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"FEDERICO II"

Veterinary Teaching Hospital
Internal Medicine Unit

PhD thesis

"Ultrasound liver monitoring as precision farming technique for the assessment of cystic echinococcosis in sheep"

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XXXII CYCLE

List of abbreviations

CE	Cystic echinococcosis
cUS	Complete ultrasonographic examination of the liver
ELISA	Enzyme-linked immunosorbent assay
HYP	Complete liver scan through hypochondrium
HYP+Z1	sum of scan in HYP and Z1
PAIR	Puncture, Aspiration, Injection, Re-aspiration
PCR	Polymerase Chain Reaction
US	Ultrasonographic examination
WHO	World Health Organization
Z1	Partial liver scan from hypochondrium to 11^{th} intercostal space
Z1+Z2	Sum of scan in Z1 and Z2
Z2	Partial liver scan from 10^{th} to 8^{th} intercostal space
Z2+Z3	Sum of scan in Z1 and Z3
Z3	Partial liver scan from 7^{th} to 5^{th} intercostal space

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Abstract

Echinococcus granulosus (*E. granulosus*) is a cestode causing cystic echinococcosis (CE) in intermediate hosts (human and livestock) and dwelling in the small intestines of the definitive hosts (canids) in its adult form. CE is a widespread zoonotic parasitic disease having a negative effect on human-animal health and livestock production. Early in vivo diagnosis, control and prevention of the infection of *E.granulosus* in sheep are crucial steps to reduce its diffusion. Currently, liver ultrasonography is one of the most reliable diagnostic techniques for CE assessment in the intermediate hosts. So, the aims of the present thesis were: (i) to evaluate the sensibility and specificity of a new fast ultrasonographic method in different sheep's breeds; (ii) to compare the latter with the protocol developed on the Italian Sarda sheep, based on a single hypochondrial acoustic window (HYP), as well as (iii) to try to define a new and fast-focused technique for CE detection under field conditions.

One-hundred-seventy-two (172) female sheep of different breeds were submitted to a complete liver ultrasound examination (cUS) starting from the right hypochondrium to the 5th intercostal space (IS). The evaluated scan area was divided in: Zone 1 (Z1, from the right hypochondrium to the 11th IS), Zone 2 (Z2, from the 10th IS to the 8th IS and Zone 3 (Z3, from the 7th IS to 5th IS). Moreover, also the HYP technique was employed. Each zone (ZONAL scan) was analysed individually along with the contiguous ones (HYP+ Z1; Z1+Z2, Z2+Z3). During each scanning, the hydatid lesions detected were localized in the corresponding zones. After the clinical procedures, the animals were slaughtered and necropsy's results were recorded (gold standard). All US techniques were compared using sensitivity and specificity as well as the number and percentage of positive zone detected. Because of the non-homogenous weight distribution, the sample population was later divided into Group 1 (G1, weight $\leq$ 50 kg: 22/172 - 13%), Group 2 (G2, 51$\leq$ weight $\leq$ 75: 69/172 – 40%), Group 3 (G3, weight $\geq$76: 81/172 – 47%). cUs showed the highest level of sensitivity and specificity, as well as the highest number of positive zones when compared to all the other techniques ($p \leq 0.01$). cUS, resulted the best technique also during the comparison by weight distribution; HYP and HYP+Z1 showed performance similar in lighter sheep (Group 1). The current thesis represents

the first investigation evaluating the use of the US as a potential fast-focused technique for CE hepatic lesions detection in different breeds of sheep under field conditions. The present investigation confirmed that ultrasonography could be considered a reliable intra-vitam technique for CE assessment. Indeed, scanning the entire organ, from the hypochondrium to the 5th intercostal space is recommended under field conditions to optimize the diagnostic performance. However, the time needed for the exam execution can represent a limit especially for screening in large flocks; further strategies to reduce the time consuming under field condition should be evaluated to improve the widespread of ultrasound use for CE diagnosis in sheep flock.

Cystic echinococcosis (CE) is a zoonotic parasitic disease caused, in its intermediate hosts (human and livestock), by the larval form of *Echinococcus granulosus,* a cestode that dwells in the small intestines of canid in its adult form (Taylor et al., 2010). This parasite is recognized as highly endemic in several parts of the world, including Mediterranean region (WHO, 2011), and it's responsible for severe economic losses in the entire livestock breeding system ($2.951.409.989/ year) and in human healthcare ($763.980.979/year) (Budke et al., 2006). In sheep this parasitosis has a strong impact on farmer causing a decrease of milk (7-20%), meat (5-20%) and wool (10-40%) productions (Battelli, 1994). Thus, the early in vivo diagnosis, the control and prevention of the infection of *E. granulosus* in sheep is a crucial step to reduce both the diffusion of CE in humans and its negative economic impacts in rural areas. Several serological tests were developed using different substrata (i.e. antigens, antibodies, recombinant protein) but none of them obtained acceptable results (Sage et al., 1998; Naidich et al., 2006; WHO, 2011; Xunhui et al., 2017). In humans, liver ultrasonography (US) is a common diagnostic procedure for hepatic CE, applicable for mass screening in endemic communities (Macpherson and Milner, 2003). In sheep, the first diagnostic imaging technique for CE detection was based on the radiological examination of lungs (Wyn-Jones and Clarkson, 1984), subsequently the US was employed to assess the prevalence of hydatid cysts in liver of sheep and goats in Turkana, Kenya (Maxson et al., 1996). During last years, several studies have established that the transcutaneous ultrasonography of the entire liver, is a reliable, simple, non-invasive method for CE diagnosis in sheep and, therefore, it can be considered the gold standard for the intra-vitam diagnosis of liver hydatidosis in small ruminants (Hussein et al., 2014; Dore et al.; 2014). Nevertheless, the time necessary to perform the examination often a limit of this diagnostic technique, especially when it is employed for mass screening of sheep flocks. Recently, Dore et al. (2014), performed on the Italian Sarda sheep a faster liver ultrasound examination using mainly a single acoustic window, placed in the right hypochondrial space, without wool shaving, reporting good sensitivity and specificity results. However, in our preliminary study the same technique, when applied to different breeds, resulted sometimes uncapable to assess the entire liver area (Borriello et al., 2019). Thus, to efficiently assess the presence of *E. granulosus* in sheep, a fast-focused ultrasonography technique applicable in field to different sheep breeds is needed. Based on the previous considerations, the aim of the present thesis was (1) to further evaluate the sensibility and specificity of the

US in different breeds and (2) try to develop a practical, fast-focused technique for CE hepatic lesions detection under field conditions.

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Chapter 1

Aetiology

1.1Taxonomy

Echinococcosis is a parasitic disease caused by cestodes belonging to the genus Echinococcus (Echìnos: spiny shell; còccos: berry) that dwells, in its adult form, in the small intestines of canid. In its larval form, instead, it will grow as metacestodic cysts (Hydatid) causing cystic echinococcosis (CE) (Zhang and Mc Manus, 2006). The genus Echinococcus includes different species, which classification has undergone considerable changes over time. In fact, at the beginning of the 20th century, 16 different species and 13 subspecies of Echinococcus were described based on host-parasite specificity but, more recently, further in-depth morphological studies reduced to five the species (Thompson and McManus, 2001; Xiao et al., 2005). Among these, the most widespread are *E.granulosus* and *E.multiloculari*, indeed the latter are characterized by a significant impact on public health because cause of cystic and alveolar hydatidosis in humans and animals (Puccini, 1975). *E. vogeli* and *E. oligarthrus,* are instead two species mainly diffuse in Central and South America and responsible for polycystic echinococcosis (McManus and Thompson, 2003). More recently, a new species was discovered in China, *E. shiquics,* that recognizes as definitive hosts the Tibetan fox (Vulpes ferrilata) and the pika (Ochotona curzoniae) (Xiao et al., 2005). Moreover, since a long time it has been proved, in equine and sheep infected by *E. granulosus*, intra-specie differences existing between isolates from different hosts in various parts of the world (Smyth and Davies, 1974). These studies provided the basis for understanding these differences considerably changing the epidemiology of *E. granulosus* introducing the nomenclature of "strain or genotype" (Thompson and McManus, 2002). Different genotypes have also an important practical consequence since they can present differences regarding the biological cycle, host specificity, development rate, pathogenicity, antigenicity, sensitivity to chemotherapy and epidemiology of CE (Thompson and McManus, 2001).

Molecular studies identified 10 genotypes/strains of *E.granulosus* on the basis of polymorphisms of the nucleotide sequence of some genes especially in the mitochondrial DNA (mtDNA). In particular, the genotypes are: G1 (ovine strain), G2 (Tasmania sheep strain), G3 (buffalo strain), G4 (horse strain), G5 (bovine strain), G6 (camel strain), G7 (pig strain), G8 (cervid strain), G9 (human strain), G10 (Finnish Scandinavian strain) (McManus, 2002; Lavikainen et al., 2003; Maravilla et al., 2004). Nevertheless, some doubts are reported about the G9 role because very

similar to G7 (Snabel et al., 2000). Recently, Huttner et al. (2007) found a strain in Uganda classified as “Lion”, which had been described previously in South Africa as *E.felis* (Huttner et al., 2007) although more studies are needed to confirm this hypothesis.
It has also been shown that there are also "variants" of some genotypes such as: G1a, G1b, G1c, G1d, G1e, G2a, G2c, G7a and G7b (Kamenetzky et al., 2002) although these are still to be considered in continuous evolution. Nakao et al. (2007) proposed a taxonomic revision of these strains based on their phylogenetic relationships evaluated by molecular analysis of the complete mitochondrial genome; whereby the G1, G2 and G3 genotypes should be grouped into the single species of *E. granulosus sensu stricto*;, while the equine strain G4 and the bovine one (G5) would represent two new species*: E. equinus* and *E.ortleppi*, respectively. Finally, the G6, G7 and G8 genotypes could be unified in a single species, *E. canadensis*, in which they could also include the G9 and G10 genotypes, since there is a close relationship between the G7 and G9 strains and between G8 and G10.

