Study of ovine sarcosporidiosis in slaughterhouses of El Harrach in north of Algeria

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ABSTRACT
Samples of the oesophagi and diaphragms of 580 sheep were collected from the slaughterhouses of El Harrach to determine the prevalence of sarcosporidiosis in the ovine carcasses and identify the implied species of Sarcocystis. All samples were analyzed by the enzymatic digestion and 335 samples were examined by the histopathological analysis. Macroscopic cysts of Sarcocystis gigantea were identified in six oesophagi (1.03%). The enzymatic digestion and the histopathological analysis showed a high prevalence (99.14% and 94.03%, respectively). The thin-walled cysts of Sarcocystis arieticanis (92.54%) remained more predominant than the thick-walled cysts of Sarcocystis tenella (43.88%). The prevalence of the thin-walled microcysts was higher in the diaphragm (80.60%) than in the oesophagus (62.69%). In addition, the prevalence of the thick-walled microcysts in the diaphragm (33.73%) was higher than in the oesophagus (25.67%). These results indicate a heavy contamination of the environment with the dog oocysts.

Keywords: ovine carcasses, Sarcocystis, diaphragms, oesophagi.

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Introduction
Sarcocystis is one of the most prevalent protozoan parasites in the striated muscles of livestock such as cattle, sheep and goat slaughtered for human consumption (4). Sheep are intermediate hosts of five Sarcocystis species: Sarcocystis tenella (synonym Sarcocystis ovicanis), Sarcocystis gigantea (synonym Sarcocystis ovifelis), Sarcocystis arieticanis and Sarcocystis medusiformis. S. gigantea and S. medusiformis are transmitted by felids and are non-pathogenic. S. tenella and S. arieticanis are transmitted by canids and are pathogenic (10). Another species, Sarcocystis mihoensis has been recently reported to occur in sheep (24). In sheep, species with the definitive canine host may cause clinical illness in the acute form of sarcocystosis such as abortion (3), and neurological signs (6). Chronic infection may cause lowering of the weight gain and poor production of milk (19). Moreover, the losses caused by discrimination of the carcasses with macroscopic cysts during meat inspection represented a serious economic problem. Despite it all, sarcosporidiosis in Algeria is underestimated, while the inspection of the carcasses for sarcosporidiosis is not obligatory. The aim of the present study is to determine the prevalence of sarcosporidiosis infection in 580 sheep in the slaughterhouses in the north of Algeria, particularly those of El Harrach, by using two techniques: enzyme digestion and histopathological examination, which is used for the first time for identification of different species of sarcosporidiosis in sheep in Algeria.

Material and Methods
Collection and preparation of tissue samples
A total of 580 slaughtered sheep were inspected for Sarcocystis cysts visible to the naked eye. From each carcase, the oesophagus and pieces of the diaphragm muscle were sampled into the separate bags. Samples were taken to the Laboratory of Parasitology and Mycology in the Superior National Veterinary School, Algiers and divided into two groups: one for enzymatic digestion and the other one for histological analysis, which was previously fixed in 10% neutral buffered formalin.
Results

Technique of enzymatic digestion
Total of 1160 samples (580 diaphragms and 580 oesophagi) were digested by enzymatic digestion using the modified method of Seneviratne as described by Latif et al. (18): 10 g of diaphragm and 10 g of oesophagus of each carcass were minced and mixed. 50 ml of the digestion solution (prepared by mixing 1000 ml of water, 7 ml of 25% HCl, 5 g of NaCl and 2.6 g of pepsin), was added to the mixed tissue samples and was homogenized. After the incubation period (at 40°C for 30 min), the digest was sieved and the filtrate was centrifuged. The pellet was taken up in PBS (pH 7.2), and the same operation was performed twice. A few drops of the pellet were deposited on a slide for the preparation of smears. Dried smears were coloured by using the technique of May Grunwald Giemsa (MGG) cited by Bussiéras and Chermette (5). Stained smears were then examined with the light microscope (Leica DMLS ®) magnification (100×, 400×, 1000×). A sample was considered positive when typical Sarcocystis bradyzoites were observed (a banana shape).

