Study of heartwater by infection of sheep with Ball 3 E. ruminantium stock in Namibia: clinical symptoms, gross lesions and molecular diagnosis

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SUMMARY
Heartwater (HW) is tick-borne rickettsiosis, caused by Ehrlichia ruminantium (in the past known as Cowdria ruminantium) transmitted by hard ticks of the Amblyomma genus. It spreads in sub Saharan Africa and its economic impact on livestock is high, secondary only to that of East Coast fever and African trypanosomosis and represents a major obstacle to ruminants production. The Amblyomma genus occurs worldwide, with the exception of Europe. Heartwater distribution is mainly affected by the distribution of the 5 main natural vectors, all of African origin. Of these, Amblyomma variegatum (Tropical Bont Tick - TBT) is most widespread. Even if the introduction and the establishment of HW and its vectors in Mediterranean area is unlikely, it can be considered at risk of introduction, since A. variegatum has been seldom reported in area, probably introduced by migratory birds. Moreover, A. variegatum and E. ruminantium were already introduced outside Africa moving cattle from Senegal into the Caribbean, where A. variegatum colonization has been boosted by favourable ecological conditions, leading to the establishment of the tick and of the infection in the region. The study described was conducted in the framework of the activities of the preparedness policy of the National Reference Centre for Exotic Diseases (CESME) at the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise (IZSAM) in cooperation with Namibian Veterinary Services. Ten selected Dorper Sheep, five years old, were infected intravenously by infective blood containing the E. ruminantium Ball 3 stock (OBP). Sheep were kept under observation till 36 day p.i. and temperature registered. Blood samples and tissues during necropsy were collected for PCR test. Respiratory symptoms and HW lesions were observed (idrotorax and idropercardium). PCR on blood was positive only in case of febrile animal, brain confirmed as choice for post mortem laboratory diagnosis using PCR test. Clinical symptoms and lesions have been observed, allowing to improve diagnostic skills for HW. Ball3 stock, improperly called vaccine, is confirmed to cause clinical disease and livestock losses if injected animals are not properly and timely treated with tetracycline.

KEY WORDS
Amblyomma, diagnosis, Ehrlichia ruminantium, heartwater, lesions.

INTRODUCTION
Heartwater (HW) is a tick-borne rickettsiosis, caused by Ehrlichia ruminantium (in the past known as Cowdria ruminantium), transmitted by hard ticks of the, Amblyomma genus. Infection involves a wide range of hosts, but clinical disease is seen only in domestic ruminants. In endemic areas, wild ruminants are usually asymptomatic, acting as reservoirs of the parasite. The disease is widespread in sub Saharan Africa and the economic impact is difficult to quantify, anyway it is certain that the economic importance of heartwater in Africa is comparable to that of East Coast fever, trypanosomosis and dermatophilosis and it represents one of the major obstacles to the improvement of livestock production in sub Saharan Africa.

Domestic cattle, in particular imported cattle breeds and as Merino derived sheep and Angora goats are highly susceptible to the disease. HW is absent from the dry south and central areas of Namibia, not favourable to the survival of Amblyomma tick, whereas it is a serious problem in the northeast areas (Caprivi strip-Zambezi Region), characterized by abundant rainfall (avg 650 mm/year). Domestic ruminants may develop four clinical forms of disease: hyperacute, acute, subacute and subclinical. The hyperacute form is characterized by sudden death occurring within a few hours from the temperature reaction. Exotic goat breeds, such as the Angora and the Boer goat, are commonly affected by this form. The acute form is most common in sheep and goats, incubation has a mean duration of 2-6 days. A temperature reaction exceeding 40°C may last for the entire course of the disease till the death of the animal. Depression, shortness of breath, dyspnoea, presence of froth from the nose and nervous signs are typical of this form; in cattle, approaching death, diarrhoea is also present.
In the subacute form, the incubation may last up to 10 days, and clinical signs are less severe, mortality is lower and death is sometimes due to secondary respiratory or digestive complications. The subclinical form is the most insidious one, difficult to be recognized in field conditions. Fever, mild apathy and rapid breathing may go undetected until recovery that occurs after a few days. 

*E. ruminantium* is detectable in the bloodstream only during the febrile period, being a parasite of endothelial cells, lesions are recorded in various organs and systems. Severe hydropsycoardiand, hydrothorax and, in some cases, ascites are striking changes in most fatal cases. The trachea and bronchi often contain a fibrinous froth and the mucosa is congested and covered with petechial haemorrhages. Lung oedema and congestion of the parenchyma and interlobular secta are often observed. Degeneration and haemorrhages on myocardium may be common in the acute and subacute forms. In cattle, pathological changes on the digestive system are regular findings, they are not common in sheep and goats. Lymph nodes are frequently oedematous with petechial haemorrhages. In most severe cases, in Angora goats, kidneys may be highly congested. Oedema of the brain and of the meninges commonly occurs in animal suffering from hyperacute and acute forms. Occasionally, the entire brain is prominently swollen, with the result that there is a partial herniation of the cerebellum through the foramen magnum.