1.2 Morphology and biology of the adult form

E.granulosus in adult form is a tapeworm 2 - 7 mm long, composed by head, neck and body. The head (scolex) has four roundish suckers placed in an equatorial position and a *rostrum* with a double crown of hooks, one smaller (22-39 microns) and one larger (31-49 microns) (Casaravilla et al., 1985). The neck is short and narrow while the body, also called the *strobilus*, consists of 3 – 6 hermaphrodite proglottids (Euzeby, 1968) provided with a single genital pore. The proglottids near the neck are sexually immature, followed by the mature ones (endowed with sexual organs suitable for reproduction) and the pregnant ones (in which the eggs are present). The last proglottid is also able to self-fertilize and to detach itself from the body of the parasite to be released into the environment through the faeces of the definitive host, once pregnant. In this phase, it is also able to disperse its eggs in the environment (in a mature or immature stage), that in their turn will develop themselves outside the definitive host in presence of appropriate microclimatic conditions (Thompson and Lymberly, 1995).

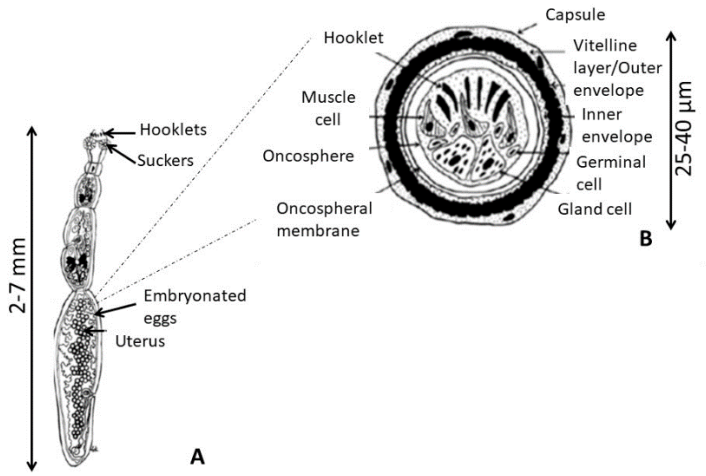

Fig 1.1. *The key-stages of the development of Echinococcus spp. in definitive hosts A) Adult worm; B) Egg (Thompson and McManus, 2012)*

Even though poor information regarding the reproduction rate of *E.granulosus* are present in literature, it has been estimated that pregnant proglottids are produced every 7 - 14 days (Smyth, 1964; Shantz, 1982) and that each of them contains about 1000 - 1500 eggs (Arundel, 1972; Rausch, 1975; Thompson and Eckert, 1982; Heath and Lawrence, 1991). The eggs measure approximately 30-50 x 22-44 microns in diameter, appear spherical or oval, morphologically indistinguishable from those of the other cestodes belonging to the *Taenidae* family. In fact, ultrastructural studies demonstrated similarities between them: proceeding from outside to inside usually the capsule, vitelline membrane, embryophores, granular layer, oncosphere membrane are found (Morseth, 1965; Sakamoto, 1981; Swiderski, 1982). The survival of *E. granulosus'* eggs is encouraged by low environmental temperatures; indeed, they remain viable up to 294 days at 7 ° C and, at temperatures below 0 ° C (from -35 ° C to -50 ° C), they can withstand for 24 hours retaining their infective capacity. At -70 ° C egg will die over a few minutes. On the contrary, at high temperatures ranged

between 60 and 100 °C, eggs can resist only 1or 2 minutes (Laws, 1968; Thompson and Lymbery, 1995), while 28 days at 21 ° C on case of adequate environmental humidity (low values usually cause eggs death (Laws, 1968).

1.3 Morphology and biology of the larval form

The metacestode (hydatid) appears as a cyst formed, and from outside to inside made of:

• **the adventitia**, a layer of fibrous tissue belonging to the host, consists of mononuclear cells, eosinophilic giant cells, endothelial cells andfibroblasts. It is a defensive reaction against the parasite.

• **the helminthic membrane**, composed of two layers: a prolific, internal and very thin germinal layer (12-15 micron) and an external membrane (laminated) rich in cells. The latter is followed by the cuticular layer or chitinous membrane, of variable thickness and characterized by concentric lamellae.

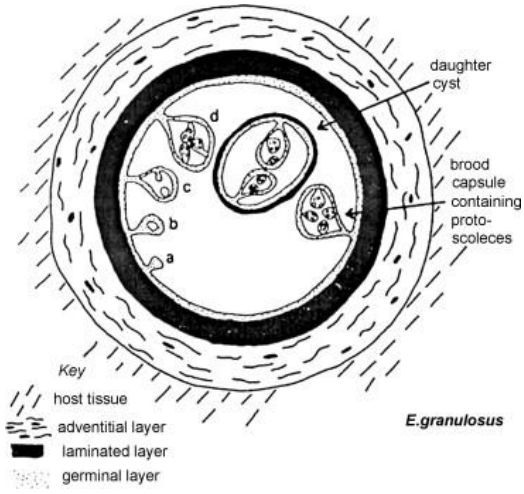

Fig.1.2 *Representation of structure of the echinococcal cyst.* ***(Thompson and McManus, 2012)***

These membranes are perfectly contiguous between them because of the pressure of the hydatid liquid and they are usually non-communicating. When fertile and able to infect canids, the germinative layer contains small structures called brood capsule (Taylor et al., 2010). The brood's capsule originates from the germinative layer during the first 5th month of life and it appears whitish, with the dimension of a grain of sand and connected with a thin peduncle to the germinative membrane or remaining free in the cystic liquid. Every brood's capsule contains from 6 to 30 proto-scolex, very similar to the adult one. The presence of these protoscolices, adherent to the germinative layer or free in the liquid constitutes the so-called "hydatid sand" (Taylor, 2010).

The hydatic cyst usually has different microscopic forms. The easiest type of cyst is the unilocular: whitish, spheroidal, with variable volume and single cavity containing both hydatid liquid and sand. This type of cyst is always fertile, even if there is a variability in the number, vitality of the proto-scolex and size of the structure. Years after its development, the hydatid may be subject to changes increasing the chances of spreading their protoscolices inside infected body and form secondary cysts.

A recognized spreading mechanism consists of the wedging of portions of the helminth membrane in the pericystic tissues overcoming the adventitial capsule. Thank to this condition, the parasite is able to generate new hydatids communicating with the main one or free and able to develop in other sites (exogenous daughter cysts). Less frequently, daughter cysts are formed within the main hydatid (endogenous daughter cysts), being fertile in most cases and able to reach considerable dimensions (10 - 12 cm). Moreover, the protoscolex can reach other organs (i.e. bronchi, bile ducts, pleurae, peritoneum) through the blood and lymphatic circulation, causing bronchitis, angiocolitis and echinococcal embolism. Because of the pressure exerted by the daughter cyst, the mother cyst may have different shapes from the spherical one, assuming a lobed or multilobed appearance or, sometimes, irregular contours. When the daughter cysts can develop in a single district, all at the same stage of development with their own adventitial, this structure is called pseudomultilocular cyst.

The cysts can regress and their cavity are usually filled with layers with laminar tissue new-formed after the collapsed and tightly pressed daughter cysts; it will form the non-fertile and hyperlaminated cysts. These cysts are often considered ex-fertile, because the findings of residue of prostocolex and brood capsule are quite common. Sterile and degenerated cysts can remain silent in the organs for many years and when degenerate they form

the hyperlaminated caseous cysts or calcified hyperlaminated cysts. When the hydatid one degenerates, it appears filled with dense, yellowish fluid and characterized by indistinguishable tissue layers. Inestad when calcification occurs, the cyst is considered dead. A less frequent case is acephalocysts, an hydatid with a germinative membrane that cannot produce proliferating vesicles, therefore characterized by a cyst fulfilled by hydatid fluid (Taylor, 2010).

1.4 Biological cycle

In its adult stage, *E. granulosus* dwells in the small intestine of the definitive hosts, in their turn infected through the ingestion of organs containing vital hydatid cysts. After the ingestion from a specific definitive host, the proto-scolex sticks to the intestinal mucosa due to the suckers and hooks of the rostrum, maturing to the adult form in 4-5 weeks (Taylor, 2010). The pregnant proglottids, releases their eggs which have remarkable environmental resistance once expelled into the environment (Laws, 1968; Thompson and Lymbery, 1995) and can be ingested by an intermediate host. In the digestive tract the proteolytic action of pepsin and pancreatin, clear the hexacantus embryo of the embryophore activating him (Taylor, 2010). At this stage, the latter can anchor itself to the epithelium with a complex muscular system and throughout the use of the penetration gland's secretion it will reach the lamina propria within 30 -120 minutes from the ingestion. Once it gets, the embryo can reach the blood and lymphatic pathways and spread throughout the intermediate host's organism preferring as target organs liver and lung, while less frequently other organs like kidneys, liver, bones, spleen. When the oncosphere reaches the targets organs, it develops a hydatid cyst in a time ranged from 1 to 14 days characterized by a growing rate of approximately 1 cm a year (Taylor, 2010)

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Chapter 2

Epidemiology

2.1 World epidemiology

The biological cycle of *E.granulosus* is often epidemiologically divided into a "domestic" and a and "wild" cycle. The domestic cycle has a greater zootechnical importance, involving domestic dogs as definitive hosts and different species of domestic ungulates as intermediate hosts, whereas the latter recognizes wild canids (i.e. wolf) and wild ungulates (i.e. deer, moose) as definitive and intermediate hosts (Eckert and Deplazes e, 2004, Thompson and McManus, 2001). However, has been proved how the two type of cycles sometimes can overlap between them, mainly due to wild canids that come into contact with livestock farms (McManus, 2002).The worldwide epidemiological pattern of *E. granulosus* in humans is comparable to that of other intermediate hosts, with an almost cosmopolitan spread and areas of particular endemicity such as the Mediterranean basin, southern and eastern Europe, southern tip of South America and areas of Central Asia, Siberia and eastern China (WHO, 2011).

