INVESTIGATIONS INTO THE VECTOR COMPETENCY OF ARTHROPODS FOR TWO EHRLICHIAS: EHRLICHIA RISTICII AND COWDRIA RUMINANTIUM

by

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Veterinary Science

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May, 1990

Blacksburg, Virginia

INVESTIGATIONS INTO THE VECTOR COMPETENCY OF TWO EHRLICHIAS: *EHRLICHIA RISTICII* AND *COWDRIA RUMINANTIUM*

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(ABSTRACT)

Three studies relating to the vector competency of several species of ticks and *Simulium* spp. (blackflies) for *Ehrlichia risticii*, causative agent of Potomac horse fever (PHF) and *Amblyomma variegatum* for *Cowdria ruminantium*, causative agent of heartwater, are described.

Dermacentor variabilis, *Rhipicephalus sanguineus*, *Amblyomma americanum* and *Ixodes scapularis* ticks were investigated for their ability to acquire and transmit PHF. Larval and nymphal ticks were exposed to *E. risticii* by feeding on mice inoculated with the organism. Molted exposed ticks were then allowed to feed on susceptible ponies or mice and were examined by light and electron microscopy. No evidence of transmission, either clinically or by seroconversion in mice or ponies was observed.

Blackflies (*Simulium* spp.) were trapped in an area endemic for PHF and inoculated into mice in an attempt to demonstrate *E. risticii*. No evidence of seroconversion by mice to *E. risticii* was observed.

Two laboratory colonies of *Amblyomma variegatum* ticks were investigated for their ability to acquire and transmit *C. ruminantium*. Nymphs from both laboratory groups were simultaneously fed on a goat that had been infected with *C. ruminantium* and was febrile. Engorged nymphs from both groups were replete from feeding on three consecutive days. Nymphs from both groups were then incubated under identical conditions until molting.

Acknowledgements

There are a number of people who have helped me over the last four years. First of all I must thank Dr. Jorgen Hansen for letting me talk him into accepting me as a graduate student. He has been a constant source of moral and academic support and I have enjoyed working with him. I also thank Dr. Anne Zajac for stepping in as my committee chairperson and for her help, friendship and contributions throughout my program.

I give my sincere thanks to Dr. Brian Perry for getting me involved in Potomac horse fever research, for enticing me to Kenya, for helping me in every way imaginable from bleeding horses on week-ends to constructive review of manuscripts to welcoming my family into his home. His help and friendship have been invaluable.

Dr. Kathy Kocan has also been an integral part of my studies. She has provided me with the technical skills and study design for a major portion of my dissertation. Additionally she has shown me that it is possible to "do it all" and remain a terrific person at the same time.

I thank the other members of my graduate committee, Dr. Gerhart Schurig and Dr. Daniel Sonenshine for their advice and critical review of this manuscript. I also thank Dr. Jay Levine for participating in my thesis defense as external examiner.

I would like to acknowledge the help of Drs. Andy Norval and Linda Logan at the International Laboratory for Research on Animal Diseases, Kenya, Dr. S. Waghela of the Kenya Agricultural Research Institute and Dr. J.D. Bezuidenhout for their advice and support on the Cowdria work, Drs. Michael Fletcher and Craig Turner for their help, advice and equipment in the PHF transmission experiments and Dr. Ed Schmidtmann for his advice and loan of equipment in the blackfly studies.

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Introduction

Vectorial competence is defined as the combined factors that affect a vector's ability to serve as a host for a pathogen (Spielman and Rossignol, 1989). A competent vector must be able to successfully take-up, maintain (possibly with development and/or multiplication) and transmit a pathogen. Vectorial competence can be determined in the laboratory under controlled conditions. The subject of this dissertation is the vector competence for several species of arthropods for the two closely related Ehrlichieae, Cowdria ruminantium and Ehrlichia risticii.

Ehrlichias are generally tick-borne and cause disease in people as well as a variety of wild and domestic animals. The first step in controlling any vector-borne disease is a clear understanding of the epidemiology of the pathogen in both its vertebrate and arthropod hosts. Amblyomma variegatum, the tick vector of C. ruminantium, causative agent of heartwater, has been identified for decades. Yet there are still a number of questions relating to the competency of this vector that remain unanswered. The vector of E. risticii, causative agent of Potomac horse fever (PHF), remains unknown. This dissertation describes several efforts to determine the vector of PHF by evaluating the vector competency of several suspect ticks, and to address specific factors pertaining to vector competence of A. variegatum.

The specific objectives are as follows:

1. To investigate the vector competence of four species of ticks (Dermacentor variabilis, Rhipicephalus sanguineus, Ixodes scapularis and Amblyomma americanum) to transmit E. risticii. This was done by one of three ways.

a. The attempted transmission of the above ticks from experimentally infected mice to susceptible mice and horses.

b. Examination by light and electron microscopy of experimentally infected ticks.

c. Inoculation of 24 hour experimentally infected replete ticks into susceptible mice.

2. To investigate the vector competency of blackflies in the transmission of E. risticii. This was done by attempting to identify E. risticii in blackflies trapped in areas endemic for PHF.

3. To investigate the vector competency of Amblyomma variegatum for C. ruminantium. Two laboratory colonies of A. variegatum ticks were compared for their ability to acquire, maintain and transmit infection with C. ruminantium.

The evolution of working on the two ehrlichias for one dissertation was brought about by calculated chance. At the onset of my studies I was searching for the vector of PHF. When offered the opportunity to work in Kenya at the excellent facility of the International Laboratory for

Research on Animal Diseases I was able to expand the scope of my dissertation and examine aspects of ehrlichial transmission in an established vertebrate host/vector relationship.

The chapters of this dissertation are presented as separate studies and have been published or submitted for publication as follows:

1. " The attempted transmission of Ehrlichia risticii, causative agent of Potomac horse fever, by the ticks Dermacentor variabilis, Rhipicephalus sanguineus, Ixodes scapularis and Amblyomma americanum." has been published under the same name in Experimental and Applied Acarology, volume 8, pages 41-50, with the following contributing authors N.E. Hahn, M.G. Fletcher, R.M. Rice, K.M. Kocan, J.W. Hansen, J.A. Hair, R. Barker and B.D. Perry.

2. "Role of blackflies in the epidemiology of Potomac horse fever" has been published under the same title in the Veterinary Record, volume 125, pages 273-274, 1989 with the following contributing authors; N.E. Hahn, B.D. Perry, R.M. Rice, J.W. Hansen and E.C. Turner.

3. "Variation in infection rates of two laboratory colonies of Amblyomma variegatum ticks experimentally infected with Cowdria ruminantium" was submitted for publication with Veterinary Microbiology in March 1990, with the following contributing authors; N.E. Hahn, R.A.I. Norval, S.D. Waghela, M. Shaw, K.M. Kocan, F. Rurangirwa and B.D. Perry.

CHAPTER 1

Literature Review

INTRODUCTION

The Rickettsiales comprise an order of bacterial organisms characterized by their intracellular location and association with arthropods. Some, most notably the Wolbachia, are maintained in their arthropod hosts and never venture into the vertebrate realm. In contrast, Neorickettsia helminthoeca, causative agent of salmon-poisoning disease in dogs, can apparently be maintained without an arthropod host at all. Many of these organisms, when transmitted to human and other animals, can be highly pathogenic.

The subject of this review is the transmission of one tribe of the rickettsias, the Ehrlichieae (see Table 1), a group that until recently was considered to cause disease in domestic animals only. However, the causative agent of human sennetsu fever in Japan, Ehrlichia sennetsu (previously Rickettsia sennetsu) has recently been included in the genus Ehrlichia (Ristic & Huxsoll,1984). Ehrlichia canis, causative agent of canine ehrlichiosis, has also been implicated as a cause of disease in humans (Maeda et al., 1987). However, this diagnosis was based primarily on serology and not identification of the causative organism: serological cross-reactions within the Ehrlichieae have been well documented (Ristic et al., 1986, Logan et al., 1986, Jongejan et. al., 1989). Until E. canis can be isolated from human cases it will not be clear whether this disease is truly caused by E. canis or some as yet unidentified agent (Ewing et al, 1987).

CLASSIFICATION OF THE EHRLICHIEAE

A. Within the Order Rickettsiales

The taxonomy of the Ehrlichieae is confusing and has been changed periodically as more has been learned about the rickettsias. There is no disagreement on the inclusion of the Ehrlichieae in the order Rickettsiales. The problem has been the relationship of the Ehrlichieae as a group with other members of the order Rickettsiales and to each other within the tribe.[1]

Inclusion of the ehrlichias into the order Rickettsiales is based mainly on morphological characteristics. The rickettsias are gram-negative, coccoid, rod-shaped to pleomorphic organisms ranging in size from 0.2 to 0.5 um in diameter and 0.8 to 2.0 um in length. They are obligate intracellular organisms and possess distinct cell walls (Weiss & Moulder, 1984).

The order is divided into three families (Table 1). Members of the family Rickettsiaceae are generally found in nucleated blood cells and/or vascular endothelial cells whereas the families Bartonellaceae and Anaplasmataceae parasitize erythrocytes. The division of the family Rickettsiaceae into tribes and genera has historically been based on pathogenicity and host susceptibility (Wolbach, 1925) and this classification is still in use (Wiess & Moulder, 1984). Organisms causing human diseases were placed into the tribe Rickettsieae, the non-pathogenic arthropod symbionts into the Wolbachieae and the diseases of domestic animals into the Ehrlichieae. These divisions are arbitrary and there are obvious inconsistencies in this classification. For example Rickettsia rickettsii

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[1]The classification of the order Rickettsiales as it appears in the most recent Bergey's Manual of Systematic Bacteriology (Weiss and Moulder, 1984) is shown in Table 1.For the purposes of this paper"rickettsia" refer to any organism within the order Rickettsiales, "ehrlichia" are organisms within the tribe Ehrlichieae

causes disease in dogs as well as people. In spite of this subsequent investigations using immunological techniques have for the most part supported these groupings.

