Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkhälsovetenskap

Echinococcus multilocularis in wild boar
- aiming at an alternative surveillance method

Tuva Vamborg

Uppsala

2011

Examensarbete inom veterinärprogrammet

ISSN 1652-8697
Examensarbete 2012:44

SLU
Sveriges lantbruksuniversitet

Echinococcus multilocularis in wild boar
- aiming at an alternative surveillance method

Tuva Vamborg

Handledare: Anna Lundén, Institutionen för biomedicin och veterinär folkhälsovetenskap

Biträdande handledare: Henrik Uhlhorn, Enhet för patologi och viltsjukdomar, SVA

Examinator: Johan Höglund, Institutionen för biomedicin och veterinär folkhälsovetenskap

Examensarbete inom veterinärprogrammet, Uppsala 2011
Fakulteten för Veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkhälsa
Kurskod: EX0239, Nivå AX, 30hp

Nyckelord: Echinococcus multilocularis, wild boar, surveillance

Online publication of this work: http://epsilon.slu.se
ISSN 1652-8697
Examensarbete 2012:44

INDEX

ABSTRACT

This study was the first part of a larger project investigating a new surveillance method for *Echinococcus multilocularis*. In this study we examined livers from wild boar (*Sus scrofa*), looking for lesions caused by *E. multilocularis*. *E. multilocularis* is a small tapeworm which has an indirect life cycle. The definitive host is usually the red fox (*Vulpes vulpes*) and intermediate hosts are rodents, for example the European water vole (*Arvicola terrestris*). But *E. multilocularis* can use many other species of carnivores as definitive hosts and other rodents as intermediate hosts. In the definitive host the parasite is found in the intestine as the adult worm. Proglottids are shed by the definitive host and ingested by the intermediate host in which cysts develop in the liver. This disease is called alveolar echinococcosis.

Other animals, including humans that are not considered normal intermediate hosts can also develop alveolar echinococcosis. These animals are referred to as accidental hosts because they are unlikely to become part of the lifecycle. This is either because the parasite does not develop fully in these animals or that these animals are unlikely to become prey to a definitive host. Examples of accidental hosts are wild boar and pig (*Sus scrofa)* and horse (*Equus ferus caballus*). The lesions found in pigs are similar to those in the intermediate hosts, but the metacestode is not fully developed. The disease in humans has a very long incubation time, varying from five to fifteen years. The case fatality rate is high but the incidence is low with approximately 18’000 new cases per year worldwide.

Surveillance methods have traditionally been based on identifying infection in the definitive host. The method mostly used in Sweden is the sedimentation and counting technique (SCT), often combined with copro-enzyme-linked immunosorbent assay (ELISA). These methods have high sensitivity and specificity but are expensive, time consuming and pose a risk of infection to persons performing it. For this study, 80 livers from Swedish wild boars were collected and examined macro- and microscopically. Four livers had lesions that looked like “white spots”, probably caused by migrating *Ascaris suum.* These lesions were tested with PCR and no *E. multilocularis* DNA was detected. No *E. multilocularis* lesions were found. It was concluded that the samples in this study could be used as a negative reference material in the continued project which in turn would investigate whether it would be possible to use the detection of antibodies to *E. multilocularis* in wild boar, using ELISA, and whether this could be used as a surveillance method.

SAMMANFATTNING

Denna studie var den första delen av ett större projekt med syfte att undersöka en ny övervakningsmetod för *Echinococcus multilocularis*. I denna studie undersöktes levrar från vildsvin (*Sus scrofa*), med avseende på lesioner orsakade av *E. multilocularis. E. multilocularis* är en liten bandmask som har en indirekt livscykel. Huvudvärden är vanligtvis rödräv (*Vulpes vulpes*) och mellanvärdar är gnagare, till exempel vattensork (*Arvicola terrestri*s). Men *E. multilocularis* kan använda många andra rovdjursarter som huvudvärd och andra gnagare som mellanvärdar. I huvudvärden finns de adulta maskarna i tarmen. Proglottider urskiljs av huvudvärden och äts upp av mellanvärden varvid cystor utvecklas i levern. Denna sjukdom kallas alveolär ekinokockos.

Andra djur, inklusive människa, som inte anses vara normala mellanvärdar kan också utveckla alveolär ekinokockos. Dessa djur kallas oavsiktliga värdar (accidental hosts) eftersom det är osannolikt att de utgör en del av livscykeln. Det beror antingen på att parasiten inte utvecklas fullständigt i dessa djur eller att det är osannolikt att de blir uppätna av en huvudvärd. Exempel på oavsiktliga värdar är vildsvin och svin (*Sus scrofa*) samt häst (*Equus ferus caballus*). De förändringar som finns i grisar liknar dem som ses hos gnagare, men metacestoderna är inte fullt utvecklade. Sjukdomen hos människa har en mycket lång inkubationstid, på 5-15 år. Letaliteten är hög, men förekomsten är låg med ca 18'000 nya fall per år i hela världen.

Övervakningsmetoder har traditionellt varit baserade på att identifiera infektionen hos huvudvärden. Den metod som används mest i Sverige är sedimentation and counting technique (SCT), ofta i kombination med copro-enzyme-linked immunosorbent assay (ELISA). Dessa metoder har hög sensitivitet och specificitet, men är dyra, tidskrävande och innebär en risk för infektion för personer som utför den. I denna studie samlades 80 levrar från svenska vildsvin in och undersöktes makroskopiskt och mikroskopiskt. PCR användes på lesioner som bedömdes kunna vara orsakade av parasiter. Fyra levrar hade "white spots", troligtvis orsakade av migrerande *Ascaris suum*. Dessa skador testades med PCR och var negativa. Inga *E. multilocularis* lesioner hittades. Slutsatsen var att proverna i denna studie kunde användas som ett negativt referensmaterial i det fortsatta projektet vilket i sin tur ska utvärdera huruvida det går att detektera antikroppar mot *E. multilocularis* hos vildsvin med ELISA och därmed undersöka huruvida denna metod kan användas till övervakning.

INTRODUCTION

Background

Echinococcus multilocularis, also known as the fox dwarf tapeworm, is a parasite that can infect humans (Eckert et al., 2001). The disease, known as alveolar echinococcosis, is rare but the case fatality rate is very high. Most commonly the parasite has a sylvatic lifecycle with red fox (*Vulpes vulpes*) as definitive host and small rodents as intermediate host. But other carnivores like the domestic dog (*Canis lupus familiaris*) and the raccoon dog (*Nyctereutes procyonoides)* can also act as definitive hosts. In addition to the natural intermediate hosts, other mammals, such as pigs and wild boars (*Sus scrofa*), can become infected but without being a part of the transmission cycle. These animals are often referred to as accidental hosts.