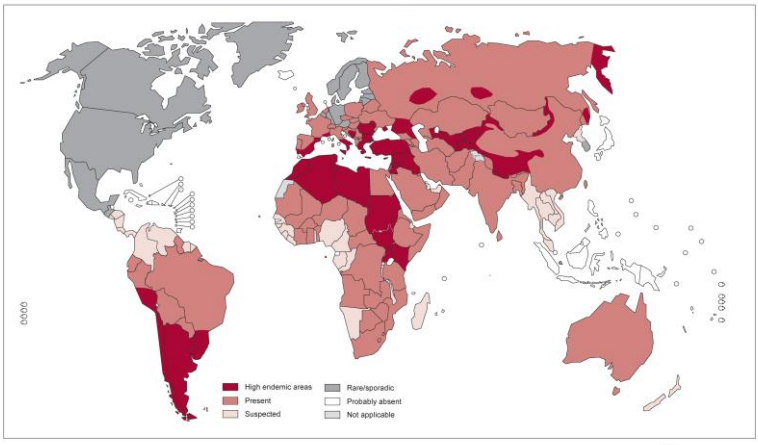

Fig.2.1 *Distribution of Echinococcus granulosus and cystic echinococcosis. (WHO, 2011)*

The sheep genotype (G1) is the most widespread in Europe (i.e. Mediterranean basin, Great Britain) it was also detected in China, where the hydatidosis has important implications for public health (McManus, 2002;

Varcasia et al., 2007, Guo et al., 2019). Furthermore, G1 is also widely described in the African continent (Bart et al., 2004; Azab et al., 2004 Lahmar et al., 2004) as well as in Asia (Iran) where, out of 200 isolates, the sheep genotype would be the one most present in the intermediate hosts observed. The G1 was also found in livestock and human in south Brazil (de La Rue M., 2011).
The Tasmania sheep genotype (G2) has instead been identified both in Europe (particularly in romanian sheep, cattle and humans) and in the Indian subcontinent (Bart et al., 2006). In the latter, apart from G1 e G2, also the G3 genotype was detected in buffaloes (Bhattacharya et al., 2007). More recently, G3 was also found in Europe (Rinaldi et al., 2008; Casulli et al. 2012). In Argentina, where hydatidosis represents a great scourge for public health, many areas of the country are endemic mainly for G1 and G2 (Rosenzvit et al., 1999). The G4 (equine genotype) was instead found in equidae (horses, donkeys and zebras) from different parts of the world (i.e. Europe, Middle East and Southern Africa) (Thompson and McManus, 2001; Daniel Mwambete et al., 2004). However, no human cases have ever been documented, suggesting the non-pathogenicity of this genotype to humans.
Regarding the G5 (bovine) genotype, it does not appear to be widespread. Indeed, sporadic cases have been described in Asia, in some areas of Africa (Magambo et al., 2006) as well as in South America. Until today, few human cases have been associated to this genotype (Sharma et al., 2013).
The G6 (camel), the G7 (pig), the G8 (cervid), the G9 (man) and the G10 (Finnish - Scandinavian) strain were rarely found in confined and specific areas; they are sometimes considered a geographical variant of a single species of *Echinococcus.*.
In northern Africa (i.e. Morocco, Algeria, Tunisia, Libya and Egypt), the G6 and G1 are the more common in both humans and animals, especially in the Turkana area where the camelid breeding is particularly developed (Bekele and Butako, 2011; Bardonnet et al., 2003).
In Europe, G7 genotype was isolated in pigs in countries such as Spain, Poland, Romania, Lithuania, Slovakia and Ukraine (Kedra et al., 1999; Snabel et al., 2000; Gonzalez et al., 2002; Bart et al., 2004); quite recently it was also described in Greek goats (Varcasia et al., 2007) and in Turkish sheep and humans (Snabel et al., 2007). Despite its wide diffusion and impact on the farm, the G7 is considered as poorly infectious for humans (Deplazes et al., 2017)..
In Canada and Alaska, a wild G8 cycles involves dogs and wolves as definitive hosts while moose, reindeer and other cervids are its intermediate

hosts has been described (Rausch, 2003). As last, as reported in the previous sections of the manuscript, the validity of the G9 strain (found only in Poland; Scott et al., 1997) has been questioned by several authors because it seems to be stacked with G7 genotype (Snabel et al., 2000), while the presence of G10 has been only confirmed in the North-East of Finland and in Sweden (Lavikainen et al., 2003), although poorly properties to infect humans (Oksanen and Lavikainen, 2004).
An important change of the *E.granulosus* wild cycle was detected in African lions, found to be definitive host; until now, it represents the only felid considered involved in its biological cycle (Huttner, 2008). In South America, an important role as final host is played both by the fox of the genus Dusicyon and most likely also by red fox (Vulpes vulpes). Both the canids are sensitive to the infection seem to have a role in the spread of the parasite in Europe (Thompson, 1983; Jenkins and Craig, 1992; Jenkins and Morris, 2003), Iran (Dalimi et al., 2002; Dalimi et al., 2006) and in the United Kingdom (Clarkson and Walters, 1992; Jones and Walters, 1992). In Australia, both cycles have been described: the first seems to involve sheep (considered the main intermediate hosts) cattle and pigs (having a marginal role in dissemination, as hosts accidental intermediates), while the sylvan cycle involves marsupial macropods (kangaroos). There is an interaction between these two cycles through several species of carnivores (e.g. domestic dogs, wild dogs, red foxes) acting as definitive hosts (Hope et al., 1992). Other wild cycles involving wolves, sled dogs, deer, moose, reindeer have been detected in mountainous regions of North America and Europe (Rausch, 1995).

2.2 Italian epidemiology

The presence of *E. granulosus* in Italy is mainly linked to areas with high density of sheep such as Sardegna, Sicilia, Campania, Lazio and Abruzzo, in which the animals are "traditionally" reared in extensive or semi-extensive systems and potentially characterized by a slaughtering carried out outside the official circuits (Garippa et al., 2004).
The first national survey on the diffusion of CE in intermediate hosts in Italy dates back to 1952 (Pellegrini and Cilli, 1955) and showed a greater spread of parasites in the islands and southern regions. The cattle were less affected in northern Italy than central and the south (4.1% - 11.4% - 13.3%), with a

peak of prevalence in Sardegna region (up to 55.09%). In sheep farming, the trend was slightly different (North: 15.9%, Central: 21.7%, South: 14.9%) with a peak of 41.7% in Toscana (central Italy) and islands (Sardegna: 68.7%, Sicily: 21.4%). In goats, the mean prevalence varied between 1.8% in the North (with a peak of 6.2%, in Piemonte), 8.1% in the Center (with a peak of 31.4% in Tuscany), 10.3% in the South, 7.5% in Sicilia and 12.7% in Sardegna. In pigs, the prevalence of CE significantly varied between the different regions with values ranging from 19.8% in Sardegna, to 2% in Emilia Romagna (Pellegrini and Cilli, 1955).
In 1977, Schiavo et al. (1979) recorded a national mean CE prevalence of 11.6% in sheep, 5.6% in goats, 1.5% in cattle, 1.1% in pigs and 0.4% in horses. Later, Romboli et al. (1980), analysing official national data of the decade 1968 – 1978 detected similar prevalence of CE in the intermediate hosts (cow: 8.1% -15.2%; sheep: 8,1%-15,3%; goats: 2.7% - 8.9% , horses: 0.4 - 0.9%) and slightly higher values for swine (0.7-1.2%). The regional trend, observed using the data of the years 1972-1978, was similar to what found by Pellegrini and (1955) with higher prevalence of CE detected in the southern regions and the isles in all intermediate hosts (Romboli et al., 1980). However, both national and regional data recorded during the years are incomplete or not homogenous, so they often underestimated the spread of echinococcosis. In northern Italy, between the years 1995 - 2005, the data collected from the Governative Service of Veterinary Health of Valle D'Aosta public health showed a decrease in the overall prevalence of CE infection in slaughtered livestock from 0.08% to 0.2%. For sheep and goats, a prevalence of 0.5% on slaughtered animals and 0.08% on total assets was described in Piemonte region (Garippa et al. 2004). The overall prevalence of CE in animals regularly slaughtered in Emilia Romagna between 1996 and 1999 was studied by Faggioli et al. (2001) with the following results: pigs 0.0009%, cattle 0.4%, sheep 0.3%, goats 0.4%, horses 0.3%. In 2007, Manfredi et al., reported in Lombardia region a CE prevalence of 0.3% in sheep and 0.1% in cattle whereas, more recently, the prevalence assessed in cattle in the same region increased to 4.99% (Manfredi et al., 2013). The data available for Central and Southern Italy are more numerous but discontinuous and not sufficient to draw a picture of the parasitic disease's diffusion and its temporal evolution. For example, in Abruzzo, the development overtime of the parasitosis in farm animals does not appear linear. Indeed, the data collected in the year 1981 by Manilla (1986) reported a percentage of 50.8% in adult sheep but, between 1972 and 1984, an overall prevalence of 10.6% (8.9% - 20%) in sheep and goats (Gargiulo et al., 1987).

In other species, between 1985 to 1989, Schiavo et al. (1992) found a decrease of positivity in cattle (3.5-2.3%), horses (3.8-1%) and a slightly increase pigs (0.3-0.6%) while in goat, even though the prevalence decreased from 16.3% to 4.5%, a peak of 22.5% was recorded in 1988. During the years of the study, prevalence in sheep did not varied, with a mean value of 17.8% (Schiavo et al., 1992). Subsequently (1985-1994) in the district of Teramo, average prevalence of 32.1% in sheep/goats and between 4-6% in cattle, horses and pigs were recorded (Tieri and Gatti, 1995). Moreover, the latest epidemiological data on intermediate hosts showed an increased values (47.0%) of sheep hydatidosis in Arezzo district (Bio and Fagiolo, 2004).In Puglia region, the prevalence values of *E. granulosus* detected in sheep and goats were of 4.9% (1975) and 3.9% (1982) respectively (Puccini and Tassi, 1983). Further and subsequent studies revealed a decreasing prevalence in sheep, from 3.4% to 0.5%, and goats 5.9% - 0.3% (Schiavo e Pansini, 1996). In a different region, Basilicata, between 1996 and 2002 the prevalence values of hydatidosis changed in the different species: 5.0% – 28.0% in sheep, 4.0% - 25.0% in goats, 2-0% - 3.0% in cattle, 0.05% - 0.50% in pigs, 0.04-0.10% in horses (Quaranta et al., 2003). Studies carried out in Campania region, on cattle, sheep, goats, pigs and horses slaughtered in the district of Avellino and Salerno detected an overall prevalence of less than 5% (Garippa et al. 2004) despite in some districts values of prevalence of 16.0% and 21.0% were found in sheep and cattle (Cringoli et al., 1998). A study conducted later by, Cringoli et al. (2007), in an area where the CE prevalence of sheep was up to 75%, showed similar values in cattle (20.0%) while, in water buffaloes reared in the same area, the prevalence was lower (12,4%). A greater number of investigations describing the epidemiological situation of the parasitosis in Sicilia region are instead reported in literature. The first research carried out dates back to 1951 (Bertocchi) in which prevalence values between 6% - 10% were reported in cattle slaughtered in Palermo, Messina, Catania districts. Noteworthy differences were found by Magliarditti and Niutta (1995) who recorded am increased prevalence in cattle (11.1%), sheep (43.2%) and pigs (4.7%). More recently, a study conducted by Giannetto et al. (2004) detected a higher prevalence in cattle (67.1%) and sheep (57.6%). In Sardinia island, although three official eradication plans have been performed overtime in this region (1960, 1978 and 1987) sheep CE prevalence remained high (83.0% – 88.0%) during the years (Conchedda et al., 2012). More recently a study conducted by Varcasia et al. (2007)

observed prevalence of CE in sheep, in cattle and in pigs respectively of 75.3%, 41.5%, 9.4%.