There are a number of characteristics that distinguish the ehrlichias from the Rickettsieae, the most important being their trophism for mammalian leukocytes. Members of the tribe Rickettsieae are found free in the cell cytoplasm. Ehrlichial organisms are found in membrane bound inclusion bodies inside the host cell. This is not unique to the ehrlichias and it is also seen in Anaplasma sp. and Coxiella sp. The Ehrlichieae differ from Rickettsieae in their inability to grow in embryonated hens eggs (with one apparent exception, Ehrlichia risticii (Rice R, unpublished data, cited in Fletcher et al., 1990), their sensitivity to sulfonamides (the ehrlichias are very sensitive), and their inability to produce Proteus agglutins as do the Rickettsieae (Smith & Ristic 1977; Marchette, 1982).

The Rickettsieae develop in all tissues of the vector tick and are transmitted transstadially (from one stage to another) as well as transovarially (from adult female to progeny) (Burgdorfer, 1985). These organisms divide by binary fission probably in all tick tissues. In contrast, the ehrlichias for which the tick vector are known, are generally transmitted transstadially but not transovarially.

Several reviewers have suggested that the development of the ehrlichias is more complex than that of the Rickettsieae, justifying a classification system more closely aligned with the chlamydias (Smith & Ristic, 1977; Uilenberg, 1983; Marchette, 1982; Scott, 1987). The chlamydias are also membrane bound, obligate intracellular organisms. In the 8th edition of Bergey's Manual of Systematic Bacteriology the chlamydias

were placed in an order separate from the Rickettsiales; this was due in part to their complex developmental cycle (Page, 1974). Briefly, the chlamydial life cycle consists of stages termed elementary bodies, dispersing forms, reticulate bodies and condensing forms, that are seen in sequential order in mammalian cells (Storz & Spears, 1977). In contrast, the rickettsias are considered to multiply by simple binary fission. With increasing knowledge of the ehrlichias and some other rickettsias it has been shown that the chlamydias are not as unique as had been thought. Anaplasma marginale has been shown to have a complex life cycle in the vector tick (Kocan, 1986). Some of the ehrlichias have been shown to have different developmental forms (Nyindo et al., 1971; Smith et al., 1976; Kocan et al., 1987a; 1987b) although no definite sequence has as yet been described for the ehrlichias. In contrast, Woldehiwet and Scott (1982) in their studies of the development of the tick-borne-fever agent, concluded that "the development of C. phagocytophila is not as complicated as previously thought. The discrete particles represent the early stages of infection and the clusters are a result of subsequent division by binary fission inside cytoplasmic vacuoles." It should be noted that most of the studies of development of the ehrlichias have been done in mammalian tissue. To date the only ehrlichias to be studied in their arthropod hosts are C. ruminantium (Kocan et al., 1987a; 1987b) and E. canis (Smith et al., 1976). The information on the development of C. ruminantium and A. marginale indicate that forms are seen in the vectors that may not be seen in the vertebrate host. It would therefore be worthwhile to study all the ehrlichias within their respective vectors.

Scott (1987) has proposed a classification scheme for the rickettsias that is based on a combination of 63 characteristics including morphology, epidemiology and cell trophism. In this system the ehrlichias are also more closely aligned with the chlamydias. Recent reports of the discovery of close genetic relationships between the Rickettsieae and certain arthropod-borne plant bacteria (Wiesburg et al., 1985) but not between Chlamydia sp. and the plant associated organisms (Wiesberg et al., 1986) shed new light on the situation but by no means clarify it. It seems, based on comparison of antigens, that the Rickettsieae are more closely related to the plant associated organisms than they are to the Chlamydia; performance of the same analysis on the ehrlichias may also yield useful information. The new DNA analytic technologies should soon allow the clarification of the taxonomy of the Rickettsiaceae.

B. Within the tribe Ehrlichieae

Three genera comprise the tribe Ehrlichieae: Ehrlichia, Cowdria and Neorickettsia. Cowdria has only one member, C. ruminantium, which causes the disease known as heartwater, or cowdriosis, in domestic and wild ruminants. Neorickettsia helminthoeca is the only named member of the genus Neorickettsia. There are several species within the genus Ehrlichia. The most recent edition of Bergey's manual (1984) lists Ehrlichia canis, E. equi, E. phagocytophila and E. sennetsu. The basis of this classification is probably morphology and antigenic relationships, although E. canis is listed as sharing common antigens with E. sennetsu and E. equi with no mention of such immunological relationships with E. phagocytophila. Included as species incertae sedis are E. bovis, E. ovina, E. kurlovi, E. platys, Cytocetes microti, C. ovis var. decani, Rickettsia delphi and

R. belgaumi. An omission to both of these lists is Cytocetes ondiri, the causative agent of bovine petechial fever in cattle. This organism is included in other reviews of the Ehrlichia (Smith & Ristic 1977; Marchette, 1982; Scott, 1987). An undisputed recent addition to the genus is Ehrlichia risticii (Holland et al., 1985b), causative agent of Potomac horse fever.

Foggie (1962) proposed a classification of these organisms based on their cytotropism and suggested that the tick-borne fever agent be placed in the genus Cytocetes together with an ehrlichia-like organism called Cytocetes microti described in the vole (Tyzzer, 1938). Other authors (Krauss et al., 1972; Scott, 1987) later agreed, adding the bovine petechial fever agent to the genus, to become C. ondiri. Under this scheme the monocytic organisms (E. canis, E. bovis, E. ovina) would remain Ehrlichia with the granulocytic organism E. equi would be placed in the genus Cytocetes. The limitation of this classification is that some of the organisms exhibit a variable trophism. For instance E. canis has been reported in lymphocytes, eosinophils and neutrophils as well as in monocytes. However, these groupings may turn out to be valid as the evidence from immunological studies show strong antigenic similarities between the predominantly monocytic organisms E. canis, E. risticii and E. sennetsu but less with E. equi (Ristic et al., 1986). Furthermore, the granulocytic organisms E. equi and E. phagocytophila have corresponding antigenic similarities (Logan, pers. comm.). A close antigenic relationship has been demonstrated between C. ruminantium and the ehrlichias (most strongly E. equi, less with E. canis but not at all with E. sennetsu, E. risticii, and 12 other rickettsias) (Logan et al., 1986), C. ruminantium and E. phagocytophila (Jongejan et al., 1989) and C. ruminantium and E.

bovis and E. phagocytophila (Du Plessis et al., 1987; Camus, 1987). Since C. ruminantium has also been found in neutrophils (Logan, 1987) the possibility that it may belong in the same genus as the granulocytic ehrlichias should be considered.

For the purposes of this review the following species will be considered as members of the genus Ehrlichia:

E. canis

E. bovis

E. ovina

E. equi

E. (Cytocetes) phagocytophila

E. sennetsu

E. risticii

C. ondiri

C. ruminantium and N. helminthoeca will also be discussed.

A summary of differing characteristics of these organisms as they relate to their transmission is presented in Table 2.

COWDRIA RUMINANTIUM

Heartwater, caused by C. ruminantium, is a severe disease of wild and domestic ruminants that has been well known in Africa for over a century. Due to its importance in African domestic livestock more is known about heartwater than the other ehrlichial diseases. It was first reported in 1898 (Anon.) in South Africa at which time it was already known as heartwater to local farmers. Lounsbury (1900) determined that it was transmitted by the bont tick Amblyomma hebraeum and in 1925 Cowdry demonstrated that it was caused by a rickettsia and it was named

Rickettsia ruminantium. In 1947 the name was changed to Cowdria ruminantium (Moshkovski, 1945).

Heartwater is characterized by fever, nervous signs, anorexia, and petechiation. The course of the disease ranges from peracute to inapparent (Van de Pypekamp et al.,1987). Hydropericardium is a common postmortem finding from which heartwater derives its name (Prozesky, 1987) but is not present in all cases (Mebus & Logan, 1988).

Cowdria ruminantium, is classically described as having a trophism for vascular endothelial cells (Ristic & Huxsoll, 1984). Recent work (Logan et al., 1987) has shown that C. ruminantium is also found in neutrophils.

Although the etiology of heartwater has been known for a long time, further studies into its epidemiology and natural history have been severely hampered due to an inability to grow the organism in vitro and develop a reliable serodiagnostic test. Recent progress in this area (Bezuidenhout et al., 1985; Yunker et al., 1988) should result in a rapid increase in the understanding of heartwater epidemiology.

Distribution

Heartwater is known to occur through most of Africa south of the Sahara. The known distribution has been reviewed by several authors (Uilenberg 1983a; Camus & Barre, 1982; Provost & Bezuidenhout, 1987). It is likely to be found wherever African Amblyomma ticks occur (Uilenberg, 1983a). Heartwater has recently been diagnosed in several islands in the Caribbean (Perreau et al., 1980), and spread to North America is considered to be a serious possibility (Barre et al., 1987). Based on a computerized climate matching model Sutherst and Maywald (1985) have

predicted that should A. variegatum be introduced to the western hemisphere it would find suitable habitat in the southern United States as well as large portions of Central and South America. Amblyomma ticks have been shown to be an important reservoir of heartwater and the introduction of infected ticks is one possible way of introducing the disease. There are already several documented cases where African ticks have been transported into the US on imported animals, most notably A. hebraeum on rhinoceros (Uilenberg, 1983a; Wilson & Richard, 1984). Fortunately the ticks were discovered and did not become established. Another possible means of establishment of heartwater is by indigenous North American ticks following the introduction of carrier domestic or exotic animals. Both A. maculatum and A. cajennense are widespread in the United States especially along the gulf coast (Walker,1987) and have been shown to be capable of transmitting heartwater under experimental conditions (Uilenberg, 1982; 1983b). The North American white-tailed deer has been shown to be susceptible to infection (Dardiri et al., 1987) and could potentially become reservoir.

