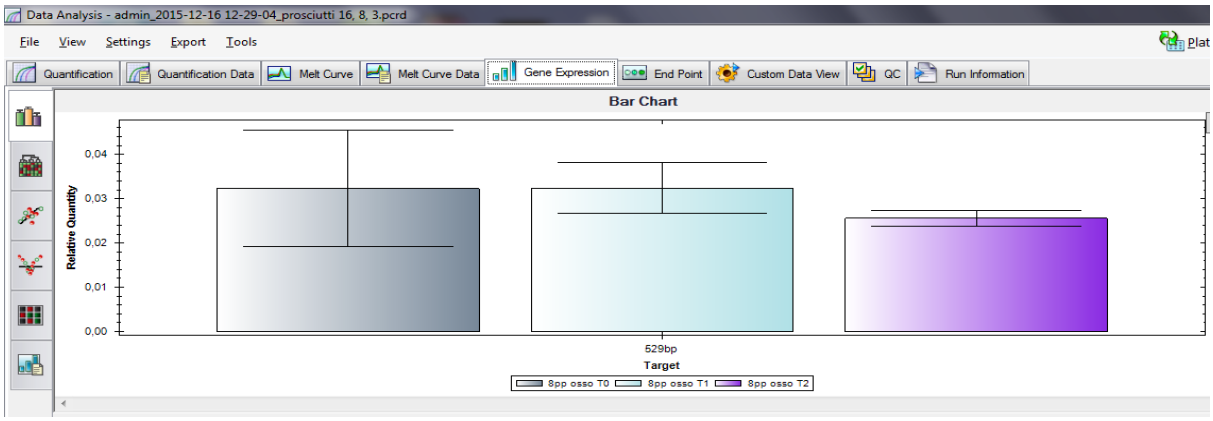
6pp rind part



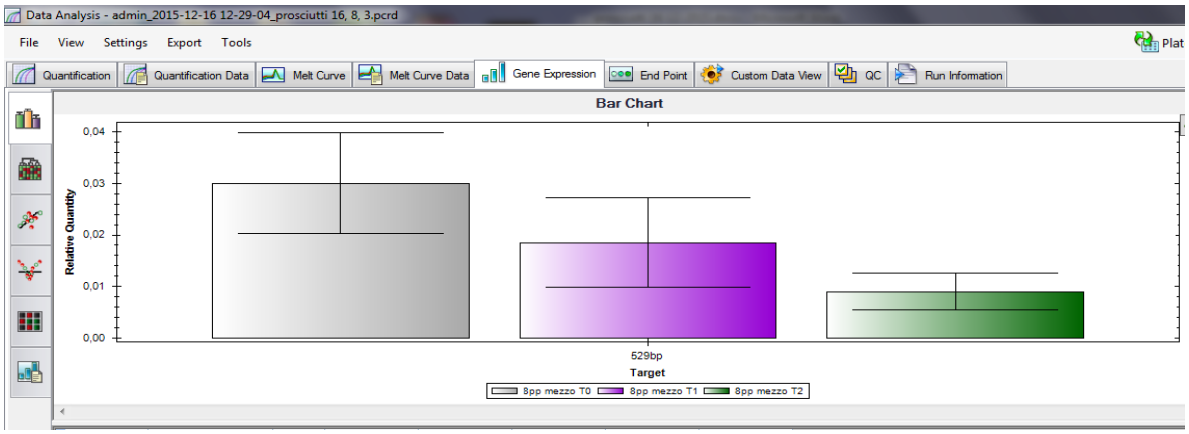
6pp middle part



8pp bone part



8pp rind part



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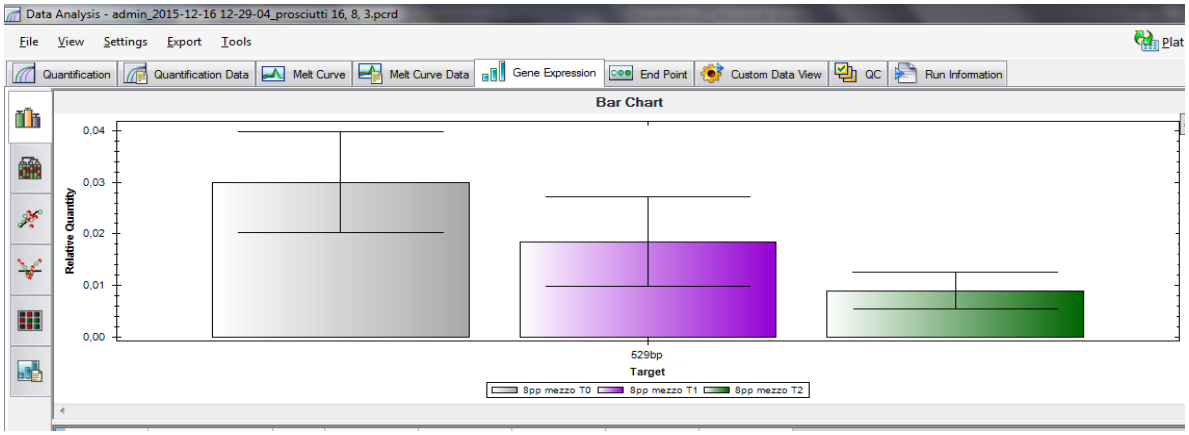
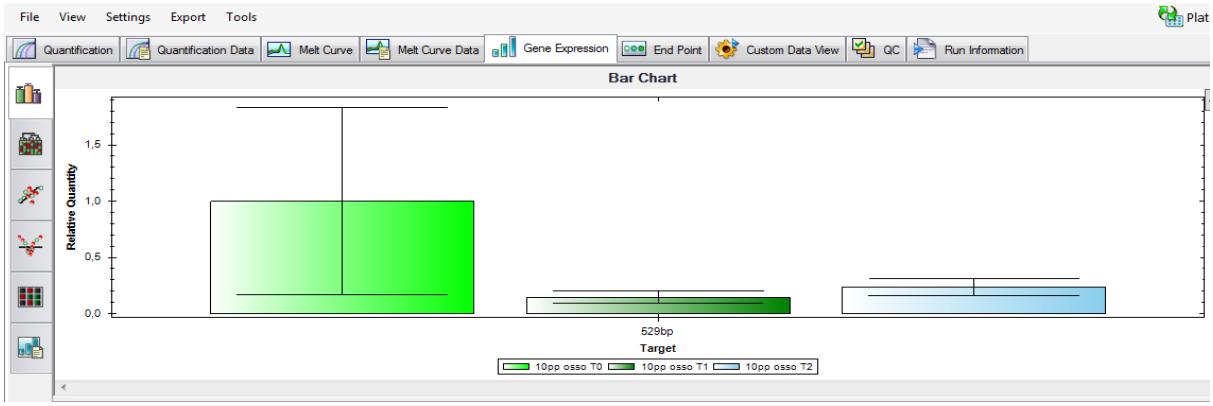