HW and its vectors have already shown their spreading capacities outside the sub-Saharan endemic areas. *Amblyomma variegatum*, the most important HW vector, has spread in the Caribbean boosted by suitable ecological conditions and by erratic movements of cattle egrets (*Bubulcus ibis*), strictly associated with grazing ruminants and natural hosts of the immature forms of *Amblyomma variegatum*. *E. ruminantium* was isolated for the first time from a goat living in the island of Guadeloupe only in 1980 and then in the nearby island of Marie-Galante; later on, seropositivity has been reported in different islands of the Region.

Even if the introduction and the establishment of *Amblyomma* ticks and HW in Mediterranean area is to be considered unlikely, there are models suggesting that suitable environmental conditions for colonization of the tick exist. *A. variegatum* in the Mediterranean area has been reported, probably introduced by migratory birds. In addition, exotic ticks may be introduced through the import and or smuggling of exotic animals other than ruminants. Import of exotic reptiles has been responsible for the introduction in the United States of *A. marmoreum* and *A. sparsum*, the latter tested positive for *E. ruminantium*.

Even though vector competence for *E. ruminantium* of tick species present in Italy has not been tested, the risk of a possible adaptation of *Ehrlichia* to indigenous species may not be excluded. Non-pathogenic strains of *E. ruminantium* to tick species different from the genus *Amblyomma* has, in fact just been reported.

The study hereunder reported, was conducted in compliance to the mandate of the National Reference Centre for Exotic Diseases (CESME) at the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise “G. Caporale” (IZSAM), in the framework of the preparedness policy; should incursions of exotic disease occur and, at the same time, it has provided diagnostic skill and tools also to the Namibian Veterinary Services.

### MATERIALS AND METHODS

Ten Dorper Sheep, five years old, were selected for the trial since their susceptibility to *E. ruminantium* is comparable to that of the Merino sheep and is higher than that of local indigenous breed, furthermore sheep are easy to handle and to keep in insect proof stable. The animals were tested for parasites and transferred to the insect proof premises provided with screened door and windows, at the ‘Bergvlug’ Veterinary Research Farm of the Namibian Ministry of Agriculture, Water and Forestry, in the Windhoek district.

### INOCULUM

On the day of infection, time zero (T0), 8 ml of infective blood containing the Ball 3 stock of *E. ruminantium*, batch 210, produced by Onderstepoort Biological Product (OBP), was slowly administered intravenously to each animal. The inoculation of infective blood containing the Ball 3 stock is usually used for HW control; the method is called “infection and subsequent treatment”. Ball 3 stock induces clinical disease and high mortality if not followed by tetracycline treatment.

### CLINICAL SYMPTOMS

Clinical signs, including rectal temperatures, were recorded daily from the T0 to the end of the trial on day 36 post-infection (pi). Blood samples were collected weekly till the development of the first clinical sign and thereafter on daily basis.

### NECROPSY

Post-mortem examination was performed on the eight animals which died within the observation time of 36 days; brain, kidney, spleen, lungs and in some sheep cephalic, mesenteric and mediastinal lymph nodes were also collected.

### PCR

Tissue and blood samples were subjected to the specific *E. ruminantium* PCR by amplifying a fragment of the pCS20 gene, according to the method described by Molia and collaborators.

### RESULTS

**Clinical signs**

The incubation period ranged from day 11 to day 15 pi, when pyrexia was first recorded (Table 1). Temperature peaks varied between 39.9°C and 41.3°C. Mortality occurred between day 17 and day 36 pi. Clinical signs observed were attributable to each of the four clinical forms described in literature. Hyperacute form, characterized by sudden death on day 17, was recorded in animal n. 5. Depression, shortness of breath, dyspnea presence of froth from the nose, typical of the acute clinical form, were
recorded in sheep n. 1 and n. 6 (Fig. 1). However, it was not possible to detect nervous signs that usually appear in the second stage of this form of disease, in no case was diarrhoea seen.

Subacute form was found in five sheep (n. 3, 4, 7, 8 e 9) in which depression and tachypnea were present before death. The subclinical form was observed in sheep n. 2 and n. 10 which survived throughout the observation time.

NECROPSY

Body cavities
Serum haemorrhagic exudate was present in the abdominal cavity of all animals, from the thorax it was possible to collect up to 1500 ml of fluid. In animal n. 8 and 9 haemorrhagic ascites and pleural effusion were respectively recorded (Fig. 3). Pleural effusion was also seen in animal n. 1.