Vector

Twelve species of Amblyomma ticks have been shown to transmit heartwater experimentally (Bezuidenhout, 1987). Amblyomma variegatum and A. hebraeum are considered to be the most important heartwater vectors based on factors such as vector efficiency, adaptation to livestock and distribution (Uilenberg, 1983; Bezuidenhout, 1987).

Transstadial transmission occurs with varying degrees of efficiency in all of the Amblyomma ticks determined to be experimental vectors. Some transmit only from larvae to nymphs, some from nymph to adult, some from larvae through to adults. Transovarial transmission has been shown

for A. hebraeum ticks only (Bezuidenhout & Jacobz, 1986). This finding was surprising as previously it was not considered possible and has not been repeated. The success of transovarial transmission was low and the authors doubt whether this mode of transmission contributes significantly to the epidemiology of heartwater. Intrastadial transmission by male A. hebraeum ticks, which was not considered to occur (Lounsbury, 1902; Alexander, 1931), has recently been shown to be of importance (Andrew & Norval, 1989). Males transferred from both live and dead hosts that had been infected with heartwater were found capable of repeatedly transmitting the disease to susceptible hosts.

It is not known whether C. ruminantium is transmitted by the salivary route or by gut regurgitation. Cowdry (1925b) first described C. ruminantium as occurring in midgut epithelial cells and occasionally in the gut lumen of A. hebraeum. He hypothesized that the transmission of C. ruminantium to its vertebrate host was by means of gut regurgitation. This hypothesis was supported by later studies which confirmed the presence of colonies of C. ruminantium in gut epithelial cells of A. hebraeum and A. variegatum by fluorescent antibody staining and light and electron microscopy but which did not demonstrate any Cowdria organisms in salivary glands (Bezuidenhout, 1984; Kocan, et al.,1987b). Gut regurgitation as the sole means of transmission has been questioned following recent studies where saliva collected from infected ticks was sometimes infective and salivary gland homogenates were consistently infective for susceptible sheep (Bezuidenhout, 1981). Kocan et al. (1987a) demonstrated by electron microscopy colonies of C. ruminantium in salivary glands of A. hebraeum nymphs that had been fed as larvae on heartwater infected sheep. These organisms were found in ticks that were fed for 4 days before removal

and dissection. Cowdria ruminantium has not been demonstrated in adult tick salivary glands.

Host range and reservoirs

Naturally contracted fatal heartwater has been found in several species of wild and domestic ungulates (Uilenberg, 1983a). Numerous wild African ruminants have been experimentally infected with Cowdria as reviewed in Oberem & Bezuidenhout (1987). Clinical signs in these animals ranged from mild to severe, with sudden death occurring in many of them. North American white-tailed deer are also highly susceptible to experimental infection with C. ruminantium (Dardiri et al., 1987).

No non-domestic vertebrate reservoir has yet been found in the wild but many investigators believe that one exists. Bezuidenhout (1985) has pointed out that because different stages of A. hebraeum ticks are active at different times of the year, that a reservoir must exist for the disease to be perpetuated. Other circumstantial evidence indicates the presence of a wildlife reservoir. Mackenzie and Norval (1980) reported cases of heartwater in livestock introduced to a game reserve in which no domestic animals had previously been pastured and none of the known heartwater vectors were present. Since the introduced livestock were from a heartwater-free area a wildlife reservoir is likely to have been involved. The difficulty in determining reservoir status in wild populations has been due to the lack of a reliable serological test for the identification of antibodies, prohibiting large scale screening of mammals for evidence of exposure to infection.

The African buffalo (Syncerus caffer) has been shown to be refractory to heartwater infection (Gradwell et al, 1976; Keffen, 1895; Andrew &

Norval, 1990). However, in experiments by Andrew and Norval (1990), A. hebraeum nymphs fed on the buffalo 161 days following experimental infection could transmit heartwater as adults. Although this has not yet been shown to occur in the field, it is likely that the African buffalo and possibly other wild ungulates will be found to be of importance in heartwater epidemiology.

Sheep and cattle experimentally infected with C. ruminantium can also act as carriers (Andrew & Norval, 1990). Sheep and cattle were found to be still infective for A. hebraeum ticks 223 and 246 days respectively after laboratory infection with C. ruminantium. This is contrary to what had been found by other investigators. Cowdria ruminantium was shown present up to 60 days in spontaneously recovered or treated animals (Neitz, 1939; Ilemobade, 1976; 1978). In unpublished studies by Bezuidenhout (cited in Bezuidenhout, 1987), ticks feeding on such animals did not become infected. In experimental tick infection only ticks replete during or within two days of the febrile response of the host became infective in the next stage (Bezuidenhout JD & Oliver JA, unpublished data 1986, reported in Bezuidenhout, 1987; Camus & Barre, 1987). Andrew and Norval attribute their ability to detect the carrier state to the use of nymphs for the acquisition phase as opposed to larvae used in previous experiments. This ability to detect a carrier state of heartwater will facilitate future studies to determine whether it occurs in host populations in the field.

Laboratory rodents are generally not susceptible to infection with Cowdria. There are a number of mouse-adapted strains that have been very important in heartwater research (MacKenzie & McHardy, 1987). However, the general lack of susceptibility of laboratory animals to Cowdria is

significant in itself in that it indicates that rodents are probably not important in the epidemiology of heartwater.

The helmetted guinea fowl (Numida meleagris) and the leopard tortoise (Geolchelone pardalis) can be experimentally infected with C. ruminantium and act as a source of infection of C. ruminantium to Amblyomma ticks (Bezuidenhout, unpublished data, reported in Oberem & Bezuidenhout, 1987). These animals were studied as potential heartwater reservoirs after immature stages of A. hebraeum were found to parasitize them (Walker & Schulz, 1984; Horak & Williams, 1986). Another source of infection is the tick itself. Camus and Barre (1982) considered infected ticks to be the principle reservoir of heartwater infection. Reports of infection rates in unfed ticks is variable but ranges from 1-7% (Uilenberg, 1971; Camus & Barre 1987; Du Plessis, 1985; Du Plessis & Malan, 1987) to up to 45% (Norval et al., 1990). The organism has been shown to survive in experimentally infected ticks up to 15 months (Ilemobade, 1976).

Adult male ticks may be considered as a reservoir separate from adult female ticks. Unlike nymphs and females, their behavior allows them to both acquire and repeatedly transmit infection without molting (Andrew & Norval, 1989). They can survive on the host for up to 8 months (Jordan & Baker, 1981), potentially on multiple hosts (Andrew & Norval, 1989). Norval et al. (1990) have suggested that the repeated infection by male ticks could act to reinforce the carrier state. The exact role of male ticks as vectors and reservoirs remains to be elucidated.

EHRLICHIA CANIS

Ehrlichia canis, the first of the Ehrlichia to be described (Donatien & Lestoquard, 1935), to date remains the member of the genus about which

the most is known. The species causes a disease in dogs most commonly known as canine ehrlichiosis. A severe form of the disease that occurs in certain breeds of dogs is referred to as tropical canine pancytopenia (TCP) (Wilkins et al., 1967; Huxsoll et al., 1970).

The clinical picture of canine ehrlichiosis is varied. This probably depends on varied individual animal response and concomitant infection with other diseases such as Babesia (Ewing, 1969). Acute, hemorrhagic, uremic, subclinical, chronic and carrier forms are recognized (Price et al., 1987). Clinical signs range from fever, lymphadenopathy and anorexia followed by rapid recovery to a severe hemorrhagic disease that often results in death. Uncomplicated ehrlichiosis is considered by some to be a mild disease except in young puppies where it can be fatal (Ewing, 1969) and in German shepherds where the severe hemorrhagic form is seen. Polyarthritis caused by E. canis infection has also been reported (Stockham et al., 1985; Bellah et al., 1986).

Ehrlichia canis is most commonly found in lymphocytes and monocytes (Donatien & Lestoquard, 1935), more rarely in granulocytes (Ewing, 1969). The most common form seen in polyarthritic dogs has been found in neutrophils (Stockham et al., 1985; Bellah et al., 1986; Madewell & Gribble, 1982). It has been suggested that this neutrophilic strain is actually E. equi, which is normally found in equine neutrophils (Madewell & Gribble, 1982). Dogs are susceptible to experimental infection with E. equi and organisms were found in the neutrophils and eosinophils of the infected dogs (Lewis et al., 1975). However, infection with E. equi did not protect against subsequent challenge with E. canis (Lewis et al., 1975). Furthermore, in one study of nine dogs with polyarthritis, all but one dog demonstrated serum antibodies to E. canis, whereas none

(only three of the same group of nine dogs were tested) had significant titers to E. equi (Stockham et al., 1985). Combined, these results indicate that polyarthritis in dogs is not caused by E. equi, but whether this form of canine ehrlichiosis is caused by E. canis, E. equi or an as yet unidentified organism remains unanswered.

Distribution

Ehrlichia canis was originally reported from Algeria (Donatien & Lestoquard, 1935). It has since been reported widely in Africa, the Near and Middle East, Southeast Asia, the Caribbean and the North America. The disease has not been reported from South or Central America although dogs seropositive to E. canis have been reported (Smith et al., 1975) and the vector ticks are reported worldwide between 50 degrees N latitude and 35 degrees S latitude (Herms, 1961).

Vector

The vector of canine ehrlichiosis has been shown to be the brown dog tick, Rhipicephalus sanguineus (Donatien & Lestoquard, 1937; Groves et al., 1975). Experimental transmission, first by inoculation of macerated ticks from infected dogs (Donatien & Lestoquard, 1937), then by transstadial transmission (Groves et al., 1975) established R. sanguineus to be a vector. To date no E. canis organisms have been isolated from ticks not associated with infected dogs (Marchette, 1982). In Kenya Haemaphysalis leachi is suspected of transmitting E. canis, but no experimental evidence is presented to support this (Price, 1986). Since the immature forms of H. leachi feed on burrowing rodents, not dogs

(Norval, 1984), it is unlikely that this tick is involved in E. canis transmission. In contrast all stages of R. sanguineus parasitize dogs.