Because of its properties as a human pathogen, and because it had not been found in Sweden until February 2011, *E. multilocularis* is notifiable (SFS, 2004:255) and prevention and surveillance is being carried out. Prevention has been through compulsory deworming of cats and dogs before arrival to Sweden (SJVFS 2010:43). Import or travel from a few European countries, considered free from *E. multilocularis*, has been excepted from compulsory deworming in these regulations. However, the requirements of deworming have ceased as of the 1st January 2012 due to the discovery of *E. multilocularis* in Sweden in 2011 (SJVFS 2011:49).

Surveillance has been carried out by the National Veterinary Institute through detection of the parasite in the definitive host, the red fox (Osterman Lind et al., 2011). The main method used is the sedimentation and counting technique (SCT) in which the content of the entire intestine is examined, which makes the method costly, time consuming and poses a risk of infection to personnel performing it. There are many reasons why surveillance should continue or increase. The discovery of the parasite in Sweden could lead to the need for intensified surveillance to be able to establish its epidemiology. As many scientists claim that climate change and changing environments might have an impact on *E. multilocularis* epidemiology it is hard to predict how or if it will spread (Mas Coma et al., 2008).

In Sweden possible definitive hosts are found throughout the country such as red fox, arctic fox (*Vulpes lagopus*), domestic dog and wolf (*Canis lupus*) (Osterman Lind et al., 2011). The raccoon dog is migrating to the North of Sweden from Finland. There is a wealth of intermediate hosts such as voles and mice. There is also an increasing awareness and fear among the public who wants to learn about the risks that this parasite entails. Even though the screening method used in Sweden today has a high sensitivity, it also has many drawbacks, as mentioned above. There is a need for a more efficient and cheaper method, which could be used for large scale screening.

Aim

This study is the first part of a larger project investigating a new potential screening method for *E. multilocularis*. This method would be based on detection of antibodies to *E. multilocularis* in wild boar by an enzyme-linked immunosorbent assay (ELISA). To produce a negative reference material for the serological analysis the first step involves examining livers from Swedish wild boar for lesions caused by *E. multilocularis*.

LITERATURE REVIEW

Wild boar

The wild boar is an omnivore, mostly eating roots, fruits, acorns etc (Markström, 2002). Animal proteins mainly come from worms, larvae and in some cases, small mammals and eggs. The wild boar was extinguished from Sweden in the 17th century, but was reintroduced as farmed wild boar in southern Sweden during the 19th and 20th centuries. Break-outs and illegal releases during the second half of the 20th century resulted in the establishment of small colonies of free-ranging wild boars. The wild boar is now considered to be a part of the Swedish fauna.

The population was estimated to be at least 150’000 animals in 2008 (Svenska jägareförbundet, 2009). The estimation is based on the number of wild boars shot (50’000) and reported by hunters that year. It is estimated that the population can double in 3 years, and this is likely to result in increased hunting. The population is distributed from the southernmost part of Sweden to the County of Dalarna, and a few groups of animals have established even further north. Wild boar of various subspecies is found in Europe, Northern Africa, and large parts of Asia but it is not found in very dry areas or in high mountain ranges (Markström, 2002). It has been introduced to Australia, New Zealand, North and South America.

Echinococcus multilocularis

Taxonomy and morphology

Echinococcus multilocularis is a cestode (eng. tapeworm) of the family *Taeniidae* (Taylor et al., 2007; Eckert el al., 2001). Within the *Echinococcus* genus there are several other species. They have similar life cycles and morphology. With new diagnostic tools such as genetic sequencing, the taxonomy of the genus is changing (Nakao et al., 2007). One example is *E. granulosus* which previously was considered to have different strains whereas now, some of these strains are considered as individual species. Previously, taxonomy was based mainly on parasite morphology and on which animals that were its natural hosts (Gemmel, 1959; Rausch, 1968). Within the *E. multilocularis* species there are small strain differences (Eckert et al., 2001). These differences, however, do not seem related to the many intermediate or definitive hosts that *E. multilocularis* uses (Vuitton & Gottstein, 2010).

E. multilocularis is a small tapeworm, measuring 2-4 mm in its adult form, and consists of three to five segments (Taylor et al., 2007). The head, which in tapeworms is called scolex, is followed by a chain of segments, the proglottids. The scolex has four suckers and small and large hooks in a double row which are

used for attachment to the intestinal wall in the definitive host. Like other tapeworms, it has no alimentary tract and instead absorbs all nutrients through its outer covering, the tegument. The third segment has genital pores and a uterus and produces eggs. The eggs are morphologically indistinguishable from eggs of other tapeworms of the genus *Taenia*.

Life cycle

Definitive hosts

The life cycle of *E. multilocularis* is indirect with a carnivore as definitive host (Taylor et al., 2007). The following canids have been described as definitive hosts: red fox, arctic fox (*Alopex lagopus*), wolf, domestic dog, coyote (*Canis latrans*), corsac fox (*Vulpes corsac*) and raccoon dog (Eckert et al., 2001). Felidae can also be definitive hosts although they usually do not shed as many proglottids. Felidae that have been identified as definitive hosts are domestic cat (*Felis silvestris f. catus*), wildcat (*Felis silvestris*) and lynx (*Lynx lynx*).

Intermediate and accidental hosts

There are many animals, rodents and other small mammals that can act as intermediate hosts. As in humans, the disease is referred to as alveolar echinococcosis. The common vole (*Microtus arvalis*), the European water vole (*Arvicola terrestris*) and muskrat (*Ondatra zibethica*) have been described as important intermediate hosts in central Europe (Eckert, 1997). Of these three species the last two are found in Sweden (Osterman Lind et al., 2011).

An accidental host is an animal that becomes infected but is unlikely to transmit the parasite to a definitive host (Eckert, 1997; Eckert et al., 2001). This is either because the parasite does not develop fully or because the animal is unlikely to become prey to a definitive host. The wild boar is an example of an accidental host. *E. multilocularis* has evolved together with its intermediate hosts (Vuitton & Gottstein, 2010). Whether the parasite can go through a complete development to metacestode depends on the balance between the damage the parasite causes and the host's immune system.

Life cycle

The definitive host sheds proglottids containing 200-300 eggs, which can be ingested by an intermediate host (Taylor et al., 2007). The eggs are very resistant in the environment (Schiller, 1955). They are well suited to cold climates. In experiments, eggs or entire proglottids have remained viable after 24 hours in −51°C and after nearly two months in −26°C. In more recent studies eggs have remained infective after more than a year at + 4°C in water (Veit et al., 1995). They are less resistant to drier and warmer conditions.