10pp bone part



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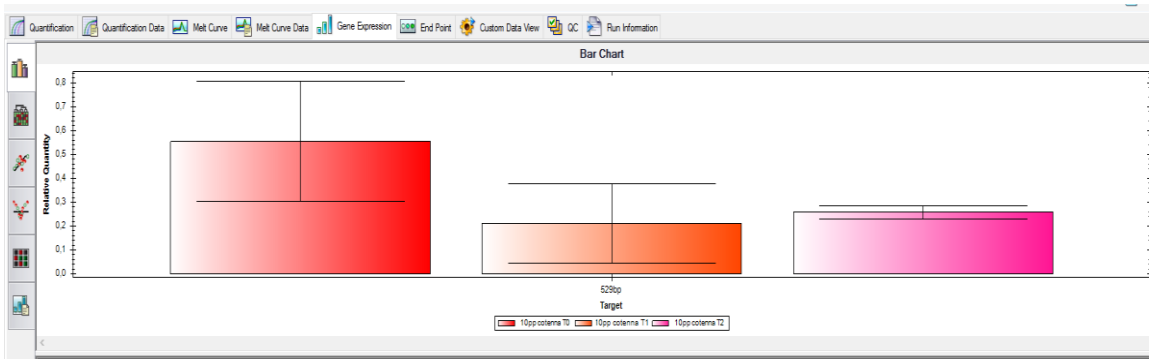
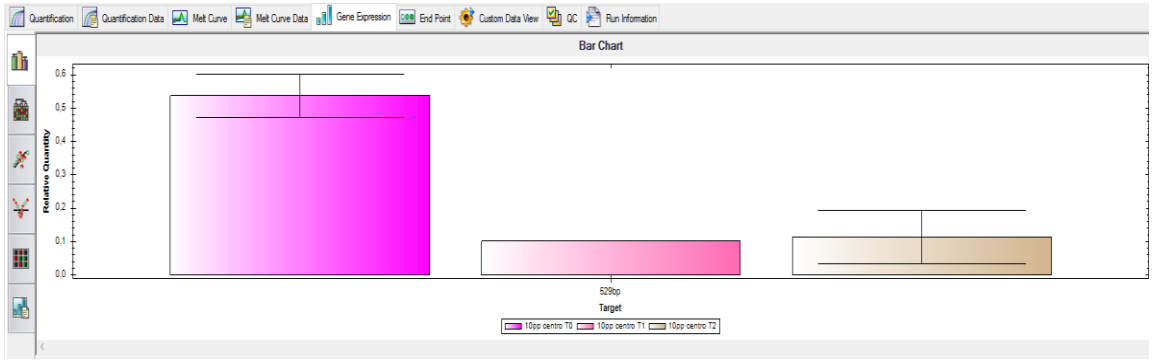


10pp middle part



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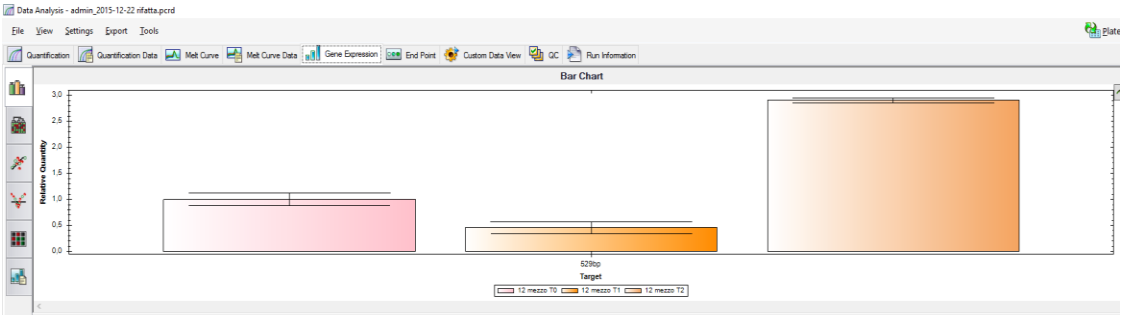
12pp rind part

12pp middle part



15pp bone part

15pp rind part (data not available for contamination)

15pp middle part