Donatien and Lestoquard (1935) concluded that transovarial and transstadial transmission of E. canis occurs (Donatien & Lestoquard, 1937). More recent work has confirmed the occurrence of transstadial transmission but failed to demonstrate transovarial transmission. Larvae from eggs of infected adult females were not able to transmit canine ehrlichiosis (Ewing & Philip, 1966; Groves et al., 1975). Furthermore, organisms were not found in the ovaries of infected female adults (Smith et al., 1976).

Host range and reservoirs

Canine ehrlichiosis has been reported in domestic canids only. In Kenya black-backed jackals (Canis mesomelas) have been shown to harbor the organism (Price, 1980), but no other wild canids have been found to be infected. Coyotes and foxes have been experimentally infected with E. canis (Ewing et al., 1964; Amyx et al., 1973), as have the wild dog (Lycoan pictus) and the black-backed jackal (Van Heerden, 1979). Wolves and wolf-dog crosses in captivity have become infected and died from the disease (Harvey et al., 1979). Ferrets are susceptible to experimental infection with E. canis (Spence et al., 1967). Other laboratory animals are refractory to infection.

Dogs that are infected with E. canis, and not treated, may harbor the organism for up to five years (Groves et al., 1975). Many of these dogs will show no signs of the disease at all, and have been termed carriers (Price et al., 1987; Groves et al., 1975). Blood from these carriers is infective when injected IV into susceptible dogs but to date

tick transmission from a carrier has not been successful (Lewis et al., 1977; Groves et al., 1975). Dogs that have been cleared of E. canis by tetracycline therapy are completely susceptible to reinfection (Buhles et al., 1974).

The report of E. canis in Kenyan jackals (Price, 1980) raises a question in the epidemiology of canine ehrlichiosis. A reasonable conclusion is that the jackal is a reservoir of E. canis. A reservoir status for the black-backed jackal has also been suggested by Van Heerden (1979) for E. canis in South Africa. He was able to infect black-backed jackals without showing signs of the disease and was also able to transmit E. canis from the jackal to a domestic dog. Transmission was performed by inoculation of whole blood, so the ability to transmit by ticks has not been established. It remains to be determined whether the jackal is a major reservoir of E. canis, acting as a source of infection to ticks or have been infected due to their proximity to domestic dogs. The jackal reservoir hypothesis would not explain the high incidence of canine ehrlichiosis in other parts of the world where jackals do not exist, although they would remain an important reservoir where they do exist. Since coyotes are susceptible and indeed there is a high incidence of canine ehrlichiosis in the south-western United States where coyotes are abundant, the coyote could fill the same role of the jackal in Africa. However, R. sanguineus is a tick that has adapted to a cosmopolitan habitat, breeding in kennels and other dwellings where dogs are present. Rhipicephalus sanguineus is generally not found unless a population of dogs is in the vicinity (Norval et al., 1983).

It has been proposed (Marchette, 1982) that in view of the persistence of E. canis in the blood of recovered dogs that the dog is the major

reservoir. As mentioned above, whole blood transmission of infection from recovered dogs has been shown possible but to date tick transmission has not been demonstrated. However, only one tick transmission experiment has been carried out and in light of the recent discoveries on the transmission and carrier state of C. ruminantium, this needs to be reexamined.

The third potential reservoir is the arthropod host, R. sanguineus itself. The life cycle of the tick and the survival of the organism in ticks is compatible with a disease that is maintained by ticks which become infected during the acute phase of the disease only. All instars of the tick are active during the same season. The organism can survive at least 155 days in the tick and still be capable of disease transmission (Lewis et al., 1977). This means that when an adult or nymphal tick infects a dog it will be very likely that other, non-exposed larvae or nymphae will be available for infection and able to transmit in the following instar.

Ehrlichia canis is probably transmitted from the tick to canine host by the salivary gland route. Organisms were identified in the salivary glands on electron microscopic examination and salivary gland homogenates were infective to dogs (Smith et al., 1976).

EHRLICHIA (CYTOCETES) PHAGOCYTOPHILA

Tick-borne fever (TBF) was originally described by Gordon et al. (1932). A febrile disease of sheep had been noted by Macleod (1932) while studying the tick transmission of louping ill virus. He found that sheep that recovered from a febrile disease were not immune to louping ill. Gordon (1932) also studying louping ill found that controls and

immune animals all responded alike when exposed to Ixodes ricinus ticks.

Uncomplicated TBF is a rarely fatal febrile disease (Woldehiwet & Scott, 1983). The most important aspect of infection with E. phagocytophila appears to be that it predisposes animals to other infections, most notably louping ill (Macleod & Gordon, 1932; Gordon et al., 1962; Foggie, 1962).

Gordon et al. (1940) characterized the organism in granulocytes and monocytes, suggesting that it was taxonomically related with the rickettsia. Based on its morphological resemblance to an organism found in granulocytes of voles called Cytocetes microti (Tyzzer, 1932), Foggie (1962) suggested the name C. phagocytophila. Bergey's Manual currently classifies the tick-borne fever agent in the genus Ehrlichia. Many authors do not agree with this classification and its taxonomic status remains unsettled.

Distribution

Tick-borne fever was originally described in Scotland but has since been reported in other parts of Europe, as well as in India and South Africa (reviewed in Woldehiwet, 1983).

Vector

Tick-borne fever is transmitted transtadially but not transovarially by I. ricinus (MacLeod & Gordon, 1933; MacLeod, 1936). The possibility of mechanical transmission was raised by Foggie (1951) but this has not been substantiated.

Salivary gland and gut homogenates both resulted in TBF when inoculated experimentally into sheep (Lewis, 1979) which indicates that TBF is transmitted by the salivary route. Lewis (1977) found organisms in

hemolymph and gut tissues of infected ticks that had fed for 5 days. This suggests some form of "activation" necessary to cause multiplication of gut organisms and possibly the migration to the salivary glands via the hemolymph.

Host range and reservoir.

Cattle and sheep are naturally affected (Hudson, 1950; Gordon et al., 1932) by E. phagocytophila and the organism has been isolated from red, fallow and roe deer (Foggie, 1962; McDiarmid, 1965) and from feral goats (Greig, 1969). As with E. canis and jackals, it is not known whether the wild animals act as reservoirs or are simply infected along with the sheep and cattle.

Sheep have been shown to harbor the organism in peripheral blood for a "variable period", up to 25 months (Foggie, 1951). As with E. canis, animals that are cleared of infection by antibiotic therapy become completely susceptible to reinfection (Synge, 1976).

Laboratory animals are generally not susceptible to infection with E. phagocytophila (MacLeod & Gordon, 1933; Hudson, 1950; Foggie, 1951) although ovine strains have been adapted to mice and guinea pigs. These strains retain their infectivity to sheep (Foggie & Hood, 1961).

EHRLICHIA RISTICII

Ehrlichia risticii is the causative agent of Potomac horse fever (PHF) and the most recently recognized of the ehrlichial diseases. (Holland et al., 1984, 1985a, 1985b; Rikihisa et al., 1984; Rikihisa & Perry, 1984, 1985). It was first recognized in Maryland in 1979 (Knowles et al., 1984), mainly in horses kept in the region adjacent to the

Potomac river. Infections often resulted in severe diarrhea and death. The relatively sudden appearance of PHF in the high density horse population of Maryland resulted in numerous investigations to determine its epidemiology and etiology. Within a very short time it was found to be caused by a rickettsial organism (Holland et al., 1984, Rikihisa et al., 1984, Rikihisa & Perry, 1984) and was subsequently named E. risticii (Holland et al., 1985).

Potomac horse fever is a naturally acquired disease in horses only. The clinical signs of PHF are variable with fever, depression and anorexia being the most common signs. Diarrhea and laminitis are frequently seen and can be so severe as to result in death.

Ehrlichia risticii can be found in glandular epithelial cells, macrophages and mast cells of the large colon. They are also found in monocytes in the peripheral blood of infected horses but only rarely .

Distribution

Although PHF was first reported in the Potomac region of the eastern United States it has since been reported in over 21 states in the U.S. and in Canada but is thought to be confined to North America. Large numbers of cases have been associated with major river valleys (Palmer et al., 1986) though no substantive proof of this association has been shown.

Vector

The vector of PHF, despite numerous investigations, remains unknown. Even before the rickettsial nature of PHF was known a hematophagous arthropod was suspected of being involved in the transmission. Potomac horse fever is a seasonal disease, with most cases occurring in the

summer months (Perry et al., 1986). It can be transmitted by intravenous inoculation of whole blood from infected animals (Jenny, 1984; Whitlock et al., 1984; Rikihisa et al., 1984; Holland et al., 1984), intravenous and intradermal injection of organism grown in cell culture (Holland et al. 1985; Rikihisa & Perry, 1985; Perry et al., 1985) and by oral inoculation of organism grown in culture (Palmer & Bensen, 1988). There is no evidence that PHF can be transmitted by direct contact. The spatial and temporal pattern of infection are consistent with a biologically transmitted vector-borne disease (Perry et al., 1986).

When the organism was found to be a rickettsia of the genus Ehrlichia, attention was focused on ticks as potential vectors because of their relationship with other rickettsial diseases. Much attention has been focused on Dermacentor variabilis as a potential PHF vector. It was the only tick to be found in the region on horses, in horse stalls and on other domestic and non-domestic animals (Carroll & Schmidtmann, 1986; Fletcher, 1987). Over 600 adult ticks from one PHF endemic area were placed on horses without transmitting disease (Fletcher, 1987; Schmidtmann et al., 1986). Experimental transmission was attempted between mice, from mouse to horse and between horses (Fletcher, 1987; Hahn et al., 1990) without success. Light and electron microscopy were performed on tissues of ticks that had been fed on infected mice, without indication of the organism being established (Hahn et al., 1990). Similar studies on three other species of tick known to be present in the area also met with negative results (Hahn et al., 1990). This failure to provide any evidence, besides circumstantial, that ticks are involved in PHF epidemiology has resulted in the conclusion that it is unlikely that PHF is transmitted by ticks.