Histological technique
For histopathological analysis, 670 samples (335 oesophagi and 335 diaphragms) were studied. Some tissue samples (n: 90) were prepared in the Laboratory of Anatomy and Cytopathology of the University Hospital of PARNET, Algiers, while the others (n: 580) were prepared in the Laboratory of Anatomy Pathological at the Superior National Veterinary School, Algiers. The fixed tissue samples were cut into 0.5 cm-thick sections, dehydrated with serial dilutions of ethanol and xylene, processed into paraffin, sectioned to 4 to 5 microns, stained with haematoxylin and eosin (H&E), and examined for microcysts with the light microscope at magnification (100×,400×,1000×). Selected tissue sections with the macrocysts were also studied by using the same technique. The wall morphology of microcysts in the muscle sections were examined by the light microscope with immersion oil (×1000). Positive slides revealed the presence of Sarcocystis cysts inside the muscle fibers having a wall of variable thickness, which depended on the species involved.

Statistical analysis
For statistical analysis, we used the software program Microsoft Excel 2010 and IBM SPSS Statistics Version 20. The comparison of the distribution of different populations were analysed by using Chi-Square test with the level of significance P <0.05.

Research of Sarcocystis by macroscopic examination
Macroscopic examination revealed the macroscopic cysts (Figure 1) in the oesophagi of 6 (1.03%) sheep (females) aged 5 years, whereas 574 (98.97%) of sheep carcasses did not reveal any macroscopic cysts. The chi-square test was very significant (P< 0.001) between the presence and absence of macroscopic cysts in 580 sheep carcasses. No cases of eosinophilic myositis were observed.

Figure 1: Macroscopic cysts of Sarcocystis spp. in oesophagus of sheep female (arrow)

Research of Sarcocystis spp. by using the technique of enzymatic digestion

Overall prevalence
Sarcocystis spp. bradyzoites were revealed in the muscles of 575 sheep (99.14%). Banana- shaped bradyzoites were found and observed under the light microscope (Figure 2).

Figure 2: Sarcocystis bradyzoite observed in samples of sheep diaphragm and oesophagus after MGG staining (1000×)

The prevalence of Sarcocystis spp., according to sex and age of sheep show no significant differences (P>0.05)

Research of Sarcocystis spp. cysts by histological technique
Sarcocystis spp. microscopic cysts were detected in 315 (94.03%) of the examined sheep. According to the cyst wall morphology, two types of microcysts were differentiated. Thin-walled Sarcocystis with the hair like projections identified as S. arieticanis, and thick-walled Sarcocystis with radial striations identified as S. tenella (S.ovicanis) were observed under the light microscope (× 1000) and shown in Figure 3 and Figure 4.
In addition, the same technique identified macrocysts with the thin wall and cauliflower-like protrusions surrounded by the secondary cyst wall corresponding to the description of *S. gigantea* (*S. ovifelis*), as shown in Figure 5. No pathological changes of the parasitic muscle fibres were observed.

The prevalence was lower in the oesophagus, 229 (68%) than in the diaphragm, 284 (85%). The chi-square test showed a very significant difference (P< 0.01).

310 (92.54%) sheep were infested with the thin-walled cysts of *S. arieticanis*, and 147 (43.88%) sheep with thick-walled cysts of *S. tenella*. The chi-square test was very significant (P< 0.01) for the presence of two species of *Sarcocystis*. Also, in two types of muscles the thin-walled cysts were predominant (Figure 6). The chi-square test was very significant (P< 0.01) for the difference between two types of *Sarcocystis* (thin-walled cysts and thick-walled cysts) for each muscle.

In both types of samples, we observed the cases of mono-infections (thin-walled or thick-walled cysts only) as well as double, mixed infections (thin-walled and thick-walled cysts simultaneously). For each muscle, the chi-square test was very significant (P< 0.01) between the infections with thin-walled cysts only, thick-walled cysts only, double mixed infestations and the absence of cysts. The prevalence for each case is shown in Figure 7.
From parasitized sheep we obtained a total of 3401 cysts of Sarcocystis spp. They were distributed in the samples of diaphragms and oesophagi according to the thickness of their walls. In the diaphragms we observed 1811 thin-walled cysts and 440 thick-walled cysts. In the oesophagi we observed 818 thin-walled cysts and 332 thick-walled cysts (Figure 8). The chi-square test was very significant (P< 0.01) between the level of infestation with thin-walled cysts and thick-walled cysts in the diaphragms and oesophagi.