The fully developed egg consists of an oncosphere covered by a shell called the embryophore, which it loses in the intestine of the intermediate host (Taylor et al., 2007). The oncosphere then uses its hooks to penetrate the mucosa and access the circulatory system and subsequently the liver. There it develops into its metacestode stage in the shape of a multilocular or alveolar cyst. The outer layer of the cyst is an acellular laminated layer and inside that is a cellular germinal layer. By asexual budding the germinal layer produces brood capsules within

which protoscolexes develops. The protoscolex is a precursor to the scolex. The cyst is made up of many compartments filled with a gelatinous matrix.

The growth of the cyst is invasive and can metastasize to other organs such as lungs, brain and muscle (Taylor et al., 2007). The invasive growth can weaken the intermediate host, increasing the risk of predation. When a predator ingests an infected intermediate host the protoscolex attaches itself to the intestinal mucosa and the metacestode develops into the adult tapeworm in five weeks. They survive in the definitive host's intestine for up to six months. They then become sterile which means that the infection in self-limiting in the definitive host (Rausch & Schiller 1956). The definitive host is usually not affected by the infection.

Human alveolar echinococcosis

Humans can also become infected with *E. multilocularis* thorugh the same route as the normal intermediate hosts, and the disease is called the same as in the intermediate hosts: alveolar echinococcosis (Torgerson et al., 2010). Humans can become infected either through direct contact with an infected definitive host or contaminated environment, or through contaminated food which has not been cooked. Infected humans are considered to be accidental hosts as fully developed metacestodes are rarely seen. However, in some cases protoscoleces have been found (Rausch & Yamashita, 1957). Investigations into whether humans can act as an intermediate host and infect a definitive host have been conducted (Rausch & Schiller, 1956). In one case, material from alveolar cysts from a human was fed to a dog. Necropsy was performed on the dog and adult *E. multilocularis* worms were identified. A similar method was used in a study in the USSR with the same results (Lukashenko, 1968).

Human alveolar echinococcosis is a rare disease but has a high case fatality rate (Kern et al., 2006). Alveolar echinococcosis is most commonly diagnosed in older people because of its long incubation period of five to 15 years (Eckert et al., 2001). Primary cysts usually develop in the liver and grow slowly but invasively, and can be mistaken for a slowly growing hepatic cancer. It can develop without clinical signs until it has reached a certain size and the liver becomes compromised. As the disease progresses the cysts may become damaged and infectious material metastasize to other organs in the body. The most common site of metastasis is the lungs.

The clinical signs of human alveolar echinococcosis are variable but include jaundice and epigastric pain (Eckert et al., 2001). The cysts are often discovered during medical examinations for less specific symptoms such as fatigue, weight loss etc. Treatment consists of a combination of radical surgery and chemotherapy. But even with treatment, relapses or other complications are frequent. Immunosuppressed individuals have a higher risk of developing the disease. Large scale screenings in human populations have shown high seroprevalence without signs of disease (Kern el al, 2003; Vuitton & Gottstein, 2010). This indicates that many humans can become infected but their immune systems fight the parasite and prevent the disease from developing. This new knowledge has also lead to research into alternative treatment methods involving vaccinations or immunomodulating chemotherapy.

Epidemiology

Echinococcus multilocularis is a parasite that thrives in colder climates and it is most prevalent in tundra regions such as subarctic regions of Canada, Russia, and Alaska (Taylor et al., 2007). But its distribution extends over most parts of the Northern hemisphere.

Europe and Asia

In Europe *E. multilocularis* was known to be endemic in some parts of France, Northern Switzerland, southern Germany, and Austria in the 1980's (Eckert, 1997). But after increased surveillance it was also found further north, east and south. By the late 1990's it was found in Belgium, Luxembourg, France, Switzerland, Liechtenstein, Austria, Germany, Poland, Turkey and the Czech Republic. However, there can be vast differences in occurrence between different areas within a country. The red fox is the natural definitive host in Europe but in areas with a high prevalence a larger proportion of the dog population is found to carry the parasite compared to areas with lower prevalence.

By 2005 *E. multilocularis* had been found in several other countries: Denmark, Lithuania, Slovakia, Hungary and Northern Italy (Romig et al., 2006). It has also been found in arctic foxes on Svalbard. *E. multilocularis* was found in Sweden in the beginning of 2011 (Osterman Lind et al., 2011). During 2011, four foxes were diagnosed with *E. multilocularis*, two in the region of Västra Götaland (OIE, 2011 a), one in Södermanland (OIE, 2011 b) and one in the county of Dalarna (OIE, 2011 c). These areas are plotted in figure 1. Today most countries in eastern and central Europe have endemic areas (Torgerson et al., 2010).

E. multilocularis is found in eight provinces in western, northern and central China (Zhenguan et al., 2008). In Japan human alveolar echinococcosis was first diagnosed in the 1930's on small islands outside Hokkaido (Rausch & Yamashita, 1957). Although definitive and intermediate hosts were examined the parasite could not be found at that time. Now Hokkaiodo is considered endemic to the parasite (Goto et al., 2010). Both the European and Asian parts of Russia and Turkey are endemic (Torgerson et al., 2010). Thus, the distribution of *E. multilocularis* in Asia stretches from the borders to Europe all the way to Japan in the east, and can be found in all Northern and Central Asian countries.

Human alveolar echinococcosis is notifiable in many countries and 2010 an article summarising these data was published (Torgerson et al., 2010). China (PRC) was estimated to have 230'000 cases with approximately 16'500 new cases each year. As the number of new cases each year in the whole world is estimated at approximately 18´000, more than 90 % of these occur in China. In other areas which are also considered endemic to the parasite the incidence is very low. In Europe most human cases are seen in endemic areas, based on data collected 1988-2000 (Kern et al., 2003)

North America

Before the 1960's *E. multilocularis* was believed to be confined to a few islands in the Arctic archipelago but then it was discovered on mainland Canada (Webster & Cameron, 1967). It is now found along the coast stretching from Alaska, through the Arctic Archipelago to Newfoundland (Torgerson, et al., 2010) *E.*

multilocularis has also spread south to the central states of USA and Canada. Even though there are such vast areas which are considered endemic to the parasite, transmission to man seems to be very rare.

Diagnostic and surveillance methods

Surveillance can be done in different ways, mostly focusing on the definitive hosts (Eckert, 1997). The prevalence in the human populations has also been monitored, for example in Japan, USA and Switzerland.