Surveys of hematophagous insects on farms in the PHF endemic region of Montgomery county, Maryland, demonstrated that blackflies were the most abundant species of Diptera to parasitize horses (Fletcher, 1987; Schmidtmann et al., 1987; Fletcher et al., 1988). Furthermore the peak of blackfly activity was highly correlated with incidence of PHF during the summer months (Fletcher, 1987). A correlation was also demonstrated between blackfly density and cases of PHF in relation to distance from the Potomac river (Schmidtmann, 1987). This may however be spurious due to the fact that horse density itself is related to distance from the river. Subsequent studies, in which over 5000 blackflies trapped in the Potomac region were homogenized and injected into susceptible mice, have failed to provide further evidence that blackflies are involved in PHF epidemiology (Hahn et al., 1989).

No animals other than horses have been shown to naturally harbor E. risticii. Dogs (Ristic et al., 1988), cats (Dawson et al., 1988), mice (Jenkins et al., 1985) and non-human primates (Stephenson et al., 1985) are susceptible to experimental infection. In study in which animals on a farm in a PHF endemic area were tested for antibodies to E. risticii cats (48 of 102 tested), a goat (1 of 3 tested) and pigs (3 of 14 tested) were found to be seropositive whereas dogs (79 tested), cattle (75 tested) and sheep (7 tested) had insignificant PHF titers (Perry et al., 1989). In another study foxes (2 of 10 tested), dogs (2 of 60 tested) and a rabbit (1 of 35 tested) but not deer (64 tested) were found to be seropositive (Sessions, 1987). House mice (Mus musculus)(28 tested), white-footed mice (Paromyscus leucopus)(40 tested), voles (Microtus pennsylvanicus) (68 tested) and rats (Rattus norvegicus) (130 tested) from farms experiencing PHF in Maryland were all found to be seronegative

(Perry et al., 1989). In a follow-up study an additional 110 white-footed mice were found to be negative (Caroll et al., 1989). Another study of seroepidemiology on two farms in Ohio found some positive white-footed mice (Gordon et al., 1987). Rodents, especially white-footed mice, are frequent larval and nymphal hosts of D. variabilis, and these ticks parasitize dogs much more frequently that they do cats. Combined, this serological data is considered further evidence against the tick D. variabilis as a vector of PHF in the Maryland region.

Fleas (Ctenocephalides felis) were investigated because cats were found to be seropositive to E. risticii, although cat fleas also parasitize dogs. Fleas were not able to transmit the disease between mice or horses (Schmidtmann et al., 1987).

EHRLICHIA EQUI

Ehrlichia equi is the cause of equine ehrlichiosis, a disease of horses only (Gribble, 1969; Stannard et al., 1969).

Equine ehrlichiosis is characterized by fever, anorexia, depression, limb edema, petechiation, ataxia and low mortality (Stannard et al., 1969; Gribble, 1969). Clinical signs in young animals are less severe (Madigan, 1987). Ehrlichia equi is distinguished morphologically from E. risticii by its localization in granulocytes (Gribble, 1969; Stannard & Gribble, 1969).

Distribution

Equine ehrlichiosis was originally described as occurring in the foothill areas of California (Gribble, 1969; Stannard et al., 1969).

Since then it has been reported in a number of states including Florida (Brewer et al., 1984) and New Jersey (Ziemer et al., 1987).

Vector

As with E. risticii the vector of E. equi remains unknown although a tick is suspected. Dermacentor occidentalis, D. albipictus (Gribble, 1969) and Ixodes scapularis (Brewer et al., 1987) have been identified feeding on horses with naturally occurring equine ehrlichiosis. Attempts to transmit the agent with adult D. occidentalis ticks that had fed as nymphs on infected horses and D. albipictus larvae hatched from eggs from adult ticks that had fed on infected horses met with failure (Gribble, 1970). Since ehrlichias are not generally transovarially transmitted, the possible role of D. albipictus deserves further investigation. Madigan (1987, cited as Gribble DH, Kirkland, Wash: Personal communication, 1986) reports transmission trials with "dermacentor ticks" unsuccessful but no details are given.

Host range and reservoirs

The range of mammals susceptible to experimental infection with E. equi is quite large. Burros, sheep, goats, dogs, rhesus macaques and baboons have been shown to be susceptible whereas cattle, rats, mice, guinea pigs, hamsters and rabbits were found to be refractory to infection (Stannard et al., 1969; Gribble, 1969; Lewis et al., 1975).

There has been no screening of other domestic and wild animals for antibodies to E. equi, nor have any animals in the wild other than horses been found infected with E. equi.

It has been generally accepted that there is no carrier state of E. equi in horses and a strong immunity is established after infection, even if antibiotics have been used during the course of the disease (Nyindo et al., 1978). This premise is based on the failure of whole blood and various tissues from convalescent horses to cause disease when inoculated into susceptible animals.

EHRLICHIA BOVIS

Bovine ehrlichiosis is one of the three ehrlichias first reported in North Africa by Donatien and Lestoquard (1936). Bovine ehrlichiosis is considered a relatively mild febrile disease (Norval, 1979) but severe cases have been noted (De Vos, 1981). It is considered a complicating factor of theileriosis and animals experimentally infected with E. bovis are more susceptible to theileriosis and babesiosis (Matson, 1967).

Ehrlichia bovis has a predilection for monocytes and in the discussion of ehrlichial taxonomy has been aligned with E. canis.

Distribution

Unlike E. canis the known distribution of E. bovis is restricted to the African continent and Sri Lanka (Marchette, 1982).

Vector

Ehrlichia bovis was described by Donatien and Lestoquard (1936) as being transmitted by Hyalomma aegyptium. This was based on reports of ticks from Iran sent to Africa and fed on susceptible cattle. Rickettsia-like organisms were then seen in the monocytes of the cattle and a febrile disease with infected monocytes was induced by inoculating whole

blood into additional cattle. Rhipicephalus appendiculatus has been shown to transmit the disease experimentally in southern Africa (Matson, 1967; Norval, 1979). In one study engorged nymphs, collected from cattle in the field, transmitted ehrlichiosis as adults. De Vos (reported as unpublished observations in de Vos, 1981) states that adult R. appendiculatus ticks are "often infected" with E. bovis in the field but does not elaborate on how this was determined.

Host range and reservoirs

Cattle are considered the natural hosts of E. bovis. Sheep develop an inapparent infection but goats and mice are not susceptible (Rioche, 1966).

EHRLICHIA OVINA

The third of the three ehrlichias described by Lestoquard and Donatien in 1936, relatively little is known about E. ovina. Ehrlichia ovina is located in monocytes and is a mild febrile disease of sheep.

Distribution

Ehrlichia ovina has been reported from Africa and Asia (reviewed in Neitz, 1968).

Vector

As pointed out by Marchette (1982) the evidence that this organism is transmitted by ticks is circumstantial or not well documented. Neitz (1956) reports that E. ovina is transmitted by R. evertsi adult ticks that had acquired the infection as larvae and nymphs but provides no

data to support this. The generally accepted belief that E. ovina is transmitted by R. bursa (Scott, 1978) is based on the work by Donatien and Lestoquard in which emulsions of engorged adult female ticks that had fed on infected sheep as nymphs were inoculated into susceptible sheep.

Host range and reservoir

Sheep are the only known host of E. ovina. Infected sheep probably harbor the organism for extended periods of time as in E. canis infection, since splenectomy of apparently immune sheep carried out 18 months following experimental infection, resulted in a relapse of clinical disease (Neitz, 1968).

EHRLICHIA SENNETSU

The disease caused by E. sennetsu, known as human sennetsu rickettsiosis (HSR) is a relatively mild, febrile disease which occurs primarily from August to November (Misao & Kobayashi, 1954; Fukuda, 1958; Hastriter et al., 1987). The organism is morphologically and antigenically similar to E. canis and E. risticii (Ristic et al., 1981; Holien et al., 1982) and is found in monocytes.

Distribution

Ehrlichia sennetsu is endemic to western Japan only (Fukuda, 1958; Misao & Kobayashi, 1955), although sera with antibodies to E. sennetsu have been reported from people in Malaysia (Holland et al., 1985a) An E. sennetsu-like agent has also been isolated from Malaysian patients (Lewis et al., 1985).

Vector

An arthropod vector of E. sennetsu is suspected, due to the seasonal nature of HSF, but has not been identified. Trombiculid mites (Leptotrombiculum fletcheri) were evaluated as potential vectors but found unable to transmit E. sennetsu (Hastriter et al., 1987). In one study raw bora fish were reported to transmit the disease when ingested but these results could not be repeated. Trematodes have been suggested as the vector (Fukuda et al., 1962), but no substantiation of this has yet been made.

Host range and reservoir

Human beings are the only known natural hosts for E. sennetsu.

EHRLICHIA (CYTOCETES) ONDIRI

Ehrlichia (Cytocetes) ondiri is the causative agent of bovine petechial fever (BPF) or Ondiri disease (Haig & Danskin, 1962). Fever, decrease in milk production, edema and petechial and ecchymotic hemorrhages are the most commonly recognized signs of clinical disease. The severity of infection can vary from mild to fatal (Haig & Danskin, 1962).

The organism has a trophism mainly for granulocytes which has led some authors (Krauss et al., 1972) to place it with E. phagocytophila, E. equi and the vole organism C. microti in the genus Cytocetes.

Distribution

Ondiri disease has only been reported from Kenya (Piercy, 1953; Danskin & Burdin, 1963) although its presence is suspected in Tanzania

(Davies, 1980). The remarkable aspect of this disease is its distinctly focal occurrence. It is only found in the Kenyan highlands above 1500 meters. Furthermore, the disease is confined to certain localities, often within a section of a paddock and usually associated with a forest edge or scrub border (Piercy, 1953; Danskin & Burdin, 1963). This focal occurrence is not unknown among the rickettsias. Cases of Rocky Mountain spotted fever occur on one side of a valley but not another. The competitive displacement of R. rickettsii by a second, non-virulent rickettsia in the vector tick is apparently responsible for this phenomenon (Burgdorfer et al., 1981).