The comparison of prevalence by two diagnostic methods

In 335 sheep analysed by both techniques, histological analysis revealed that 94.03% of sheep were infected, and the technique of enzymatic digestion revealed 100% of affected sheep. The chi-square test showed no significant difference (P> 0.05).

Discussion and conclusions

In Algeria, Nedjari (20) failed to confirm presence of macroscopic cysts of sarcosporidiosis. Also, a study by Hinaidy and Egger (14) from another country revealed the similar results. O’Donoghue and Ford (21) observed macroscopic cysts in 6.7% of the samples, but detected 2 types of species: S. gigantea (4.5%) and S. medusiformis (3.1%). Furthermore, another studies revealed a high prevalence of infections (1,2,3,4,8). On the other hand, Vercruysse and Van Markc (27) and Fassi and al. (11) failed to observe macroscopic cysts. Six 5-year-old females infested with the cysts probably lived close by the cats and were infected with S. gigantea by ingesting the oocysts from the cat feces. The adult sheep are most often infected by the macrocysts (1). For this reason, all infected females were more than 5 years old, which is in accordance with the Algerain regulations stipulating slaughtering after that age. However, Beyazit and al. (4) found cysts of S. gigantea in 3 sheep under 1 year, and suggested that macrocysts might develop in less than 1 year (4). All cysts were collected from the oesophagi supporting the results of Oryan and al. (22) and Beyazit and al. (4) who found that the oesophagus was the most affected organ. No inflammatory lesions were observed in the evaluated sheep. Same results were observed by Beyazit and al. (4) and Tinak (26). However, Jensen and al. (16) reported that all carcasses were discriminated for eosinophilic myositis indicating that opened sarcocysts killed the myocyte-host by causing the granuloma formation.

In Algeria, Nedjari (20) established the prevalence of 64.38% for sheep infected with Sarcocystis spp bradyzoites. Data from the various studies suggested different prevalence, which ranged from 25- 100 % in different countries (8,11,26,27). Such differences may be attributed to the sample size, studied organs, and especially the presence of sporocysts in the environment. The high prevalence of Sarcosporidia is related to close contacts between the final hosts (carnivores) and intermediate hosts (sheep). Small ruminants are often bred in the areas settled by the stray dogs and cats, as observed by Tinak (26). Some studies have shown that no significant difference was found in the prevalence between both sexes of sheep (25), and the lack of association between the prevalence and sex (4). However, according to Fassi and al. (11), the ewes were less infected than the rams, which may be contributed to the variable muscular tropism of Sarcocystis with the preferential localization in the uterus than the muscles for females. On the other hand, some authors (8,13,22) found that infections were more common in females than males. We found that the prevalence was not related to age. However, some authors established the influence of age on the prevalence of Sarcocystis spp. Diez-Banos (8) observed the absence of the infestation in the young lambs, and its presence in the older animals. Some authors observed a gradual increase in infestation with age (4,25). In our study, the climatic conditions in the north regions of Algeria are favourable for survival of the oocysts, and the
environmental contamination by the infesting forms exposes both young and old animals to infestation with Sarcocystis.

Prevalence rates of Sarcocystis spp. microscopic cysts of 86-100% have been reported by multiple studies (4,11,21), while the lower rates (9%) were reported by Kudi and al. (17). This study confirms the presence of muscular sarcocsporidiosis in sheep as well as the global distribution of Sarcocystis. No inflammatory cell reaction around the Sarcocystis cysts was observed. Vererreysse and Van Mark (27) found the same result, while Dubey and al. (10) noted that the rupture of sarcocysts might have caused a strong immune response in the muscle fibres. However, some authors were not convinced that the observed myositis was caused by sarcocysts because they found cysts without inflammatory reactions in some histological sections, while in the others they noted inflammatory reactions without the presence of cysts (26,27).

Abo-Shehada (1) reported the diaphragm to be the most infected organ, while some studies reported the oesophagus to be more often affected (11,17). Otherwise, Tinak (26) noted that the tongue was the most affected muscle in longitudinal section, and the diaphragm in cross-section. In both sections, the oesophagus appeared to be the least affected muscle. Beyazit and al. (4) showed that the prevalence in the oesophagus, heart, and diaphragm was similar, and the lowest prevalence was in the skeletal muscles and tongue. According to Abo-Shehada (1), the diaphragm was seemingly an earlier station in the migration route of Sarcocystis with the higher prevalence in the young age group. For this reason, the diaphragm is more helpful in detection of an infection at earlier stage than the oesophagus (1).