Sedimentation and counting technique

The SCT is the most accurate quantitative method for diagnosis in the definitive host and is considered the gold standard to which other methods are compared (Eckert, 2003; Torgerson & Deplazes, 2009). The basic principle for the technique is as follows: intestine from the definitive host is kept in deep-freeze for a number of days (Eckert, 2003). The intestine is incised longitudinally and submerged in physiological saline solution. The mucosa is stripped between two fingers and the fluid containing the intestinal contents is sedimented several times. The supernatant is decanted until the sediment is clear. The sediment is examined with a counting grid in small portions under a microscope with a magnification of 120x. The sensitivity is close to 100% but the method is costly, time consuming and entails a small risk of infection to the person performing it.

Other counting methods

An adaption of the SCT is the segmental sedimentation and counting technique (SSCT) (Umhang et al., 2011). The difference to SCT is that only 40% if the intestine is examined. This method also has a very high sensitivity, 98%, but it is qualitative rather than quantitative as the SCT. The intestinal scraping technique (IST) is based on deep mucosal scrapings, usually 15 per intestine which are examined under microscope (Eckert et al., 2001). This method is not as time consuming but has a much lower sensitivity of 76-78 % (Eckert, 2003).

Fecal examination

One method used for detection in the definitive host is copro-antigen ELISA (Eckert, 2003; Torgerson & Deplazes, 2009). This method is designed to detect antigen in faeces, and has a good sensitivity in cases with moderate to high numbers of parasites but it is not as sensitive for light infections. The sensitivity lies between 84 and 95% and the specificity is over 95%. This test can be used on both live and dead animals. There is a possible cross reactivity with antigens from other *Taenia* species. Detection of DNA from *E. multilocularis* with copro-PCR has a sensitivity of at least 89% but this is a very expensive method and therefore not suitable for large scale screening

Diagnosis in the intermediate host

In epidemiological studies intermediate hosts can be collected and different methods used for diagnosis of *E. multilocularis* (Torgerson & Deplazes, 2009). Necropsy is the most commonly used method, and fully developed metacestodes can easily be identified microscopically. Together with PCR methods the sensitivity is very high.

Surveillance in human populations

Mass screening of the human population has been carried out in a few endemic areas (Eckert, 1997). This has been done using serological tests and/or imaging techniques. In Japan it was found that cases of alveolar echinococcosis were discovered much earlier and prognosis for the patients improved through mass screening. In Switzerland another approach has been to perform serological tests on people who are at high risk of coming into contact with the parasite, for example farmers and veterinarians.

Surveillance in Sweden

In Sweden, SCT and copro-antigen ELISA have been used for surveillance (Osterman Lind et al., 2011). In a few cases PCR was used on *Taeniid* eggs. When the first *E. multilocularis* parasite was discovered in February 2011, surveillance was intensified. Instead of SCT, SSCT was used as this is less time consuming but still has a high sensitivity.

Accidental hosts of *E. multilocularis*

As mentioned above, there are animals that can become infected with *E. multilocularis* in the same manner as an intermediate host but without being a part in disease transmission (Eckert et al., 2001). These hosts are referred to as dead end, incidental, aberrant or accidental hosts, by different authors. These terms have slightly different meanings. In this paper they are referred to as accidental hosts.

Experimentally infected animals

To investigate the development of *E. multilocularis* and the tissue reaction in the intermediate hosts, many different rodents were experimentally infected during the 1950's and 1960's (Ohbayashi et al., 1971). Experimental infections were also carried out on other animals that had never been found as natural intermediate hosts. Among these were a horse (*Equus ferus caballus*) and goats (*Capra aegagrus hircus*) that were fed gravid segments of *E. multilocularis*. They were found to be unsuitable as intermediate hosts as the metacestode did not develop fully, but liver lesions could be found. Through histological examination it was determined that these were caused by *E. multilocularis*.

Rhesus monkeys (*Macaca mulatta*) were infected with gravid segments injected directly into the stomach (Ohbayashi et al., 1971). The monkeys developed lesions that were similar to those in man. This was consistent with the findings in a similar study performed earlier in North America (Rausch & Schiller, 1956). Authors of both articles concluded that in these primates the lesions develop slowly and that protoscoleces are rarely, or never present. In another study, lambs (*Ovis aries*), pigs and calves (*Bos primigenius*) were fed *E. multilocularis* oncospheres (Lukashenko, 1971). Lesions developed which were diagnosed as *E. multilocularis* lesions that had been disrupted by the inflammatory response of the host.

In more recent studies, pigs have been experimentally infected with *E. multilocularis* (Deplazes et al., 2005). The pigs, which were ten weeks old and parasite-free before the study started, were fed large amounts of *E. multilocularis*

eggs. Seven months later the animals were euthanized and examined. All had developed liver lesions. The lesions differed greatly in size, number and morphology. Lesions from all livers tested positive for *E. multilocularis* by PCR. During the study, the pigs were tested with serum-ELISA and after one month they were all seropositive. They remained strongly positive throughout the study that lasted seven months. The animals that turned out to have several small lesions had higher antibody titers than the animals with just a few large lesions.

Spontaneously infected species

A few animal species have been found to be spontaneously infected with *E. multilocularis* but without developing infective protoscoleces and therefore not able to infect a definitive host (Eckert et al., 2001). These animals are infected in the same way as a normal intermediate host, i.e. by ingestion of proglottids. Species described are domestic pigs (*Sus scrofa domesticus*) (Sakui et al., 1984), horse (*Equus ferus caballus*) (Miyauchi, 1984), wild boar (*Sus scrofa scrofa*) (Pfister et al., 1993), domestic dog (*Canis lupus familiaris*) (Eckert et al., 2001), and a few different species of primates.

Infection in pigs and wild boar

In 1984, in Japan, lesions were found for the first time in the livers of pigs and diagnosed as *E. multilocularis* by histology (Sakui et al., 1984). At Japanese slaughterhouse inspections such lesions were continuously diagnosed (Kamiya et al., 1987). The first finding of *E. multilocularis* lesions in wild ungulates was in wild boars in southern Germany, in an area endemic to the parasite (Pfister et al., 1993). Infections in domestic pigs or wild boars have also been described in France (Boucher et al., 2005) Switzerland (Sydler et al., 1998), Lithuania (Bružinskaitė, 2009) and recently in Poland (Karamon et al., 2011). All these areas are considered as endemic to the parasite.