Vector

The vector of BPF is unknown. As with PHF the rickettsial nature and epidemiology of the disease have led investigators to assume that an arthropod vector is responsible for the transmission of BPF, although cases are reported from areas where acaracides are used intensively (Davies, 1980). The numerous investigations to identify the vector have been reviewed by Davies (1990). Attempts were made to transmit BPF with R. appendiculatus, R. simus, R. pulchellus, R. evertsi and A. variegatum by feeding nymphs on infected cattle and the adults on sheep (Reports, 1937). Approximately 2600 mosquitoes of 6 different species, 450 blackflies (Simulium spp.) and 600 tabanid flies were collected at a site endemic for the disease and inoculated into susceptible cattle without causing disease (Reports, 1957). Walker et al. (1974) inoculated pools of trombiculid mites (700 total), collected by flagging BPF endemic pastures, into sheep without transmission.

Host range and reservoir

Breeds of cattle (Bos taurus) exotic to Africa are most susceptible to BPF. Sheep and goats in the field are apparently not affected although they are susceptible to experimental infection (Krauss et al., 1972; Danskin & Burdin, 1963). Laboratory mice, guinea-pigs and hamsters are not susceptible to infection (Cooper, 1973). Experimental transmission of BPF can be established by both subcutaneous and intravenous inoculation of whole blood from infected animals but not by mouth. Transmission by direct contact does not occur (Reports 1933).

The organism has been isolated from wild bushbuck (Snodgrass, 1975; Davies, 1980) and cattle, sheep, impala, Thompson gazelle and wildebeest were susceptible to the organism isolated from bushbuck from one BPF endemic area (Snodgrass, 1975). The fact that cattle can contract BPF within 2 weeks of being placed on pasture unused the previous 2 years (Danskin & Burdin, 1962) indicates the presence of a wild mammal/vector cycle which is independent of cattle. The bushbuck is most likely a reservoir for infection to ticks.

NEORICKETTSIA HELMINTHOECA

Neorickettsia helminthoeca is the causative agent of salmon poisoning disease (SPD), a highly fatal disease of dogs (Cordy & Gorham, 1950; Philips et al., 1953). Salmon poisoning disease, unique among all rickettsial diseases due to its transmission by a trematode, is reviewed by Philips (1955) and Milleman and Knapp (1970). The organism infects the lymphoid cells of nodes and other tissues and may be found in circulating monocytes.

Distribution

The disease is only seen in the Pacific northwestern US. The distribution of SPD is limited by the range of the snail Oxytrema (Gonobias) silica, the first intermediate host of the trematode vector. Dogs have been known to contract the disease outside this range when ingesting ocean or river salmon (Milleman & Knapp, 1970).

Vector

Dogs contract SPD by ingesting Nanophyetus salmincola, a trematode of fish-eating mammals. The encysted trematode is found in the kidneys, muscle and fins of fish of the salmon family and so is commonly fed to dogs along with the remains of fish cleaning. The life-cycle of the trematode is completed when it is ingested by a number of fish-eating birds and other non-canine mammals.

Neorickettsia helminthoeca has been found in the fluke eggs, indicating that transovarial transmission occurs (Nyberg et al., 1967).

Neorickettsia helminthoeca has been experimentally transmitted by R. sanguineus (Philip et al., 1954), although naturally infected ticks have never been found.

Host range and reservoir

The fact that SPD is a highly fatal disease in dogs suggests that the association between dogs and N. helminthoeca is a relatively recent one (Marchette, 1982). Raccoons, mink, cats, bobcats, bear, guinea pigs, hamsters and mice are all refractory to experimental infection (Simms et al., 1932; Cordy & Gorham, 1950; Philip et al., 1954). As transovarial

transmission in the trematode does occur this organism is most likely obligate with the trematode, not requiring the dog host at all.

Table 1.

Order Rickettsiales as presented in Bergey's Manual 1984.

Order Rickettsiales

Family Rickettsiaceae

Tribe Rickettsieae

Genus	*Rickettsia*
Genus	*Coxiella*
Genus	*Rochalimeae*

Tribe Wolbachieae

Genus	*Wolbachia*
Genus	*Rickettsiella*

Tribe Ehrlichieae

Genus	*Ehrlichia*
Genus	*Cowdria*
Genus	*Neorickettsia*

Family Bartonellaceae

Genus	*Bartonella*
Genus	*Grahamella*

Family Anaplasmataceae

Genus	*Anaplasma*
Genus	*Aegyptionella*
Genus	*Haemobartonella*
Genus	*Eperythrozoon*

Table 2. Summary of characteristics relating to the transmission of Ehrlichieae.

	E. canis	*E. risticci*	*E. bovis* *E. ovis*	*E. sennetsu*	*E. phagocytophila*	*E. equi*	*C. ondiri*	*C. ruminantium*	*N. helmin thoeca*
Cell trophism	mono-cytes	mono-cytes	mono-cytes	mono-cytes	granulo-cytes	granulo-cytes	granulo-cytes	neutro-phils	lympho-cytes
Distribution	World wide	North America	Africa	Japan	Europe	North America	Kenya	Africa	North America
Vector	*R. sanguineus*	unknown	*Rhipicephalus*	unknown	*Ixodes ricinus*	unknown	unknown	*Amblyomma* spp.	*N. salmincola*
Natural host	dogs	horses	cattle sheep	humans	ruminants	horses	ruminants	ruminants	dogs
Reservoir	dogs ticks	unknown	cattle	unknown	ruminants	unknown	bushbuck	buffalo* ticks cattle* sheep*	trematodes
Transovarial transmission	no	unknown	no	unknown	no	unknown	unknown	rarely	yes
Carrier	yes	no	no	no	yes	no	yes	yes	no
Lab animals susceptible	no	yes	?	?	no+	no	no	no+	no

\+ laboratory rodents susceptible to adapted strains
* determined in experimentally infected animals

CHAPTER 2.

The attempted transmission of Ehrlichia risticii, causative agent of Potomac horse fever, with Dermacentor variabilis, Rhipicephalus sanguineus, Amblyomma americanum and Ixodes scapularis.

INTRODUCTION

As soon as the epidemiological investigations demonstrated that PHF was seasonal and infectious but not contagious (Perry et al., 1986), a search for a hematophagous arthropod vector began. When the causal organism was determined to be a rickettsia of the genus Ehrlichia, attention was immediately focused on ticks as potential vectors.

Surveys of ticks on farms experiencing PHF demonstrated that the three-host tick Dermacentor variabilis was the only adult tick to be found on repeated inspection of horses, horse stalls and other domestic and non-domestic animals (Carroll & Schmidtmann, 1986; Fletcher, 1987). Based on the results of studies of the population dynamics of arthropod species in one PHF endemic area (Fletcher, 1987) and the fact that D. variabilis is a vector of Rocky Mountain spotted fever, another rickettsial disease in the eastern and southeastern United States (Burgdorfer, 1975), D. variabilis was considered the most likely candidate vector. However, a series of laboratory and field studies have subsequently failed to incriminate D. variabilis as the vector of E. risticii (Schmidtmann, 1986; Fletcher, 1987). In the first of these, adult D. variabilis ticks were collected from pastures on PHF endemic farms and fed on susceptible ponies without subsequent disease transmission

(Schmidtmann, 1986; Fletcher, 1987). Laboratory transmission by ticks was then attempted between mice, from mouse to horse and between horses (Fletcher, 1987). No clinical evidence of disease transmission was observed in either of these trials. However, the numbers of ticks feeding to repletion were sometimes low and in the mouse study serology was not performed on the tick-challenged mice (Fletcher, 1987).

Previous tick surveys of the Maryland region have shown that other ticks are present in the area besides D. variabilis. These include Amblyomma americanum, Dermacentor albipictus, Ixodes scapularis and Rhipicephalus sanguineus (Strickland et al., 1976). All these ticks are known to transmit other rickettsial or bacterial diseases. Dermacentor albipictus and adult I. scapularis are not active during the summer months when PHF is known to occur. Larval and nymphal forms of I. scapularis are, however, active in the summer (Strickland et al., 1976) and therefor are potential PHF vectors.

The laboratory mouse has been shown to be a good model for PHF (Jenkins et al., 1985; Fletcher, 1987; Rikihisa et al., 1987). Balb-C and Sprague-Dawley CF1 mice show clinical signs of the disease including lethargy, squinty eyes, rough hair coats and hunched backs 10 to 12 days after experimental infection. They consistently produce antibodies to E. risticii, detectable by indirect fluorescent antibody test (IFAT) (Rikihisa & Perry, 1985; Ristic et al., 1986) by 21 days post experimental communication (RM Rice, personal communication, 1987). Studies on the availability of E. risticii in the circulation during experimental infection have also shown that the peak rickettsemia in Sprague-Dawley CF1 mice occurs between days 8 and 10 post-inoculation (Fletcher et al.,

1987). In these studies clinical signs of disease were not observed until day 11 of infection in CF-1 mice.

In this study transstadial transmission of E. risticii was attempted using nymphal and adult D. variabilis and R. sanguineus that had been exposed to E. risticii infected mice in previous instars. In addition, similarly exposed nymphal stages of I. scapularis and adult A. americanum were investigated.

MATERIALS AND METHODS

Ticks:

Long-established colonies of D. variabilis, R. sanguineus, A. americanum and I. scapularis ticks were used. At all times, except when on animals, they were kept at 90% humidity and 25° C.

Ponies:

Ponies were purchased from local auctions in southwestern Virginia, vaccinated for eastern equine encephalitis, western equine encephalitis, equine influenza, rhinopneumonitis and tetanus and treated with anthelminthic six weeks prior to the experiment. Prior to this study all ponies were tested by IFAT for antibodies to E. risticii and found to be negative.