Data regarding the presence of *E. granulosus* in the definitive host are more incomplete. In northern Italy (Piemonte region), a prevalence of 24.6% and 26.2% was found in dogs and wolves (Garippa, 2006). Study performed in central Italy (Abruzzo region), showed a positivity for *E. granulosus* in 4% of examined dogs including stray and shepherd dogs (Di Ventura et al., 1995). Regarding southern Italy, (Puglia region) the spread of *E. granulosus* in dogs showed a decrease in prevalence from 12.9% to 5.73% between the years 1955 and 1975 (Puccini et al., 1975). In our region (Campania), the area of Naples revealed a prevalence of 1% in 500 dogs examined by means necropsy (Capurso et al., 1968). Additional considerations are possible regarding the epidemiological data of definitive hosts in the two Italian islands. The overall prevalence of hydatidosis in dogs of some Sicilian districts ranged from 4.6% in the east of the region (Messina district) to 3.4% the north west (Palermo district) (Panebianco and Sciutteri 1955; Gallo and De Girolamo, 1960); conversely, higher prevalence values were found in shepherd dogs examined in Agrigento (23.2%) and Palermo (16.2%) districts as recorded by Giannetto et al.(1997). Finally, regarding the second Island (Sardegna), In some surveys a prevalence of echinococcosis was found in 1.18% in foxes and of 16.9% in wolves (Arru et al., 1986; Guberti et al., 1983). Regarding domestic and stray dog, the prevalence values observed were of 11% and 25.42 % respectively, with a total regional prevalence of 16.2% (Arru et al., 1990).

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Informal Working Group on cystic and alveolar echinococcosis surveillance, prevention and control, with the participation of the Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health

Chapter 3

Clinical presentation and diagnosis

3.1 Distribution of the lesions and clinical presentation

Echinococcosis in definitive hosts dwells in the small intestines without causing significant pathology, even in the presence of massively infected animals. Minor changes may occur in the intestinal mucosa, such as a slight cellular infiltration or increased mucus production, but they are usually associated with a long, subclinical course (Eckert, 2004). In intermediate hosts, the clinical presentation is strongly related with the distribution and the number of hydatid cysts. In sheep, the latter are usually located within the liver and lungs although a possible distribution in organs like spleen, heart, kidney has been described (Orlando, 1997); liver and lung are also the main development sites of the CE in goats (Rausch, 1995) and pigs (Rausch, 1986). Different is the situation reported for cows, where unilocular sterile cysts care usually found, although, if an infection with the G5 genotype (bovine) occurs, the hydatid will be fertile (Eckert et al., 2001). As reported for cattle, also in water buffalo the 90% of the cysts are usually sterile and mainly localized in lungs and liver, although sporadic cases in which the lesion has been observed in the spleen, kidney, heart, brain, diaphragm and uterine musculature are present in literature since a long time (Thompson, 1977). Regarding the horses, cysts seem to mainly grow in the liver but with a very low growth rate, indeed the cysts found in 11-16 years old horses did not exceed 4 cm in diameter. However, in the rare cases in which larger cysts have been found, they resulted often asymptomatic (Thompson and Smith, 1975). Finally, in humans more than 90% of cysts are usually localized in the liver and/or in both lungs; kidney, spleen, peritoneal, muscles and heart hydatids are instead seldom reported (Menghebat et al., 1993).

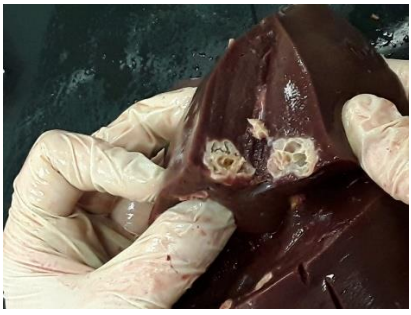

Fig. 3.1 *Hydatid cyst in sheep liver.*

Echinococcosis is often asymptomatic for many years. during almost the whole life-span of the host. However, in animals, such symptoms may be overlooked, especially in the flock or herd situation, so it has been postulated that symptoms experienced by humans infected with hydatid cysts may also occur in infected animal (Eckert et al, 2001). In sheep she symptoms are mainly the lowering of the conversion index, fertility problems, a drop in the production of milk, wool or leather (Battelli, 2004) whereas, in horses, the clinical signs reported in literature are not specific (i.e. tightened respiratory noise, inappetence, emaciation) and associated to massive infection (Hermann et al., 1988). In humans, the initial phase of primary infection is always asymptomatic, and small (<5 cm) well-encapsulated cysts located in organ sites, where it does not induce major pathology (Pawlowski et al., 2001) and after an incubation period of several months or years, if the cysts exert pressure on adjacent tissue, different symptoms can occur. Common symptom of CE infection in human are respiratory difficulties, coughing, abdominal pain, anorexia, emaciation (Pawlowski et al., 2001). The presence of cysts in the bone can often determine fractures while an encephalic localizations manifest focal neurological deficits or signs of intracranial hypertension (McManus, 2003). Moreover, after the sudden rupture of the cysts (traumatic and spontaneous), the secondary dissemination of protoscolex can cause a hypersensitivity reaction triggered by the high immunogenic power of antigens. In this situation the detectable symptoms may be eosinophilia, urticaria, pruritus, dyspnoea, asthmatic crisis up to anaphylactic shock (Vuitton et al., 2004).

3.2 Diagnosis

Considering the wide diffusion of hydatidosis, the fast, reliable detection of *E. granulosus* in its hosts has a crucial importance to set up epidemiological studies and control programs (Pawloski, 2001). The diagnosis of echinococcosis in definitive hosts is still problematic and only partially solved. In past, the diagnosis was reached by means of the macroscopic confirmation of the parasite in the intestine during the necropsy examination or the use of laxative, allowing the expulsion of the adult parasites with the faeces. Related to the necropsy examination is the "sedimentation and counting technique" (SCT) (Eckert et al., 2001). The latter represents a procedure consisting in sedimentation of the intestinal contents and consequent count of the adult parasites by light microscopy. Even if is very effective, the method is considered laborious and ethically questionable,

since it is not applicable in live canids. The only in vivo treatment was the administration of arecoline hydrobromide (parasympathomimetic), which increases the intestinal peristaltic movements, favouring the detachment of cestodes and therefore, their detection within the faeces produced immediately after the treatment. Although this method has been widely used in research studies, it has several drawbacks raising several concerns regarding animal welfare, environmental contamination, high risks for the operator, high percentage of subjects poorly sensitive to the laxative action (Eckert et al., 2001). In modern parasitological diagnosis, a technique widely used in the definitive hosts is the copromicroscopic examination after enrichment (flotation) with the aim to detect and identify the parasite's eggs. However, the efficacy of this technique is invalidated by the inability to distinguish *E. granulosus* eggs from those of *E. multilocularis* and other species of Taenia. A further restriction is given by the inconstant emission of eggs in faeces during the infection (Eckert, 2001). To overcome these limitation, coprological examination can be associated with more specific techniques, such as immunological or biomolecular methods (Varcasia, 2004). Specific systemic antibodies (IgG, IgA and IgE) against *E. granulosus* protoscolex antigens can be detected in the serum of infected dogs (Gasser et al., 1993) by immunoenzymatic techniques (ELISA) but their persistence even after the elimination of the parasite does not allow to reach a correct diagnosis through these methods. Furthermore, an adequate systemic immune response is not always triggered by *E. granulosus* infection and this has always questioned the real potential of this diagnostic test (Eckert et al., 2001). Further improvement of sensibility have been reached by Gasser et al. (1992) researching antibody against the worm excretory/secretory antigens (WES) an against protoscolex somatic antigens (PSM); nevertheless, it has been shown that some dogs suffering from severe infections can be serologically negative, compromising the real effectiveness and reliability of these tests (Craig et al., 1995). Immuno-coprological diagnosis usually guarantee an improvement of the results allowing the identification of the so-called faeces coproantigens (CA: represented by somatic antigens of the adult parasite released by proglottids), by immunoenzymatic methods. (Allan et al., 1992; Deplazes et al., 1992). This technique shows good results in term of sensitivity (> 96%) and specificity (> 96.5%); moreover it results to be approximately 2.5 times more sensitive than serological techniques because able to assess the CAs presence also when eggs are absent (Craig et al. , 1995; Allan et al., 1990; Deplazes et al., 1990). Indeed, it was experimentally proved that infected