Test of viability of the parasitic material and confirmation of diagnosis in pigs has been done by intraperitoneal injection in Mongolian gerbil (*Meriones unguiculatus*), an intermediate host, which developed typical *E. multilocularis* cysts containing protoscoleces (Kamiya et al., 1987). For lesions found in wild boars, the same method was used but only cysts without protoscoleces were found in the intermediate host (Pfister et al., 1993). However, when material from these cysts was injected subcutaneously into new gerbils, cysts with protoscoleces did develop. The first group of gerbils had been euthanized six weeks after inoculation and the second group 32 weeks after inoculation which might explain why metacestodes had not developed fully in the first group. Viability has not been proven in all studies with accidental hosts (Eckert, 1997)

Pathological findings in wild boars and pigs

According to Sydler et al. (1998) *E. multilocularis* lesions in the liver of pigs or wild boars are easily detectable and distinguished from common “white spots” caused by migrating *Ascaris suum* larvae. The author described lesions found in spontaneously infected fattening pigs that were up to six months of age and kept outdoors. The lesions were 0,5 to 1,5 cm in diameter and were distinct, dense and found close to the surface of the livers. The histological examination revealed lesions that resembled *E. multilocularis* cysts in natural intermediate hosts and

had a distinct PAS-positive laminated layer. Some lesions had cysts with a lumen while others were collapsed. There were no protoscoleces but cells of the germinal layer were found. Calcification and necrosis was often seen next to the laminated layer. Inside the cysts was a necrotic centre with neutrophils and detritus surrounded by inflammatory cells. Lymphatic follicles were often found.

The description of *E. multilocularis* lesions in experimentally infected pigs differs slightly from that by Sydler et al. (Deplazes el al, 2005). The experimentally induced lesions were not as prominent and were more variable in size, morphology and numbers. One pig with large lesions measuring 3-8 mm only had eight lesions in total, whereas a pig with small lesions, 0,5-1,5 mm had more than 50. The small lesions had blurred borders with obvious fibrous infiltration. Histological examination showed fibrosis with lymphatic follicles, sometimes with central necrosis and calcification. Lesions that were between 1,5 and 3 mm were more distinct. Histology showed a calcified necrotic centre. The largest lesions were well demarcated and had central necrosis and calcifications. Surrounding this were epitheloid cells and foreign body giant cells with connective tissue and lymphatic follicles. Some lesions, both small and large, had remnants of a laminated layer which was PAS positive. No protoscoleces or germinative cells were seen.

In the livers of the naturally infected wild boar described by Pfister et al. (1993) a distinct laminated layer was seen in contrast to experimentally infected pigs where this was not seen. The authors suggested this may be due to differences in immunological response or in different strains of *E. multilocularis*.

MATERIALS AND METHODS

Collection of wild boar samples

This study comprised livers from 80 wild boars. The study was conducted at the National Veterinary Institute in Uppsala. Forty-six of the animals were part of another project performed by the National Veterinary institute to evaluate wild boar traps. These animals had been captured and then euthanized by shooting. Eight wild boars had been shot because of suspected disease or found dead. The animals of these two categories underwent necropsy. The remaining 26 animals had been shot by hunters and processed at an approved abattoir where the livers had been taken out, frozen and sent to the National Veterinary Institute. These wild boars underwent inspection by the veterinarian on site. All livers were stored at −20°C before examination. Muscle tissue samples were also collected from all animals and kept at −20°C for collection of tissue fluid and subsequent detection of antibodies to *E. multilocularis* (not included in the present study). The collection of samples took place between October 2010 and April 2011.

Data about the wild boars were documented. The data included coordinates where they had been shot or found, estimated age of the animals and post mortal changes of samples upon arrival. The ages were estimated from their body weight or appearance and categorized into 0, 1 or ≥2 years. The coordinates were calculated with a GPS or estimated from the location stated by the hunter or finder.

Pathological gross and histological examination

The pathological examination of the livers was performed by the author with the help of experienced wildlife pathologists. The post mortal changes were documented. The surfaces of the livers were examined for pathological lesions. Then they were sliced in ≤1 cm thick slices that were examined for lesions in the parenchyma. All lesions were documented regarding to size, number, location and appearance. New instruments were used for each liver to prevent any cross-contamination.

Samples for histology were taken from the parietal surface of all livers and also from pathological lesions. The samples were fixed in 10% buffered formalin and embedded in paraffin. They were stained using hematoxylin and eosin. Paraffin blocks were saved if other (e.g. PAS) staining would to be indicated. All histology samples were scanned for pathological lesions. This examination was performed by the author, again with the help of pathologists. Lesions were described with reference to size, cellular involvement, destruction of tissue, etc.

PCR

All pathological changes that were morphologically indistinguishable from parasitic lesions were excised and stored at -20°C for PCR analysis. The lesions that showed these characteristics in histological examination were investigated with PCR. The material was diluted in TE buffer solution and homogenized. Nucleic acid was extracted from the homogenate in an extraction robot. The eluate was then analysed with an in-house PCR method based on SYBR green.

RESULTS

The ages the wild boars in this study ranged from under a year to more than two years. Of the 80 wild boars, 60 were estimated to be less than a year old, 16 were estimated to be between one and two years old and the remaining 4 were estimated to be two years or more. Their geographical distribution is plotted in figure 1.