Mice:

An outbred strain of Sprague-Dawley CF1 mice (Harlan Sprague-Dawley, Indianapolis, Indiana) were used. The mice were all female and aged between 6 and 8 weeks at the time of tick feeding or infection with E. risticii.

Tick feeding procedures and transmission studies:

Fifteen mice were inoculated intraperitoneally with yolk sac culture suspension (Oaks et al., 1977)(20% yolk sac/SPG buffer) of E. risticii. The suspension was derived from yolk sacs inoculated with blood from a clinically ill horse (RM Rice, personal communication, 1987). After 9 days post-injection the mice were killed and 0.5 ml of whole blood was taken from each mouse. These samples were pooled and diluted 1:10 with Hanks balanced salt solution (BSS) (GIBCO Laboratories, Grand Island, N.Y.) One ml. of this pooled, diluted sample was then injected intraperitoneally into each of 45 susceptible mice.

Mice were placed in wire restraint cages (Fletcher, 1987). At day 4 post-inoculation D. variabilis, R. sanguineus and A. americanum nymphs were placed on mice (40 nymphs per mouse, 10 mice per tick species). At day 5 post-inoculation D. variabilis, R. sanguineus and I. scapularis larvae were placed on mice (approximately 200 larvae per mouse, five mice per tick species). Larval and nymphal D. variabilis and R. sanguineus were similarly placed on uninfected mice as controls. Mice were kept in the restraint cages for 12 hours after tick infestation before being put into standard laboratory mouse cages equipped with wire bottoms. They were given food and water ad libitum. Ticks were allowed to engorge and drop off. Then they were placed in incubators as described above and allowed to molt. The mice were killed four weeks post-inoculation and whole blood was examined for antibodies to E. risticii by IFA (Rikihisa & Perry, 1985; Ristic et al., 1986; all serology was performed by Dr. Robert Rice, Walter Reed Army Institute, Rickettsial Diseases Research).

The resulting nymphs and adults were used for subsequent transmission studies 6-8 weeks post-molt (see Tables 3-6). Since successful infection

of these ticks with E. risticii was unproven at this stage the term "exposed" will be used to describe a tick that fed upon E. risticii infected mice in a previous instar. The exposed D. variabilis and R. sanguineus nymphs were allowed to feed on susceptible mice. Table 1 shows that only 3 D. variabilis nymphs were still alive 8 weeks post-molt although over 500 larvae were recovered and were alive 4 weeks post-molt. The ticks were allowed to feed to repletion and drop off. The numbers of exposed nymphs fed on and recovered from susceptible mice is presented in Table 3. Between 4 and 6 weeks post-feeding the mice were killed and samples of whole blood were tested by IFAT for antibodies to E. risticii.

The exposed adult ticks were fed on ponies, with one pony being used for each tick species. Exposed I. scapularis nymphs were also fed on a pony. Ticks were placed on two shaved patches on the pony's shoulders and contained by stockinette as previously described (Fletcher, 1977). The ticks were allowed to feed to repletion and drop off. The numbers of ticks placed on and recovered from ponies are shown in Tables 5 and 6. The ponies were monitored by daily rectal temperature and whole blood was drawn 3 times weekly for a period of 8 weeks. The whole blood was separated and the sera were tested for antibodies to E. risticii by IFAT.

Eight weeks after all ticks were off the ponies were challenged with E. risticii that had been cultured in mouse macrophages (provided by Dr. Wayne Roberts, University of Kentucky, Livestock Disease Center, Lexington KY).

Detection of rickettsia in ticks:

The detection of E. risticii in ticks was attempted in 2 ways;

1. Dermacentor variabilis and R. sanguineus larvae and D. variabilis nymphs were injected into susceptible mice 24 hours post repletion in the following manner; the blood meal and internal organs of 50 D. variabilis larvae were removed by decapitating the larvae and pushing the contents out the resulting opening. This material was then diluted with equal parts horse serum (GIBCO) and Hanks BSS resulting in a total volume of 2.5 mls. One-half ml of the mixture was then injected intraperitoneally into each of five mice. Five D. variabilis nymphs and 25 R. sanguineus larvae were each similarly inoculated into 5 mice. The mice were followed clinically for four weeks at which time they were killed. Whole blood was taken from each mouse, an approximately 1.5 cm. blot of whole blood was put on filter paper and analyzed for antibodies to E. risticii by IFAT.

2. Four to five weeks after molting exposed D. variabilis and R. sanguineus adults and nymphs (20 of each) were dissected and processed for transmission electron microscopy (TEM). Ten individuals of each group were incubated at 37° C for 48 hours prior to dissection. Whole guts and salivary glans were removed and placed immediately in cold 2% glutaraldehyde in a 0.2M sodium cacodylate buffer with 2% sucrose, postfixed in 2% osmium tetroxide in the same buffer, and processed for electron microscopy according to the procedure of Kocan et al. (1978). Thick sections (1 um) were cut with a microtome, stained at 60° C with Mallory's stain for 2 minutes and viewed by light microscopy. Ultrathin (silver-reflective) sections were cut with an ultramicrotome, collected

on 200 mesh copper grids, stained with uranyl acetate and lead citrate and viewed with an electron microscope (JEM 100 CYSTEM).

RESULTS

Transmission studies:

The numbers of replete larvae, nymphs and adults recovered from mice and ponies are presented in Tables 3-6. All larvae and nymphs had dropped off between days 8 and 10 post-inoculation. All the mice that were inoculated with whole blood from E. risticii infected mice developed antibodies to E. risticii and the clinical signs of E risticii infection in mice described above. In all cases clinical signs developed after the ticks had dropped off, which was consistent with previous experiments (Fletcher et al., 1990). Death occurred in some mice but it was always four to five days after the onset of clinical signs. Control mice did not develop clinical signs of disease or antibodies to E. risticii.

No signs of clinical disease were seen in any mouse or pony except pony "G", that had been fed on by exposed R. sanguineus. This pony became clinically ill at about six weeks post-infection and died. However, no serological evidence of exposure to E. risticii was present and the postmortem lesions were not consistent with those of PHF. Death was attributed to other causes.

It should be noted that although 100 I. scapularis nymphs were placed on a pony (Table 6), none were actually recovered. These nymphs were seen to feed and engorge. Because of their small size and the fact that the adhesive holding the stockinette was irritating to this particular pony, causing her to bite at the stockinette, the nymphs were lost as they dropped off.

There was no evidence of antibodies to E. risticii in whole blood samples from mice that were fed on by exposed ticks or serum samples from ponies tested by IFAT.

Ponies that were challenged after being fed on by exposed ticks developed clinical signs of PHF, including fever, depression and anorexia within two weeks of inoculation. The animals were euthanized and postmortem lesions were compatible with those of PHF.

Detection of rickettsia in ticks:

All five of the mice that were injected with contents of replete D. variabilis nymphs developed clinical signs of E. risticii infection and also developed antibodies to E. risticii. Inoculation of replete D. variabilis and R. sanguineus larvae did not result in clinical disease or E. risticii antibody production.

No rickettsial organisms resembling E. risticii were seen in either D. variabilis or R. sanguineus nymphs or adults when examined by light and electron microscopy. Rickettsia-like organisms were seen in the ovaries of both species of adult ticks, however their structure was consistent in appearance with that of the symbiotic rickettsia that have been described in these ticks (Hayes & Burgdorfer, 1981).

DISCUSSION

To date the candidate vectors of PHF that have received the most attention are undoubtedly ticks. However, the evidence of this vector ability is so far only circumstantial. Despite repeated attempts of tick transmission, there is no evidence that D. variabilis, or any other

tick, naturally harbors, or is able under laboratory conditions, to transmit E. risticii.

Seroepidemiological evidence does not support ticks, most notably D. variabilis as the vector of PHF. Dermacentor variabilis is frequently found in high numbers on dogs in PHF endemic areas. Dogs, when experimentally infected with E. risticii have been shown to seroconvert but not show signs of clinical disease (Ristic et al., 1988). The is one field report of dogs being seropositive to E. risticii (Sessions, 1987) but other investigators have failed to identify positive dogs in the Maryland region where PHF is endemic (Perry et al., 1989). Small rodents such as the white-footed mouse, meadow vole and house mouse are the most common hosts of immature stages of D. variabilis. Surveys of rodents in the PHF endemic region of Maryland have failed to discover any seropositive rodents out of 260 sampled (Perry et al., 1989). White-footed mice and meadow voles, in a region of Connecticut that has had many cases of RMSF, have been reported to having a 16.7 to 21.2% prevalence of antibodies to Rickettsia rickettsii (Magnarelli et al., 1981; 1983) and may be considered a good indicator species of rickettsial activity in the D. variabilis ecosystem (Perry, 1987). Many cats on PHF endemic farms, surprisingly , have antibodies to E. risticii (Perry et al., 1989) but D. variabilis are infrequent parasites of cats. Dermacentor variabilis nymphs are apparently able to take up E. risticii from experimentally infected mice as is evidenced by the serological and clinical response of the mice to injection of replete nymphs. However, E. risticii is apparently, under the conditions of this experiment, unable to survive in the tick since no evidence of its presence could be found in exposed D. variabilis adults, either by transmission studies or TEM. No evidence

has been produced so far that indicates that D. variabilis is able to transmit PHF under experimental conditions. Combined with the seroepidemiological data, the conclusion can be made that D. variabilis is an unlikely vector of PHF.

A potential problem with the methods used in this study is the strain of E. risticii used to infect the mice. The organism that was used was grown in egg yolk sac culture. There are well known circumstances in related disease agents where culture results in loss of their original characteristics (Uilenberg, 1983a), i.e. an organism that was originally highly pathogenic to its vertebrate host when grown in culture or passaged in laboratory animals loses that pathogenicity or ability to be transmitted by a vector. Potentially this could account for the failure of the E. risticii used in this study to be transmitted. Although this is possible, it is unlikely as the clinical response, when injected into both mice and horses is identical to that of organism derived freshly from clinically ill horses. Furthermore E. risticii strain differences in horses have yet been shown to occur. The transmission studies of Fletcher (1987) were done with an organism freshly derived from whole blood from a horse clinically ill with PHF.