dogs have levels of CA detectable as early as 5-10 days after infection, and they can be negative in 2-5 days after elimination of the parasite (Deplazes et al., 1992; Sakashita et al., 1995). A similar diagnostic technique is the sandwich ELISA for the research of CA, which uses two murine monoclonal antibodies (EgC1 and EgC3) for the detection of secretion-excretion antigens produced by *E. granulosus* (Casaravilla et al., 2005). The technique seems able to overcome the possible cross-reactions with other infection due to cestodes belonging to the Taeniidae family. Regarding the PCR (Polymerase Chain Reaction) is useful for diagnosis in definitive hosts especially in case of mild infection (Christofi et al., 2002). However, this technique is reported to be useful for discriminating *Echinococcus* infections from the other tapeworms allowing a certain diagnosis of the problem even in presence of a few proglottids or eggs in the faecal material (Cabrera et al., 2002; Abbasi et al., 2003; Dinkel et al., 2004; Stefanic et al., 2004). Two different methods to isolate DNA are reported in literature: the first provides a simple DNA extraction from feaces, the second one requires instead the use of floating solutions to concentrate the eggs (Mathis et al., 1996) and has the advantage of removing the substances present in the faeces that inhibit PCR (Stefanic et al., 2004). A recent study showed that, combining the flotation and the concentration of the eggs by mean of the FLOTAC using a zinc sulphate solution with the employment of QIAmp Stool Kit for DNA extraction, is the more efficient method to obtain a high number of PCR positivity (Maurelli et al., 2018). However, although PCR can be considered a very sensitive technique, it is worth considering that the extraction of parasitic DNA from faeces results complex and does not overcome the problem represented by the inconstant emission of eggs by the parasite (a negative PCR result cannot exclude the parasite presence) (Varcasia, 2004).In intermediate hosts, as well as in definitive hosts the diagnosis of hydatidosis was mainly based on necropsy findings, with the detection of cysts in different organs. Instead, for a correct in vivo hydatidosis detection, it is necessary to employ serological or diagnostic imaging techniques represented by ultrasound (Dore et al., 2014, Hussein et al., 2014). The serological diagnosis seems to show a poor reliability due to both the antigenic cross-reactivity with other Taenia species (*T. hydatigena, T. ovis*) and the low antibody response produced by intermediate hosts (McManus et al., 2003; Kittleberger et al., (2002) compared several ELISA techniques using the 8 kDa subunit of AgB, the recombinant EG95 protein pertaining to the oncosphere and a crude preparation of protoscolices as antigens. More precisely the latter, defined as protELISA, proved to be the most reliable but

did not obtain satisfactory sensitivity (62.7%). Recent studies have shown conflicting results but, in any case, the levels of specificity and sensitivity were suboptimal (McManus et al., 2014). More interesting results, instead, were obtained on sheep with the development of a rapid test based on the recombinant HSP70 protein, although its effectiveness on a large scale has not been tested (Xunhui et al., 2017). Detection of circulating antigens does not appear to be useful for diagnostic purposes (McManus et al., 2003) as well as the search for a cell-mediated response through the evaluation of interferon-gamma and circulating IL5 have not yielded the desired results (Kittelberger, 2002). So**,** even if the development of a serological diagnosis of *E. granulosus* and other cestodes would be of crucial importance, the results available at present are far to be valid for a correct diagnosis. Different are, instead, the results observed with the diagnostic imaging techniques. A first study was based on the radiological examination of lungs (Wyn-Jones and Clarkson, 1984) and only subsequently the US was employed to assess the prevalence of hydatid cysts in liver of sheep and goats (Maxson et al 1996). The US technique is based on the use of a probe capable of emitting ultrasound into different tissues that, due to different reflection proprieties, will generate echoes displayed as an image. On the basis of the nature of the tissue encountered, the image will be composed of a scale of gray that will go from the hypoechoic (black), typical of the liquid, to the hyperechoic (white), given by tissues such as those bone that reflects almost all the ultrasound (Arsenopolus, 2017). In sheep, ultrasonography allows to examine different organs (i.e. reproductive, gastrointestinal) and is able to show alterations such as ascites, abscesses and modification in the parenchyma of different organs (Scott, 2017). This technique is also effective for the diagnosis of various parasites. In fact, it has been proved that examining the liver, the presence of *Fasciola Hepatica* can be assessed within the bile ducts as well as that of *Dicrocoelium denditricum,* trematodes usually responsible of ectasia, calcification of bile vessel and modification of the liver parenchyma (Arsenopopolus, 2017). By means of ultrasonography, the hydatid cysts can be detected as a rounded structure with hypoechoic centre when young, while more hyperechoic and irregular when degenerated. Although the technique seems to show undoubted advantages it presents some limitation: a low number of cysts, a small size (<9 mm) and the position of the lesions can make the examination difficult (Arsenopopolus, 2017).

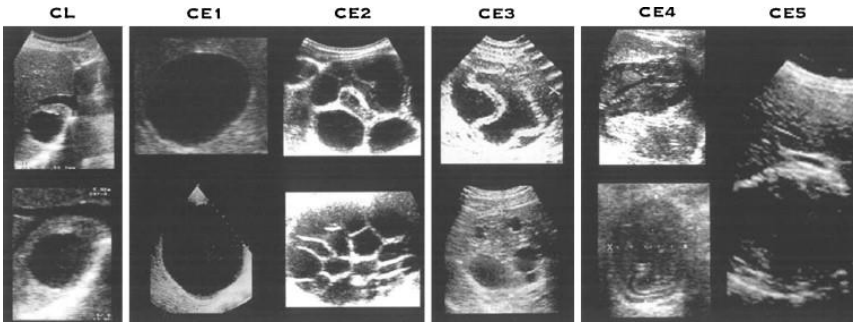

Fig.3.2 *WHO-IWGE classification of cystic echinococcosis. (Brunetti et al.2010).*

Despite these limitations, the transcutaneous ultrasonography of the entire liver, through the right intercostal spaces, is considered a reliable, simple, non-invasive method for CE diagnosis in sheep (Wyn-Jones and Clarkson, 1984). Moreover, according to the World Health Organization (WHO) the ultrasonography can be used as a tool able also to assess the stage of hepatic hydatid cyst. Hussein et al. (2014), performing a liver ultrasound examination of Baladi sheep, in standing position, shaving the right-side, obtained values of sensitivity and specificity of 80% and 100% respectively. A further study on the topic (Dore et al., 2014), carried out on 129 Sardinian sheep, obtained slightly different results. Placing the animals on dorsal decubitus and carrying out scans from the right hypochondrium, without shaving the area (in order reduce the examination time), the sensitivity and specificity values were of 88.7% and 75.9% respectively to. Since the lungs are often interested by hydatid cysts, ultrasonography could be used to obtain a diagnosis also in these organs. This technique is experimentally used to assess other lung lesions (i.e. adenocarcinoma) but the time required for the restrain, preparation, shaving of both sides and the examination could be not suitable for the usual routine of commercial sheep farm (Cousesn et al., 2015). Some experimental studies have also evaluated the use of computerized axial tomography (TAC) in sheep showing how it is actually possible to make a correct diagnosis, but this technology can be hardly used in field screening (Mao et al., 2017). Diagnostic imaging is also widely used in humans for the detection of cystic hydatidosis. Radiology (pulmonary, bone tissue or muscular cysts), ultrasound (abdominal or muscular cysts) CT (all organs) and magnetic resonance (post-surgical study of residual lesions and recurrences) (Pawlowski et al., 2001) have proved their efficacy in the diagnosis of CE. However, these techniques are not always conclusive, since

the cysts may present an "atypical aspect" which does not often differentiate them from abscesses or tumors (Siracusano et al., 2006). For this reason, even in humans, several researches have been performed on immunodiagnosis although their found much limited use (Ito, 2002). Indeed, the failure to standardize the techniques, associated with a low specificity and sensitivity values due to high number the false positive (neoplasms, cirrhosis hepatic), false negative results (recent cysts in the involution phase) as well as the possible cross-reactions with antigens of other parasites negatively influenced their application. (McManus et al., 2003). Furthermore, the antibody response of the host is related to the localization and the vitality of the cysts. Indeed, cerebral or splenic cysts will elicit a poor antibody response, especially if sterile, senescent or cracked (Pawlowski et al., 2001). However, in humans the serological methods used in the follow up of the post-surgical or post-pharmacological phase, aimed to measure in how much the antibodies titers tend to disappear after the therapy (Pawlowski et al., 2001). Moreover, according to McManus et al. (2003), also the detection of serum's interleukin 4 can be use in post-treatment follow up as this cytokine tends to reduce its levels in case of effective therapy. In last years, the serological analysis aimed to improve the detection of antibodies, antigens and also miRNA and other marker for the diagnosis of CE but they still lack of standardization and do not overcome the cross reactivity between different parasitic infections (Sarink et al.,2018)

3.3 Economic impact of cystic echinococcosis

Cystic echinococcosis not only causes severe disease and possible death in humans, but also results in economic losses from treatment costs, lost wages, and livestock-associated production losses.

In humans, costs are mainly related to diagnosis, hospitalizations and surgical treatments, loss of working days, transport costs for the care, (Budke et al., 2006). Animal-associated economic losses, instead, arise from the destruction of infected organs, decreases in carcass weight, milk production and fertility rates (Budke et al., 2006). Estimation of the economic burden in humans and livestock is important and should be part of any cost–benefit programme for the control of parasitic zoonoses. In sheep positive to CE, losses in milk production (7 – 10%), meat (5% – 20%) and wool (10% – 40%) and the weight of lamb born from infected mother (20% – 30%) have been recorded (Battelli, 2004). In all livestock this

parasite has a negative effect also on reproduction. The effects of this infection could severely affect the communities whose economy mainly relies on livestock. For instance, in Sardegna, one of the Italian regions with the larger population of sheep, data regarding the year 1989 recorded losses related to milk production due to CE infection equal to $13.700.000. A precise estimation and comparison of the cost in different countries could be problematic due to the non-homogenous methods for data collection or the underreporting of the clinical case. However, according to Budke et al. (2006), who managed to estimate the global costs of echinococcosis, when no underreporting where considered the estimated human burden of the disease is of US $193.529.740 / years, whereas estimating also the underreports, the cost arises to US $763,980,979 (Budke et al., 2006). Similar situation was found in livestock production, in which the losses correspond to at least US$ 141,605,195 and possibly rises up to US$ 2,190,132,464. However, even when underreport are considered, these data are partial, and the real costs could be higher suggesting the importance of the global monitoring and control of CE. Further work is required to evaluate the cost–effectiveness and cost–benefit of any control programmes implemented, and to guide decision makers and stakeholders on the best approach to take with the resources available. In regions of Italy where CE is epidemic, mass screening studies using ultrasonography would improve estimates of the actual prevalence of undiagnosed or asymptomatic cases.