Respiratory system
Congested and blood engorged lungs were seen in all sheep (Fig. 4); oedema and haemorrhages of the tracheal mucosa in animals n. 4, n. 8 and n. 9, pulmonary haemorrhages and hepatisation of the lungs in n. 8 and n. 9.

Cardiovascular system
Degeneration and haemorrhages on the myocardium, together with petechial haemorrhages on the sub endocardium were recorded in all animals. No lesions were observed on pericardium and endocardium however, 100 ml of exudate was present in the pericardium sac of animal n. 1 (Fig. 2).

Gastro-intestinal apparatus
Petechial haemorrhages on the small intestine, degeneration, congestion and haemorrhages on the liver together with congestion of mesenteric vessels were observed in animal n. 6. Lesions on the gastro-intestinal apparatus are not common in small ruminants.

Lymphatic system
In all sheep splenomegaly and colliquation of the splenic pulp were seen. Petechial haemorrhages were recorded on cervical lymph nodes of sheep n. 3, 5 and 6 and on thoracic ones of animals n. 8 and 9.
Table 1 - Body temperatures and PCR results from blood samples taken from sheep infected with *E. ruminantium* Ball 3 stock.

<table>
<thead>
<tr>
<th>Time of infection</th>
<th>Sheep 1</th>
<th>Sheep 2</th>
<th>Sheep 3</th>
<th>Sheep 4</th>
<th>Sheep 5</th>
<th>Sheep 6</th>
<th>Sheep 7</th>
<th>Sheep 8</th>
<th>Sheep 9</th>
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<td>ND</td>
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†: animal death; ND: Not done.

**Nervous system**

Meningeal and cortex congestion were the only lesions commonly observed.

**LABORATORY TEST**

PCR performed on blood samples were positive for all 10 animals when temperature was ≥ 39.1°C. Among the tissue samples tested, brain was for the most frequently positive detecting *E. ruminantium*. Results are reported in Table 1 and 2 respectively.

**DISCUSSION AND CONCLUSION**

The experimental infection of sheep with Ball 3 stock of *E. ruminantium* enabled to observe the clinical symptoms, the pathological lesions, the dynamic of the infection and to evaluate the performance of the molecular test in case of HW, enhancing diagnostic skills for the diseases in both Italian and Namibian laboratories. Variability of the incubation period, differences in the severity of the clinical symptoms...
and pathological lesions recorded, were in agreement with what is described in the literature in naturally infected animals. Virulence of the stock used for infection, susceptibility of the animal breed and the interaction with the immune system may influence the course of the disease.

Clinical symptoms and pathological lesions were typical of acute and sub-acute forms, that are the most commonly described in free population or in naïve animals and that would likely be the forms seen if the disease enter into free territory, like Italy. In the case of hyperacuta form, seen in sheep n. 5, clinical signs were absent. The presence of cutaneous exudate is confirmed as the most relevant finding.

Given the economic importance of HW and its spreading capacity, research on methods of controlling the disease is constantly on going, at present, however, the only prophylactic methods available is that of “infection-treatment”, based on the administration of a calibrated dose of infected blood followed by a treatment with tetracycline in those animals which develop a febrile reaction. This prophylactic method, improperly called vaccine, in addition to innocuity problems increase the number of possible carrier.

The Ball 3 is the most commonly used stock for the “infection-treatment” immunization method. The main reason for choosing this genotype is that it produces an early warning temperature rise. It differs in this respect from the highly virulent Welgevonden isolate which often causes death very shortly after a rapid temperature rise and is therefore not suitable for use in infection and treatment. Even if, the intravenous administration of the Ball 3 stock minimizes variations in the incubation time reducing losses to non-timely administration of the antibiotic, in the field conditions death of injected animals are continuously reported.

The attempts to identify a non-pathogenic strain that may be sufficiently immunogenic to protect susceptible stock has been unsuccessfully pursued over the time. Inactivated, live attenuated and recombinant vaccines that in vitro may have shown satisfactory results, when tested in the field have not been able to induce an acceptable level of protection. Our observations in experimental conditions confirm that, nevertheless the Ball 3 stock is the most used for the immunization by “infection-treatment” in southern Africa, it is able to cause clinical disease and livestock losses if injected animals are not properly and timely treated with tetracycline.

Furthermore results confirm that PCR is a reliable diagnostic assay when applied on blood samples taken from febrile animals, E. ruminantium, unlike the other Ehrlichia, is a parasite of endothelial and not of white blood cells and therefore bloodstream is only a means of dissemination to the various organs. Ehrlichia ruminantium is very fragile in the environment and it is difficult to recover it from dead animals 6 hours after death once putrefaction has begun. Thus, diagnosis in the field by traditional test (brain smears) may be problematic. Our results confirm that testing tissues from dead animals by PCR, allows to increase diagnostic capacity for the disease, and that brain is the target also for laboratory diagnosis by PCR.

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