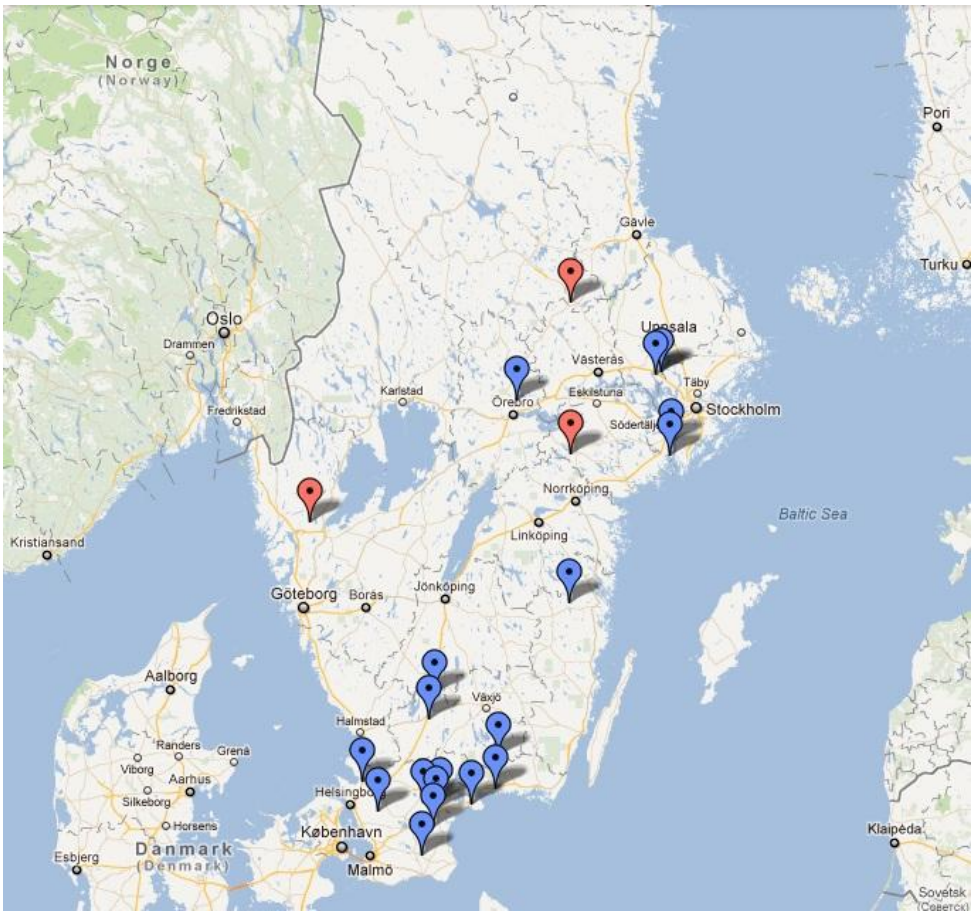

Figure 1. Origin of wild boar samples used in the study (blue markings) and the three areas (red markings) where E. multilocularis has been found in Sweden.

Gross and histological examination

34 of the livers had lesions which macroscopically were indistinguishable from parasitic lesions. Through careful histological examination, all but four samples from the wild boars of these were written off as post mortal changes. The four samples showed focal mild to moderate infiltration of lymphocytes and eosinophils, mild periportal and interlobular fibrosis and multifocal aggregates of lymphocytes (see figure 2 and 3). The lesions were diagnosed as poorly demarcated inflammatory reactions, likely of parasitic origin, so called “white spots”.

There were no characteristics indicative of *Echinococcus multilocularis* lesions, no laminated layer, no germinal layer, no protoscoleces etc.

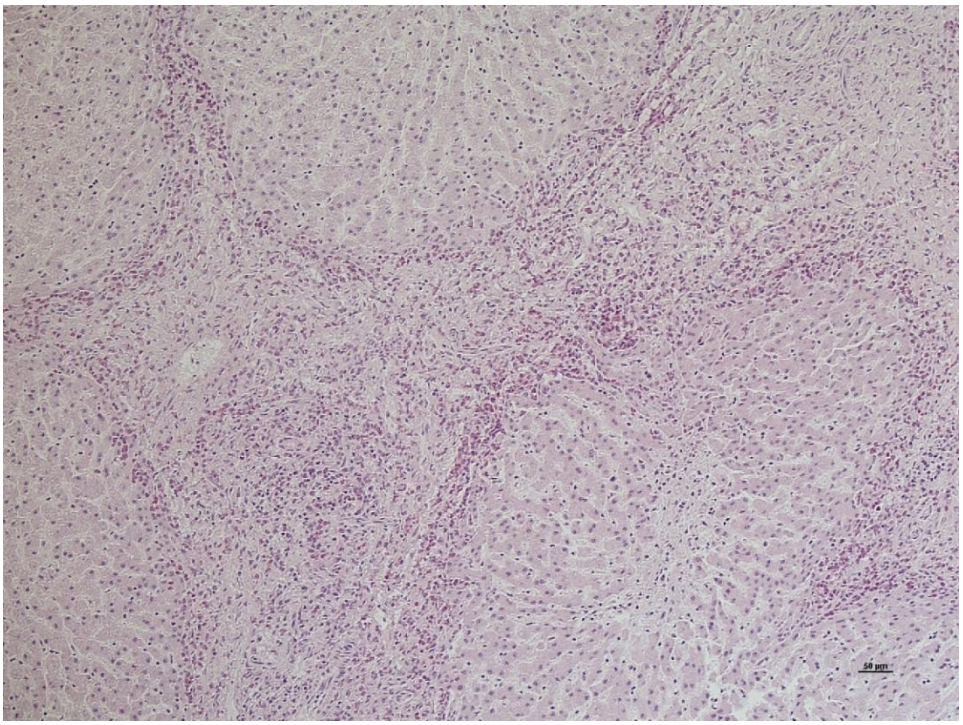

Figure 2. White spot lesion in approx. 6 months old wild boar showing interlobular fibrosis with moderate infiltration of lymphocytes and eosinophils

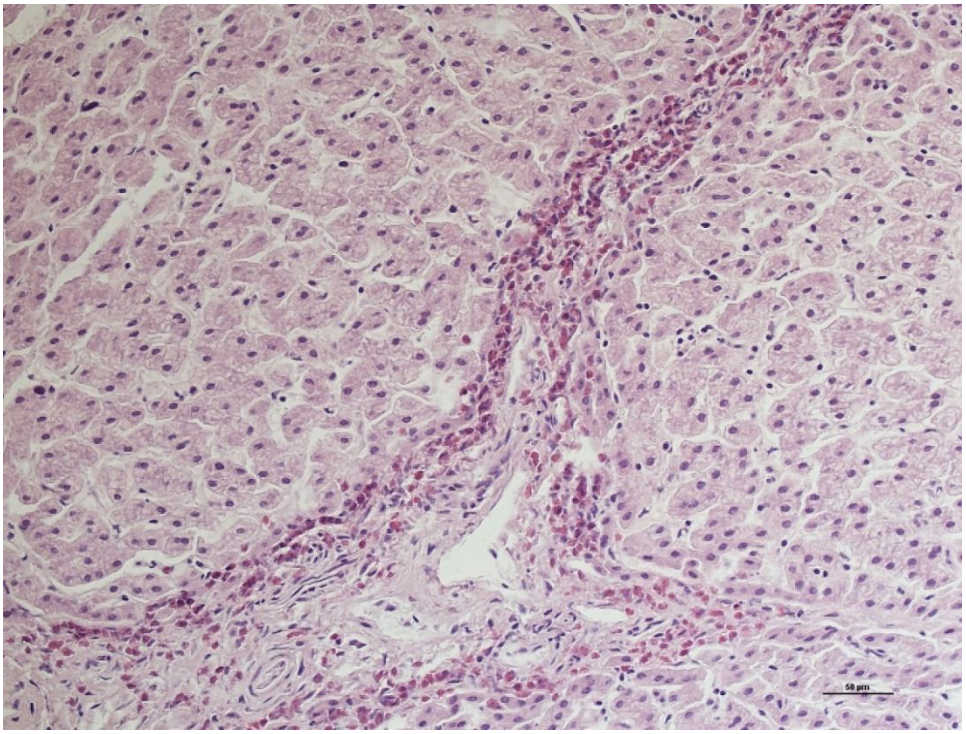

Figure 3. White spot lesion in approx. 6 months old wild boar in higher magnification showing mild interlobular fibrosis and moderate infiltration of eosinophils and smaller numbers of lymphocytes

PCR

The four samples diagnosed as "white spots" were all negative for *E. multilocularis* DNA when tested with PCR.