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Chapter 4

Therapy

4.1 Therapy in definitive hosts

The therapy of cystic echinococcosis varies significantly between the intermediate and definitive hosts due to the differences in morphology, the localization and the clinical manifestations of the adult and larval stages. In definitive hosts the therapy for *E.granulosus* is pharmacological. In the past, the main treatment carried out through use laxatives based on arecoline hydrochloride, despite having the strong limitation of requiring up to 9 treatments to eliminate 99.9% of the parasites (Eckert, 1986). The elected drug for echinococcosis treatment in dog is praziquantel, an isoquinolone pyrazine derived at a dose of 5.0 mg/kg per os and 5.7 mg/kg. in cats (Eckert, 1986). At these dosages, the drug is highly effective towards the intestinal localization stages (immature and mature) of *E. granulosus* and other cestodes (WHO, 1984; Oakley, 1991; Bauer, 1994) even though it does not present an ovicidal action (Thakur et al., 1979). In several studies, a single oral administration of praziquantel (5.0 mg/kg) was effective in all treated dogs and rarely a residual, albeit low, parasitic charge has been reported after the treatment (Oakley, 1991; Bauer, 1994). Praziquantel is characterized by a high safety index , and even in pregnant animals, high dosages are tolerated for long periods without causing side effects or reproductive disorders (Thomas et al., 1978; Andrews et al., 1983). Epsiprantel is a molecule structurally very similar to praziquantel, available in tablets for administration to dogs (5.5 mg/kg per os) and cats (2.75 mg/kg), highly effective towards several Taenia species as well *Dipylidium caninum* in dogs and cats (Corwin et al., 1989; Manger et al., 1989; Oakley, 1991), and also towards *Echinococcus spp*.. In fact, in two studies (Arru et al., 1990; Thompson et al., 1991), a single administration of epsiprantel at a dose of 5.0 mg/kg eliminated on average 99.9% of *E. granulosus* from dogs. However only 2 of the 10 dogs resulted completely free from *E. granulosus* after treatment. Epsiprantel is well tolerated in dogs and cats (safety indices of 90 in the dog and 36 in the cat) and, unlike the praziquantel it is only partially absorbed by the host; therefore, its action is probably towards directly against the cestodes (Manger et al., 1989). Other molecules such as nitroscanate and various benzimidazoles showed a partial efficacy towards *E. granulosus*, but none of them can be compared to the isoquinolones. To control echinococcosis in dogs, Cabrera et al. (1996) suggest repeated treatment at 6 weeks intervals, as the pre-patent period of this tapeworm exceeds 42 days. However, given the possibility of low residual parasitic

charges, a second treatment is recommended to be repeated 1-7 days after the first. The results of drug treatments should be evaluated with copro-ELISA or PCR. Regarding the immune response of the definitive hosts to both the infestation of *E. granulosus* and other tapeworms, several studies have also been carried out aimed at finding a vaccine. However, despite the attempts made with different antigens, the results obtained so far are not satisfactory (Heath et al., 1986, 1995). More recently a vaccination with adult-worm specific proteins (EgM9 and EgM123) obtained god results regarding the reduction of worm burden and egg production however his efficacy on large scale has not yet be proved.

4.2 Therapy in intermediate hosts

Several groups of cytostatic drugs, antibiotics, sulphonamides, antiprotozoans and many anthelmintic have been tested for the treatment the metacestode stage of Echinococcus. The benzimidazoles obtained the most encouraging results. The first studies on the anthelmintic effects of this group of substances were conducted by Thienpont et al. (1974), which described the effects of mebendazole on *T. taeniformis* in mice. Subsequently, several studies were carried out using other benzimidazole derivates, such as albendazole, fenbendazole and flubendazole. The latter were tested on mice with secondary hydatidosis experimentally induced by intraperitoneal injection of *E. granulosus* protoscolics. (Eckert, 1986; Amman et al., 1995). Studies have been carried out using only mebendazole in sheep and pigs with a dosage of 50 mg/kg administered daily for 3 months (Gemmel et al., 1995). The effects of praziquantel were tested, even on intermediate host cysts, obtained mixed results. In fact, if administered for a few days before the infection, it inhibits the development of cysts in the mouse up to 97%, while if administered after the infection, the efficacy considerably decreases up to 78%. Even in sheep experimentally infected with *E. granulosus* and treated with praziquantel, either in case of subcutaneous (50 mg/kg) or oral (100 mg/kg) administration no visible effects on cysts were detected (Richards et al., 1988). Significant progress has been made in the prophylaxis of infection of intermediate hosts with larval stages of the cestode in Australia and New Zealand. In fact, a recombinant vaccine for *T. ovis* has been developed in sheep, using antigens derived from oncospheres (Johnson et al., 1989; Lightowlers, 1994; Lightowlers et al., 1995). More recently, using the same principle, a recombinant vaccine against *E. granulosus* (EG 95) tested in sheep and

cattle showed levels of protection obtained against eggs of 97-98% and 89-99% respectively (Ligntowlers et al., 1999; Heath et al. 2003). A high level of immunity persists for 6 months and in pregnant sheep high levels of antibodies are transferred to lambs (Heath et al., 2003). In recent years Larrieu et al. (2019) conducted an 8-years study in the district of Rio Negro, Argentina, testing the efficacy of EG 95 on large scale. With a vaccination plan consisting of 3 dose/year for each lamb, the number of the sheep positive to *E.granulosus* decrease to 56.3% to 20.3% and, as a consequence, also in the number of the dog positive decreased to 9.6% to 4.5%. In addition, it has to be notice that to improve the efficacy of the vaccination, the dog population in the same area of the intermediate hosts should be submitted to treatment with praziqeuantel every three months (Larrieu et al., 2019). In humans the therapy is mainly surgical, radical or conservative (Eckert et al. 2001). The radical surgery consists in removing the entire cyst and the surrounding tissue (i.e. lobectomy) and there may be a recurrence in the 5% - 25%, of the patient. The conservative therapy consists of the endocystectomy, with the removal of the inner layer. The outer layer, which remains in the liver, is one of the causes of complications, potentially leading to the formation of biliary fistula or local recurrence (da Silva, 2005). However, the pre-operative chemotherapy treatment with benzimidazole, reduced the need for surgical procedures too invasive, as it softens the cysts and reduces their internal pressure whereas the intra surgical administration of protoscolicides in doubtful. In fact, the lack of a substance effective but sure for the patients could damage the affected organ (Eckert et al. 2001). Another treatment for human echinococcosis is also the PAIR (Puncture, Aspiration, Injection, Re-aspiration) (Brunetti et al., 2004). This method involves percutaneous ultrasound-guided puncture, aspiration of the cyst content and injection of scolicidal substances (i.e. ethanol), and finally re-aspiration of the cystic content after 5 minutes. If a hypertonic solution of NaCl (at least 15%) is used as a scolicidal substance, the action is slower, therefore the re-aspiration must take place after 15-20 minutes (Eckert et al., 2004). This technique is more effective with liver cyst classified as CL, CE1, CE2 and CE3 with a diameter of more than 5 cm (Eckert et al., 2004), on patient treated with benzimidazoles (sometimes associated with praziquantel) four days before the treatment and, to reduce the post surgical recurrences, the patient should be treated for at least one month after the PAIR (Eckert et al., 2004).

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Chapter 5

Experimental part: Ultrasound diagnosis of ovine echinococcosis

5.1 Material and Methods

5.1.1 Animal selection

Between May 2017 and September 2019, one hundred-seventy-two (172) female sheep of several breeds, scheduled for slaughtering and originating from 21 farms were enrolled. All the farms were located in Campania region, (Southern Italy), within an area showing a CE prevalence up to 75% (Deplazes et al., 2017). All the farms enrolled had a previous diagnosis of CE and a history of grazing on open pastures shared with wild animals and shepherd dogs. The sheep enrolled were submitted to a complete clinical examination before the US and weighed by means of a scale before the slaughtering.

Table 5.1 *Sheep breeds enrolled*

Breed	**n°**	**Mean weight (± SD)**
Mixed	105/172 (61%)	79.7 ± 10.9
Merinizzata	24/172 (14%)	79.1 ± 2.3
Bagnolese	21/172 (12%)	68.9 ± 4.1
Comisana	10/172(6%)	54.8 ± 5.0
Sarda	12/172 (7%)	43.0 ± 1.5

n°: number of sheep; **SD**: Standard deviation.

5.1.2 Ultrasound examination and fast scanning technique

In each animal enrolled, an area between the right hypochondrium and the caudal margin of the homolateral scapula was shaved (Econom 2, Aesculap Suhl GmbH, DE); the US was performed with a Mylab® Alpha device (Esaote SPA, IT) using a microconvex multifrequency transducer (6-10 MHz). The sheep were placed in standing position, not-sedated and handled by a single operator; the time needed to perform each exam (from clipping to US end) was also recorded (minutes). According to the technique described by Braun et al. (1992), a complete liver ultrasound examination (cUS) was performed starting from the right hypochondrium, proceeding from 12th to 5th intercostal spaces (IS), each one examined from dorsal to ventral using longitudinal and transverse complete scans. The evaluated scan area was divided in: right hypochondrium (HYP), caudally to the last rib, as described by Dore and collaborators (2014), Zone 1 (Z1), from the right hypochondrium to the 11th IS, Zone 2 (Z2) from the 10th IS to the 8th IS and Zone 3 (Z3) from the 7th IS to 5th IS (Fig. 5.1). Each zone (ZONAL scan) was analysed individually; moreover the contiguous zones were paired and analysed (HYP+Z1; Z1+Z2, Z2+Z3). During each scanning, all the hydatid lesions were always localized and characterized according to WHO (2011).

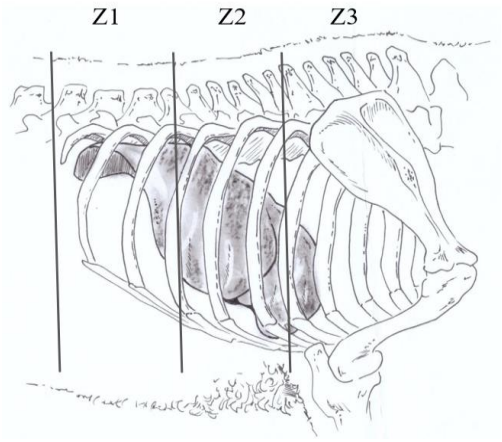

Fig.5.1 Liver area division for the development a fast scanning technique. **Z1:** partial liver scan from hypochondrium to 11th intercostal space; **Z2:** partial liver scan from 10th to 8th intercostal space; **Z3:** partial liver scan from 7th to 5th intercostal space

Within 24 hours from US, the sheep were slaughtered and the liver and lungs of each animal were collected, palpated and dissected along their longitudinal axes in <1 cm strip to identify cysts at the necropsy facilities of the Department of Veterinary Medicine and Animals Production of the University of Naples Federico II. Hydatids were differentiated from other cyst-like parasitic structures (*Taenia hydatigena* metacestodes), and distended bile ducts caused by the liver *flukes (Fasciola hepatica* and *Dicrocoelium dendriticum*) by means of macroscopic examination and, in case of diagnostic doubts, light microscopy was also used.