Two types of microcysts were differentiated on the basis of the cyst wall morphology, as described by Beyazit and al. (4). Similar results have been reported by O’Donoghue and Ford (21). In Algeria, there is very little data on the species of ovine Sarcocystis spp. except for the study of Nedjari (20) who noted the predominance of S. tenella (60.63%) than S. arieticanis (39.36%) by using the compression and agitation technique and coprology. Some researchers have found that S. tenella was the predominant species (2,3,9). However, Vererreysse and Van Mark (27) proved the presence of S.tenella only. Sheep become infected by ingesting oocysts eliminated through the dog feces to contaminated food and water (13). In addition, the cycle is kept going when the final host ingests the uncooked meat from slaughtered sheep in the slaughterhouse, and especially during the feast of Eid Al-Adha, which is celebrated by the Muslims every year, and when the stray dogs have easy access to sheep offal. Some results have revealed sheep infected by either only one or both Sarcocystis species (4). Gokpinar and al. (13) proved that the homogeneous population of S.tenella (91%) was more prevalent than the mixed infections with S. tenella and S. arieticanis (18.7%). However, Beyazit and al. (4) noted that 31.5% of sheep were infected by S.tenella only, 55 % had mixed infection and no sheep was infected by S.arieticanis only.

In USA, Dubey and al. (9) noted that the mean number of S. tenella sarcocysts in tissue sections was approximately 10 times higher than that of S. arieticanis.

The study of Beyazit and al. (4) found that both diagnostic methods had similar sensitivities (p> 0.05), and the high prevalence of ovine Sarcocystis increased the chance of detecting Sarcocystis in the muscle (4).

The high sensitivity of the peptic digestion technique that we have noted in our study could be explained by the fact that the mature cysts of Sarcocystis contained thousands of bradyzoites (7), which could be released by digestion of the cysts, and the typical banana shape could be easily seen in the microscopic examination. In our study, the search for Sarcocystis with the enzymatic digestion method was carried out in 20 g of muscles thus increasing the chances for finding the parasite, whereas the histological analysis detected the cysts on a surface area of 1 cm² of tissue only. The histological analysis is less sensitive (15), sometimes due to the low infestation level and the size of the cysts, which makes it easy to miss the cysts with the microtome cuts and to detect them. Enzymatic digestion, considered by Poli and al. (23) as a reliable method for detection of Sarcocystis remains the most sensitive technique having the best results in the cases of muscular sarcocsporidiosis. However, histology can be used for identification of Sarcocystis (12), assessment of the infestation level and the study of the tissue lesions generated by this parasite.

The present study has revealed a very high prevalence of ovine Sarcocystis in the slaughterhouses in El Harrach (Algers) by enzymatic digestion method. Macroscopic cysts of S. gigantea were found in the oesophagi. In histological sections, two types of Sarcocystis cysts were identified, S. tenella and predominant S. arieticanis. Such results indicate that the environment is heavily contaminated with the dog oocysts. The role of cats on transmitting the disease seems minimal. Other studies must ensue in order to determine the true prevalence of sheep sarcocsporidiosis in different regions in Algeria so to recognize the importance of Sarcocystis species.

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References

Uzorci jednjaka i dijafragmi 580 ovaca su sakupljeni iz klaonica u El Harrach-u za određivanje prevalence sarkosporidioze i identificiranje vrsta Sarcocysta. Svi uzorci su analizirani metodom enzimatske digestije, a 335 od ukupnog broja je ispitano histopatološkom analizom. Makroskopske ciste Sarcocystis gigantea su pronađene u šest jednjaka (1.03%). Enzimatska digestija i histopatološka analiza su pokazale visoku prevalence sarkosporidioze ovaca (99.14% i 94.03%). Ciste tankog zida vrste Sarcocystis arieticanis (92.54%) su ostale predominantne u odnosu na ciste debljeg zida vrste Sarcocystis tenella (43.88%). Prevalenca mikrocistih tankog zida je veća u dijafragmi (80.60%) nego u jednjaku (62.69%). Dodatno, prevalence mikrocistih debljeg zida u dijafragmi (33.73%) je veća nego u jednjaku (25.67%). Ovi rezultati ukazuju na to da je okolina teško kontaminirana oocistama porijeklom od pasa.