DISCUSSION

Evaluation of this study and the results

The 80 wild boar livers that were examined in this study showed no sign of *Echinococcus multilocularis* infection. During 2011, *E. multilocularis* surveillance was extensive with almost 3000 foxes being examined (SVA, 2012). But to this date, *E. multilocularis* has been diagnosed in only four foxes in Sweden. Two were shot on in close proximity of each other in Västra Götaland, one in Södermanland and one in Dalarna (see figure 1). Even though *E. multilocularis* has been found in more than one area in Sweden, the incidence is low and general contamination of the environment with *E. multilocuaris* eggs is therefore assumed to be low. In addition to this, the wild boars examined in this study did not originate from areas where *E. multilocularis* has been found. With this taken into account, the likelihood of finding *E. multilocularis* in the wild boar samples examined in this study was low.

The condition of the livers varied at the time of examination. Some of them showed extensive post mortal changes. This could be a possible source of error if those changes made it impossible to correctly assess the livers and because of that, extensive histological examination of observed lesions was performed, excluding all but four as post mortal changes. The method used in this study was based on studies described by other scientists such as Bružinskaitė et al. (2009). The method was straight forward and easy to perform, with little risk of error. If there were lesions smaller than one cm these could be missed when the livers were sliced. However, according to descriptions of *E. multilocularis* lesions, most appeared on the surface of the liver and were easily detectable (Bružinskaitė et al,. 2009). With only a small risk of error in this study, and a low probability of Swedish wild boar being infected with E. multilocularis, the negative results in this study can be evaluated as true.

The four livers that were diagnosed with "white spots" (mild focal inflammatory lesions deemed to be of parasitic origin) all came from wild boars younger than one year old. There are no published data on the incidence of "white spots" or *A. suum* in Swedish wild boar. But "white spots" caused by migrating *A. suum* is not an uncommon finding in young domestic pigs and migrating *A. suum* larvae are a likely cause of the lesions in these four animals.

New surveillance method

As mentioned in the introduction, this study was part of a larger project. The aim of that project was to evaluate a potential new surveillance method. This method would be based on an ELISA, testing for antibodies against *E. multilocularis* in wild boar. The next step is to explore whether it is possible to use meat juice instead of serum for the ELISA. The reason for using meat juice is that it could be obtained from samples sent in for the compulsory testing for *Trichinella spp*.

carried out on wild boar muscle tissue. The results from this study means that samples from the studied wild boars can be used as a negative reference material.

An uncertainty of this method at present is the limited knowledge of how long *E. multilocularis* lesions in wild boar livers persist and how long wild boars have a detectable amount of antibodies against *E. multilocularis* which increases the uncertainty in the assessment of the area contaminated. Another factor that is not well researched is how using meat juice instead of serum for ELISA will affect the outcome.

The strengths of the method would be: that it is safe for persons performing it, cheaper and faster than SCT. Collection of samples would be easy as it could be done with the obligatory trichinosis testing as mentioned above. Advantages of using samples from wild boar are the fact that they are likely to ingest *E. multilocularis* eggs in a contaminated environment and the wild boar population is increasing fast and spreading fast over the country and is now covering southern Sweden, which is also the most likely place to find *E. multilocularis.* The hunting bag is likely to increase, which would mean increased opportunity to gather blood or muscle tissue. Another possibility would be to use blood or muscle juice from out-door domestic pigs but these are not very common in Sweden.

CONCLUSION

There was no evidence that the wild boar used for this study were infected with *Echinococcus multilocularis*. Therefore, samples from these animals can be used as negative reference material when assessing the *Echinococcus multilocularis* ELISA in the continued project.

REFERENCES

Boucher, J.M., Hanosset, R., Augot, D., Bart, J.M., Morand, M., Piarroux, R., Pozet-Bouhier, F., Losson, B., Cliquet, F. (2005) Detection of Echinoccous multilocularis in wild boars using PCR techniques against larval form. Veterinary Parasitology 129, 259-266

Bružinskaitė, R., Šarkūnas, M., Torgerson, P.R., Mathis, A., Deplazes, P. (2009) Echinococcosis in pigs and intestinal infection with Echinococcus spp. in dogs in southwestern Lithuania. Veterinary Parasitology 160, 237-241

Deplazes, P., Grimm, F., Sydler, T., Tanner, I., Kapel, C.M.O. (2005) Experimental alveolar echinococcosis in pigs, lesion development and serological follow up. Veterinary Parasitology 130, 213-222

Eckert, J. (1997) Epidemiology of Echinococcus multilocularis and E. granulosus in Central Europe. Parassitologia 39, 337-344

Eckert, J. (2003) Predictive values and quality control of techniques for the diagnosis of Echinococcus multilocularis in definitive hosts. Acta Tropica 85, 157-163

Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (Eds.) (2001) WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. World Organisation for Animal Health (OIE) and World Health Organization (WHO), Paris, France

Gemmel, M. A. (1959). The fox as a definitive host of Echinococcus and its role in the spread of hydatid disease. Bulletin of World Health Organisation 20, 87-99

Goto, Y., Sato, K., Yahagi, K., Komatsu, O., Hoshina, H., Abiko, C., Yamasaki, H., Kawanaka, M. (2010) Frequent Isolation of Echinococcus multilocularis from the Livers of Racehorses Slaughetered in Yamagata, Japan. Japanese Journal of Infectious Diseases 63, 449-451

Kamiya, M., Ooi, H. K., Oku, Y., Okamoto, M., Ohbayashi, M., Seki, N. (1987) Isolation of Echinococcus multilocularis from the liver of swine in Hokkaido, Japan. Japanese Journal of Veterinary Research 35 (2), 99-107

Karamon, J., Sroka, J., Cencek, T. The first detection of Echinococcus multilocularis in slaughtered pigs in Poland. Veterinary Parasitology. In press 2011

Kern, P., Bardonnet, K., Renner, E., Auer, H., Pawlowki, Z., Ammann, R. W., Vuitton, D. A., Kern, P., European Echinococcosis Registry. (2003) European Echinococcosis Registry: Human Alveolar Echinococcosis, Europe, 1982–2000. Emerging infectious diseases 9 (3)

Kern, P., Wen, H., Sato, N., Vuitton, D. A., Gruener, B., Shao, Y., Delabrousse, E., Kratzer, W., Bresson-Hadni, S. (2006) WHO classification of alveolar echinococcosis: Principles and application. Parasitology International 55, 283-287

Lukashenko, N. P. (1968) Comparative Biologic and Pathologic Studies of Alveococcus multilocularis. Archives of Environmental Health 17, 676-680

Lukashenko, N. P. (1971) Problems of epidemiology and prophylaxis of alveococcosis (multilocular echinococcosis): a general review – with particular reference to the U.S.S.R. International Journal of Parasitology 1, 125-134.