5.1.3 Statistical analysis

All the data were analysed by standard descriptive statistics, normality was assessed using Shapiro Wilk tests. Data were expressed as absolute numbers, percentage, ranges and mean ± standard deviation (SD). Values of sensitivity, specificity, negative predictive values (NPV), positive predictive values (PPV), number and percentage of hydatid cyst detected were calculated for each scan, using as gold standard the necropsy's results. A comparison between each technique was also performed (i.e. cUS vs. HYP; Z1 vs. Z2+Z3; Z1+Z2 vs. cUS, etc). To assess the differences in number and percentage of positive zones found by each technique, a $\chi 2$-test was used. Probabilities ≤ 0.05 were considered as significant, all statistical analyses were performed using dedicated software (SPSS, Version 17.0, Chicago, IL). The sensitivity, specificity, NPV and PPV were calculated in order to identify the most effective US technique. In detail, sensitivity=[true positives/(true positives + false negatives)]; specificity=[true negatives/(true negatives + false positives)]; NPV=[true negatives/(true negatives + false negatives)]; PPV=[true positives/(true positives + false positives)]. When the post-mortem examination confirmed the presence of at least one hydatid detected by US, the zone was classified as true positive, whereas if there were negative findings in both post-mortem examination and US it was classified as true negative. Instead, a false positive was defined as a zone resulted positive for CE at US, negative during the post-mortem exam; a false negative was defined as a zone resulted negative for CE to US, positive during the post-mortem exam.

5.2 Results

The composition of the sheep population examined is reported in table 5.1; briefly, 61% (105/172) animals were mixed breed, showing a mean weight of 79.7 ± 10.9 kg whereas the remaining 39% were pure-breed, with a mean weight of 64.5 kg.
Because of the non-homogenous weight distribution due to different size of the breeds examined, the sheep were divided into three groups: G1 (weight ≤ 50 kg: 22/172 - 13%), G2 (51≤ weight ≤ 75: 69/172 – 40%), G3 (weight ≥76: 81/172 – 47%).
The mean cUS time needed for the evaluation of a single animal was of 7.1 ± 1.56 minutes; however, approximately the 60% of this time was spent to shave of each sheep; less time was needed employing about the of the other techniques (HYP: 1.7 ± 0.79; Z1: 1.3 ± 0.58; Z2: 1.3 ± 0.59; Z3: 1.3 ± 0.55). Of the 172 animals examined 46% (80/172) were positive at post-mortem examination; the cUS was able to detect the higher number of positive animals (73/172 – 42%) when compared with HYP and ZONAL (Table 5.2).
In all the animals enrolled, the highest values of sensitivity (Se) were observed when complete US was employed (Table 5.3 – 5.4), whereas PPV and NPV values resulted of 80% and 91%. This technique showed high values either of Se or specificity (Sp) also when the different groups were considered. High values of Se and Sp were observed with HYP scan in G1. When paired, the contiguous zones showed an improved value in Se and Sp, especially in G1 for all the scans and in in G3 for Z2+Z3 (Table 5.3).

Table 5.2 *Positive animal detected with each scan technique*

	cUS	**HYP**	**Z1**	**Z2**	**Z3**	**HYP+Z1**	**Z1+Z2**	**Z2+Z3**
TP (n°and%)	73/172 (42%)	48/172 (28%)	38/172 (22%)	51/172 (29%)	35/172 (20%)	46/172 (27%)	62/172 (36%)	63/172 (37%)

TP: true positive; **cUS:** complete liver scan through intercostal spaces; **HYP:** complete liver scan through hypochondrium **Z1:** partial liver scan from hypochondrium to 11th intercostal space; **Z2:** partial liver scan from 10th to 8th intercostal space; **Z3:** partial liver scan from 7th to 5th intercostal space; **HYP+Z1:** sum of scan in HYP and Z1, **Z1+Z2:** sum of scan in Z1 and Z2; **Z2+Z3:** sum of scan in Z1 and Z3

The percentages of the true positive zones detected with the different techniques and in different groups are reported in figure 5.2 and 5.3; in particular cUS was able to reveal a significantly higher number of positivity (124/136 - 91%) when compared to other technique ($p \leq 0.01$) (Fig 5.2). Group G1 showed a significantly higher ($p \leq 0.01$) percentage of positive zones than Group 2, 3 for HYP scan (Fig. 5.3). Similarly, a significantly higher value ($p \leq 0.01$) was found in G1 when HYP + Z1 was considered (Fig. 5.3).

Table 5.3 *Values of sensitivity, specificity, of US in the different groups*

	cUS		**HYP**		**Z1**		**Z2**		**Z3**	
	Se(%)	**Sp(%)**	**Se(%)**	**Sp(%)**	**Se(%)**	**Sp(%)**	**Se(%)**	**Sp(%)**	**Se(%)**	**Sp(%)**
G1	91	82	91	82	55	91	64	82	36	91
G2	91	89	61	91	47	94	70	92	36	94
G3	92	96	47	96	44	98	58	96	53	98
G1+G2+G3	91	80	60	91	48	96	64	92	44	82

cUS: complete liver scan through intercostal spaces; **HYP:** complete liver scan through hypochondrium **Z1:** partial liver scan from hypochondrium to 11th intercostal space; **Z2:** partial liver scan from 10th to 8th intercostal space; **Z3:** partial liver scan from 7th to 5th intercostal space; **Se**: sensitivity; **Sp**: specificity **G1**: weight ≤ 50 kg; **G2:** wight between 51 kg and 75 kg; **G3:** weight ≥ 76 kg; **G1+G2+G3**: total population

Table 5.4 *Values of sensitivity, specificity, of scan of paired zones for each group*

	HYP+1		**Z1+Z2**		**Z2+Z3**	
	Se (%)	**Sp (%)**	**Se (%)**	**Sp (%)**	**Se (%)**	**Sp (%)**
G1	91	82	91	82	91	82
G2	64	94	82	83	76	88
G3	56	98	69	96	81	96
G1+G2+G3	58	91	78	92	79	91

HYP+Z1: sum of scan in HYP and Z1, **Z1+Z2:** sum of scan in Z1 and Z2; **Z2+Z3:** sum of scan in Z1 and Z3; **Se**: sensitivity; **Sp**: specificity; **G1**: weight ≤ 50 kg; **G2:** wight between 51 kg and 75 kg; **G3:** weight ≥ 76 kg; **G1+G2+G3**: total population

By means of necropsy examination, 136 positive zones were detected, their distribution showed a slightly higher presence of hydatid lesion in Z2 (39% - 53/136) than Z1 (30% - 41/136) and Z3 (31% - 42/136). A total of 301 cysts were found, of which 259 (86%) were detected through US and therefore classified according to WHO as CE2/fertile (3/259 – 1.2%), CE3/transitional (13/259 – 5%), CE4/degenerated (4/259 – 1.5%) and CE5/inactive (239/ 259 – 92.3%) (Fig. 4). Only one of the cysts classified as CE3 (1/13) showed a marked multilocular aspect. Hydatid lesions in the lungs, recorded through post-mortem examination, were often associated with hydatid liver lesions (63/80 – 79%); only in three animal (3.75%) hydatid lesions were present only in the lung parenchyma.
Finally, in 61 sheep signs of trematodes lesion, as hyperechoic areas (linear or spot) irregularly distributed in the liver parenchyma (ductal phase), were detected in both CE positive (25/80 - 31%) and negative (27/92 – 29%) animals. In 2 sheep a liver abscess was detected; the lesions appear as a circular anechoic area containing multiple hyperechoic dots surrounded by a capsule (Fig.5.4).

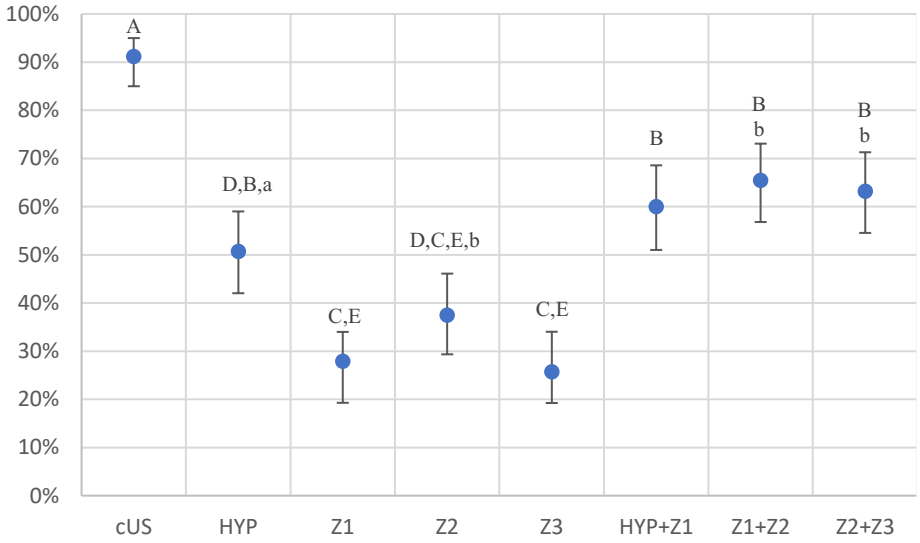

Fig. 5.2 *Percentage of true positive zones detected with each scan technique in the entire population:* ***cUS:*** *complete liver scan through intercostal spaces;* ***HYP:*** *complete liver scan through hypochondrium* ***Z1:*** *partial liver scan from hypochondrium to 11th intercostal space;* ***Z2:*** *partial liver scan from 10th to 8th intercostal space;* ***Z3:*** *partial liver scan from 7th to 5th intercostal space,* ***HYP+Z1:*** *sum of scan in HYP and Z1,* ***Z1+Z2:*** *sum of scan in Z1 and Z2;* ***Z2+Z3:*** *sum of scan in Z1 and Z3;* ***ABCDE:*** *percentage with the same letter are not significantly different ($p \leq 0.01$);* ***a,b: :*** *percentage with the same letter are not significantly different ($p \leq 0.05$)*