Markström, S. (2002) Vildsvin. Stokholm: Jägareförlaget.

Mas Coma, S., Valero, M.A., Bargues, M.D. (2008) Effect of climate change on animal and zoonotic helminthiases. Scientific and Technical Review - International Office of Epizootics 27 (2), 443-452

Miyauchi, T., Sakui, M., Ishige, M., Fukumoto, S., Ueda, A., Ito, M., Ohbayashi, M. (1984) A case of Multilocular Echinococcosis in a horse. Japanese journal of Veterinary Research 32 (3), 171-173

Nakao, M., McManus, D. P., Schantz, P. M., Craig, P. S., Ito, A. (2007) A molecular phylogeny of the genus Echinococcus inferred from complete mitochondrial genomes. Parasitology 134, 713-722

Ohbayashi, M., Rausch, R.L., Fay, F.H. (1971) On the ecology and distribution of Echinococcus spp. (Cestoda: Taeniidae), and characteristics of their development in the intermediae host. II. Comparative studies on the development of larval E. multilocularis leuckart, 1863, in the intermediate host. Japanese Journal of Veterinary Research 1971 Jul;19:Suppl 3:1-53.

OIE – World Organisation for Animal Health [online](2011-02-16a) available from: http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=10263 [2012-04-05]

OIE – World Organisation for Animal Health [online](2011-05-11b) available from: http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=10549 [2012-04-05]

OIE – World Organisation for Animal Health [online](2011-06-15c) available from: http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=10700 [2012-04-05]

Osterman Lind, E., Juremalm, M., Christensson, D., Widgren, S., Hallgren, G., Ågren, E. O., Ulhorn, H., Lindberg, A., Cedersmyg, M., Wahlström, H. (2011) First detection of Echinococcus multilocularis in Sweden, February to March 2011. Euro Surveillance [online] 2011;16(14):pii=19836. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19836 [13 January 2012]

Pfister, T., Schad, V., Schelling, U., Lucius, R., Frank, W. (1993) Incomplete development of larval Echinococcus multilocularis (Cestoda: Taeniida) in spontaneously infected wild boars. Parasitology Research 79, 617-618

Rausch, R. L (1968) Taxonomic Characters in the Genus Echinococcus (Cestoda: Taeniidae). Bulletin of the World Health Organization 39, 1-4

Rausch, R. L. & Schiller, E. L. (1956) Studies on the Helminth Fauna of Alaska. XXV. The Ecology and Public Health Significance of Echinococcus sibiricensis Rausch & Schiller, 1954 on St Lawrence Island. Parasitology 46 (3/4)

Rausch, R. L., & Yamashita, J. (1957) The Occurrence of Echinococcus multilocularis Leuckart, 1863, in Japan. Proceedings of the Helminthological Society of Washington 24 (2)

Romig, T., Dinkel, A., Mackenstedt, U. (2006) The present situation of echinococcosis in Europe. Parasitology International 55, S187-S191

Sakui, M., Ishigie, M., Fukumoto, S., Ueda, A., Ohbayashi, M. (1984) Spontaneous Echinococcus multilocularis infection in Swine in North-Eastern Hokkaido, Japan. Japanes Journal of Parasitology 33 (4), 291-296

Schiller, E. L. (1955) Studies on the Helminth Fauna of Alaska. XXVI. Some observations on the Cold-Resistance of Eggs of Echinococcus sibiricensis Rausch and Schiller, 1954. Journal of Parasitology 41, 578-582

SFS 2004:255, Smittskyddsförordningen (2004). Stockholm.

SJVFS 2010:43, Föreskrifter om ändring i Statens jordbruksverks föreskrifter (SJVFS 2004:51) om införsel av sällskapsdjur och hund- och kattsperma samt hundar, katter och illrar avsedda för handel (2010). Jönköping.

SJVFS 2011:49, Statens jordbruksverks föreskrifter om införsel av sällskapsdjur och hund- och kattsperma samt hundar, katter och illrar avsedda för handel (2011). Jönköping

SVA. SVA:s undersökningar av rävens dvärgbandmask 2011- slutrapport [online] (2012-01-02) Available from: http://sva.se/sv/Djurhalsa1/Zoonoser/Ravens-dvargbandmask/Slutrapport_dvargbandmask/ (2012-04-21)

Svenska jägareförbundet (2009) Vildsvinsförvaltning i samverkan. [online]. Available from: http://www.jagareforbundet.se/Global/Policys/vildsvinsf%C3%B6rvaltning%20i%20samverkan.pdf [2011-12-11]

Sydler, T., Mathis, A., Deplazes, P. (1998) Echinococcus multilocularis in the livers of pigs kept outdoors in Switzerland. European Journal of Veterinary Pathology 4 (1), 43-46

Taylor, M. A., Coop, R. L., Wall, R. L. (2007) Veterinary parasitology. 3 ed. Oxford. Blackwell Publishing

Torgerson, P.R., & Deplazes, P. (2009) Echinococcosis: diagnosis and diagnostic interpretation in population studies. Trends in Parasitology 25 (4), 164-170

Torgerson, P. R., Keller, K., Magnotta, M., Ragland, N. (2010) The Global Burden of Alveolar Echinococcosis. PLoS neglected tropical diseases 4(6): e722. doi:10.1371/journal.pntd.0000722

Umhang, G., Woronoff-Rhen, N., Combes, B., Boué, F. (2011) Segmental sedimentation and counting technique (SSCT): An adaptable method for qualitative diagnosis of Echinococcus multilocularis in fox intestines. Experimental Parasitology 128, 57-60

Veit, P., Bilger, B., Schad, V., Schäfer, J., Frank, W., Lucius, R. (1995) Influence of environmental factors on the infectivity of Echinococcus multilocularis eggs. Parasitology 110, 79-86

Vuitton, D. A. & Gottstein, B. (2010) Echinococcus multilocularis and Its Intermediate Host: A Model of Parasite-Host Interplay. Journal of Biomedicine and Biotechnology 2010, Article ID 923193

Webster, G. A., & Cameron, T. W. M (1967) Epidemiology and diagnosis of Echinococcosis in Canada. Canadian Medical Association journal 96, 600-607

Zhenghuan, W., Xiaoming, W., Xiaoqing, L. (2008) Echinococcosis in China, a review of the Epidemiology of Echinococcus spp. Eco Health 5, 115-126