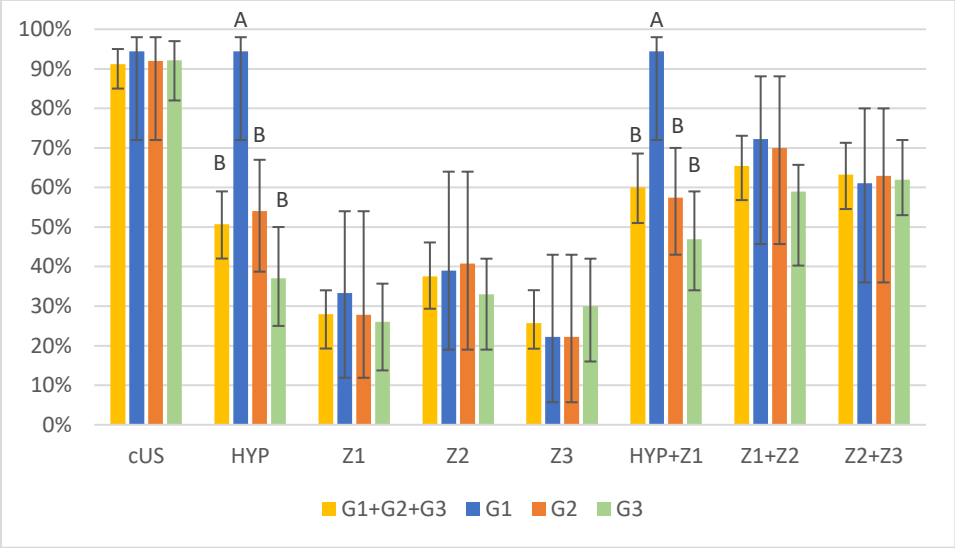

Fig. 5.3 *Percentage of true positive zones detected with each scan technique in the entire population and groups:* ***cUS:*** *complete liver scan through intercostal spaces;* ***HYP:*** *complete liver scan through hypochondrium* ***Z1:*** *partial liver scan from hypochondrium to 11th intercostal space;* ***Z2:*** *partial liver scan from 10th to 8th intercostal space;* ***Z3:*** *partial liver scan from 7th to 5th intercostal space,* ***HYP+Z1:*** *sum of scan in HYP and Z1,* ***Z1+Z2:*** *sum of scan in Z1 and Z2;* ***Z2+Z3:*** *sum of scan in Z1 and Z3;* ***G1****: weight ≤ 50 kg;* ***G2:*** *weight between 51 kg and 75 kg;* ***G3:*** *weight ≥ 76 kg;* ***G1+G2+G3****: total population;* ***A,B:*** *percentage with the same letter are not significantly different (p ≤ 0.01).*

5.3 Discussion

For the purposes of the analysis outlined in this manuscript, the Se and Sp of the US for CE detection and the development a practical, fast-focused US technique for their diagnosis has been performed in different sheep's breeds. Estimation of the reliability of this instrumental diagnostic technique, against lesions due to *E. granulosus,* represents an interesting challenge in species like sheep, where this parasitosis is common but the diagnostic possibilities are quite poor. The on-farm CE diagnosis made by liver ultrasonography has a crucial importance to identify infected animals, manage the negative effects of this parasite on sheep health and economy of

the entire dairy-food chain. In this thesis, the complete ultrasound examination of the liver has been confirmed as a reliable diagnostic method to assess CE infection in alive sheep. Indeed, the investigation revealed how the cUS scan failed in only 7 cases; in all groups considered, Se and Sp values similar to what recently reported in the literature by Dore et al (2014) (88.7% - 75.9%), as well as by Hussein et al. (2014) (80% - 100%). The other techniques employed, even if failed to detect an acceptable number of positive animals (low Se, range: 44% - 79%), could efficiently assess sheep negative for liver CE due to an higher Sp (82% - 96%) (Table 5.3 – 5.4). Despite the small number of subjects involved, only in G1 the HYP and the paired scans reached values of Se and Sp similar to cUS. These results observed only in lighter animals are like those described by Dore et al., (2014) in Sarda sheep, a typical Italian milk breed with a mean weight of 40 kg (female sheep) (Bigi and Zanon, 2010). However, the significantly higher number of positivity found by HYP and HYP + Z1 scans in G1 ($p \leq 0.01$) than those observed in the other groups, suggests that these two scans may be the most efficient only in animal of small size. So HYP scan appeared to be clearly influenced by the weight of animals scanned, indeed also when heavier animals were considered (G3), this scan was not able to reach acceptable values of Se.

In this thesis, the necroscopic and ultrasonographic analysis of the positivity distribution did not reveal a predominant concentration in none of the zones considered. In the entire population a lower and not acceptable Se were obtained either with all the single (Z1:48%, Z2: 64%, Z3: 44%) or paired scans performed (HYP + Z1: 58%; Z1+Z2: 78%; Z2+Z3: 79%) (Tab. 5.3 - 5.4); therefore, none of them resulted suitable for an on-farm flock screening for CE. Moreover, the non-specific lesions' distribution did not allow a development of a real fast scan technique, since the scanning a partial liver's areas may easily lead to false negative results. Even in the zonal scan some important differences were recorded, when different groups were considered. The higher Se of Z3 found in the heavier group (G3) could be a consequence of the larger dimension of the sheep and their intercostal space, which makes the ultrasonography more comfortable in this region. On the other side, the low Se value obtained in this heavier group regarding Z1+Z2 could be the direct consequence of the reasonable difficulties due to the scanning either of wider thorax or of bowel interposition during the caudal scansion.

Although a low number of positive animals for fertile cysts were found (2/80 – 2.5%), in each zone considered and with all the US technique used, the Se

improved significantly (100%) when fertile or transitional hydatid (CE2-CE3) were considered. The higher Se obtained indicates the US may have an important role in the control of *E. granulosus* lifecycle in a flock since this technique reach its maximum efficiency in the diagnosis of fertile and capable of infection cysts.
Regarding the overall number of cysts detected by US (259/301 - 86%), the investigation revealed how the major part of them was sterile (CE5) and degenerated (CE4), suggesting a chronic status of the lesions; Instead, active (CE2) and transitional (CE3) one were probably a consequence of an infection occurred in more recent times. Apart from hydatid cysts, US allowed the detection of further lesions with a different origin; most of them were indeed related to liver flukes in both positive (25/80 - 31%) and negative (27/92 – 29%) animals. Furthermore, in one sheep a multiple lesion showed a US appearance indicative of both active and sterile cysts although post-mortem exam revealed the presence of multiple abscesses. The presence of these structures, probably originated from hydatid complications, flukes or pyogenic microorganism infection (i.e. *Corynebacterium pseudotuberculosis)* (Scott, 2017) could lead to a misdiagnosis, thus representing a potential restriction of US employment a diagnostic test in alive animals under field condition.

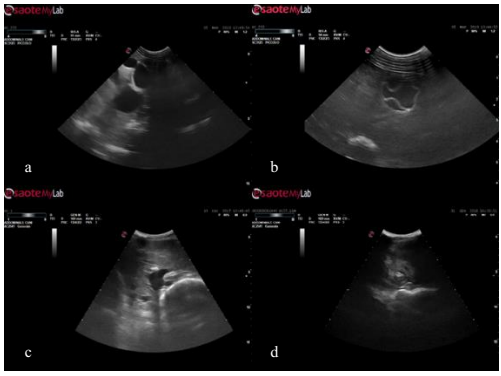

Fig.5.4 *Liver lesion detected through US: (a) CE2, multivesicular, multiseptated cysts, active;(b) CE3, unilocular cyst, with detachment of laminated membrane from cyst wall, could contain daughter cysts, transitional; (c) CE5, thick calcified wall that is arch shaped, producing a cone shaped shadow ,inactive.: (d)liver abscess.*

Regarding the simultaneous presence of CE in liver and lungs, it was frequently observed at post-mortem examination time (63/80 – 79% of cases) similarly to what reported in literature (Dore et al., 2014); case in which the lungs were instead the only target organs of echinococcosis infection was only a sporadic event (3/80 – 3.7% of cases). In our opinion, even if a low number of animals showed only lung lesions, this may represent a further restriction of the exclusive liver US examination. Indeed, if on one side, the only focusing on this organs may sensibly reduce time consuming under field condition and may give encouraging results as screening test for positive animals detection, on the other side, the complete lack of information regarding the lung's status may lead to underestimate the disease's prevalence within the flock, limiting the potential effectiveness of clinical strategies for prevention and control.
Furthermore, under the practical point of view it is important to underline how the preparation for the method (handling and shaving) and the execution of the cUS on a single animal, did not require a large amount of time for single animal but, when the procedures are applied to an entire flock, it may require a sensible increase of time potentially limiting the use of this diagnostic approach as precision farming technique. However, the major part of the time was spent for wool shaving (approximately 60%) so, in order to reduce the total time of the procedure, the Authors may suggest the use of this important diagnostic tool during or immediately after the shearing season.

5.4 Conclusion

The current study represents the first investigation evaluating the use of US as potential fast-focused technique for CE hepatic lesions detection in different breeds of sheep under field conditions. The investigation confirmed that the ultrasonography could be considered a reliable intra-vitam technique for assessment of hydatid cyst in the liver. The HYP scan was not always able to correctly assess the presence of the parasite in sheep of medium or heavy weight (>51 kg) whereas it can correctly detect positive sheep weighing less (<51 kg). Considering the results observed, the scanning the entire organ, from the hypochondrium to the 5th intercostal space is there recommended under field condition to optimize the diagnostic performance. However, the time needed for the exam execution can represent a limit especially for screening in large flocks; further strategies to reduce the time

consuming under field condition should be evaluated to improve the widespread of ultrasound use for CE diagnosis in sheep flock.

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