The recovery of engorged exposed D. variabilis females from ponies was low (14.3%) and the mortality of exposed pre-fed nymphs was high (almost 100%). Fletcher (1987) also reported poor success with feeding exposed adult female D. variabilis ticks and suggested that it might be due to the lack of proper photoperiod. Dermacentor variabilis adults normally feed in the summer, whereas the transmission study of Fletcher (1987) was carried out in the winter (albeit in heated stalls). This could be a potential problem in the present study since it was carried

out during October and November. This would not, however, explain the high mortality rate among exposed nymphs. Possibly there was an adverse affect of the rickettsia on the ticks themselves that resulted in the reduced performance. This poor performance was not seen in the other species of ticks which were kept under identical conditions. Colony and control D. variabilis nymphs kept in the same incubator also did not experience high mortality. The R. sanguineus and A. americanum adults, which are also normally active during the summer months and were fed and housed under the same conditions as the D. variabilis adults successfully fed to repletion (60% and 33.3%, respectively).

The negative data presented for the other ticks also indicates that they are unlikely vectors of PHF. The evidence against D. variabilis as a vector of PHF has come from a variety of sources but the only evidence against the other species of ticks discussed is that presented in this paper. Although D. variabilis was the only adult tick found on horses, this does not rule out the possibility that nymphal or larval ticks were present on animals but not see. It may be that a situation exists with PHF where the "normal" host is another animal, as yet unidentified, and the horse (and laboratory mouse) is an accidental, dead-end host. If this were the case then the only way to demonstrate transmission in the laboratory would be to identify the natural reservoir host and use that animal for transmission studies.

Table 3.

Numbers of larval and nymphal ticks recovered from feeding on mice infected with *Ehrlichia risticii*

Tick	No. larvae recovered	No. nymphs recovered
D. variabilis	> 500*	155
R. sanguineus	> 200	110

* over 500 larvae were recovered, but all except 3 were dead by 8 weeks post-molting.

Table 4.

Numbers of exposed nymphs placed on and recovered from susceptible mice.

Tick	No. nymphs placed on mice	No. nymphs recovered
D. variabilis	3	3
R. sanguineus	50	15

Table 5.

Numbers of exposed adult ticks placed on and recovered from susceptible ponies.

Tick	No. males placed on pony	No. females on pony	No. engorged females recovered
D. variabilis	43	35	5
R. sanguineus	20	10	6
A. sanguineus	5	15	5

Table 6.

Numbers of exposed I. scapularis nymphs placed on and recovered from a pony.

Tick	No. nymphs placed on pony	No. nymphs recovered
I. scapularis	100	none*

*see text

CHAPTER 3.

The role of blackflies in the epidemiology of Potomac horse fever.

INTRODUCTION

Ticks were initially the prime vector candidates of PHF owing to their role in the transmission of other rickettsial diseases. However, all attempts to demonstrate any association between PHF and the ticks known to inhabit one PHF endemic area have met with failure.

Studies in the endemically infected region of Montgomery County, Maryland showed that blackflies were the most abundant insect species feeding on horses; of these Simulium jenningsi comprised approximately 99 per cent and S. vittatum 1 per cent of the population (Fletcher, 1987; Schmidtmann, 1987). Blackflies are not known to transmit rickettsial organisms, but they are the vectors of Onchocerca volvulus in humans and of Leucocytozoon species in birds. Blackflies breed in running water, and an association between PHF and large rivers has been reported in some parts of the USA (Palmer et al., 1986). Furthermore, it has been observed that the incidence of the disease corresponds with the recorded blackfly density; the number of cases of PHF and the number of blackflies both decrease with increasing distance from the Potomac river (Schmidtmann et al., 1987). However the horse population is not evenly distributed in the area and this association may be spurious. This objective of this study was to further investigate the role of blackflies in the epidemiology of PHF.

MATERIALS AND METHODS

Blackflies were collected on three farms in Montgomery County, maryland during May to August, 1987, by two different methods. The first was a simple hand-held, battery-operated vacuum aspirator equipped with a collection bag. A horse was tethered outside the stables and blackflies were drawn into the vacuum as they landed on the horse. The second method used a simulated-ear-insect-trap (Schmidtmann, 1987). This trap consisted of a hollow styrofoam horse head with a felt "ear" that was placed on a tripod approximately 1.2 m above the ground and filled with dry ice. The blackflies were attracted to the simulated ear and the vaporized dry ice as it leaked out of the container. Blackflies were caught in a sweep net as they swarmed around the net. Both methods of trapping were used daily when weather permitted.

The trapped blackflies were stored immediately in liquid nitrogen. They were homogenized manually in a 50:50 mixture of horse serum (GIBCO laboratories) and Hanks BSS. An initial homogenate of 90 blackflies/ml was made and diluted in Hanks BSS to give homogenates of 72, 54, 36, and 7.2 blackflies/ml. One ml of each homogenate was then injected intraperitoneally into groups of 5 mice. Mice which received 72 flies or more died of peritonitis or were moribund within 24 hours, whereas 15 mice which received 54 or fewer flies did not. Samples containing approximately 50 flies were subsequently injected intraperitoneally into 99 Sprague-Dawley CF1 mice, none of which showed signs of peritonitis.

Two sets of controls were used. In the first, mice were injected with blackfly homogenates mixed with E. risticii. The organism was derived from pooled blood samples from three mice taken nine days after infection with E. risticii grown in embryonated egg yolk sac culture

(Fletcher et al., 1990). The pooled blood was diluted 1:10 and 1:100 in Hanks BSS and 1 ml samples of each dilution were injected intraperitoneally into five mice. A second set of controls was inoculated with E. risticii infected whole blood alone at the same dilution using five mice per dilution. The response to E. risticii was measured by the number of deaths within ten days after inoculation or by seroconversion. Four to six weeks after inoculation the surviving mice were killed by cervical dislocation and a drop of blood was taken from the axilla, placed on filter paper and stored at -70°C. These samples were then tested for antibodies to E. risticii by the indirect immunofluorescent antibody test. A titer of 1:40 or greater was considered positive.

RESULTS

The total number of blackflies injected into mice was 5385 (822 trapped by vacuum and 4563 by the ear-trap). All mice in both control groups showed evidence of E. risticii infection (Table 7). None of the 99 mice died or seroconverted. One of the five mice that were inoculated with 7.2 blackflies per mouse developed antibodies to E. risticii. All others were negative.

DISCUSSION

The detection of E. specific antibodies in only one of 114 mice is difficult to interpret but was probably a non-specific false positive. Supporting evidence is provided by the fact that the positive mouse was inoculated with a solution containing 7.2 flies/ml diluted from a homogenate of 90 flies/ml. None of the mice receiving more than 7.2 flies/ml

seroconverted. In addition, it was the only mouse of a cohort of five to have a positive reaction.

The data presented in this paper do not support the seasonal and geographical evidence that blackflies are directly involved in the transmission of Potomac horse fever. However, it is possible that if E. risticii was transmitted by S. vittatum, an insufficient number of blackflies was caught to detect infection. If it is assumed that one infected fly can cause disease when inoculated into a mouse, that the IFAT has a high sensitivity and that 1 per cent of the blackfly population is S. vittatum, a sample of 50 blackflies would theoretically detect a 1 per cent or greater infection rate of E. risticii in the vector population with a 99 per cent level of confidence (Cannon & Roe, 1982). Using the same assumptions, a sample of 5000 S. jenningsi should be able to detect a 0.1 per cent or greater infection rate with 99 per cent certainty. It is extremely difficult to assess the validity of these assumptions, but if they are accepted it can be concluded that it is unlikely that the blackfly is the biological vector of PHF.

Table 7.

Response of control mice inoculated intraperitoneally with E. risticii alone or in blackfly homogenates.

	E. risticii alone		E. risticii and blackfly homogenate	
Dilution	1:10	1:100	1:10	1:100
Number of deaths*	3	1	3	2
Number of seroconversions	2	4	2	3
Total mice	5	5	5	5

* Mortality measured 10 days after inoculation

CHAPTER 4.

Variation in infection rates of two laboratory colonies of Amblyomma variegatum ticks experimentally infected with Cowdria ruminantium.

INTRODUCTION

Heartwater is an often fatal rickettsial disease of wild and domestic ruminants that is caused by Cowdria ruminantium and transmitted by several tick species of the genus Amblyomma. The disease has been well known for over a century in Africa where it limits livestock production. Heartwater is a problem in exotic as well as indigenous stock when naive animals are introduced into an endemic area (Provost & Bezuidenhout, 1987). Its recent introduction into the Caribbean presents the serious possibility of the introduction of heartwater onto the North American mainland where potential vectors are already established (Uilenberg, 1982a; Barre et al., 1987).

In Africa the major known vectors of heartwater are A. hebraeum and A. variegatum (Bezuidenhout, 1987). Cowdria ruminantium has been detected in ticks by a number of methods including light and electron microscopy (Cowdry, 1925b; Bezuidenhout, 1984; Kocan et al., 1987a; 1987b; Yunker et al., 1987), feeding of suspect ticks on susceptible ruminants (Norval et al., 1990), inoculation of emulsified ticks into ruminants (Camus & Barre, 1987) and inoculation of emulsified ticks into mice followed by detection of antibodies to Cowdria (Du Plessis, 1985; Du Plessis & Malan, 1987; Camus & Barre, 1987).

Rates of infection in ticks collected in heartwater endemic areas vary greatly from 1-2% (Camus & Barre, 1987) to 0.0-44.9% (Norval et al., 1990). It is difficult to compare directly these infection rates since the methods of detection were different. The relatively high infection rates of up to 44.9% were from experiments using field ticks fed in pools on susceptible ruminants, whereas the other studies used an indirect method in which the serological response of mice was measured following their inoculation with macerated ticks. The assays that used ruminants exposed to tick inoculation or feeding to determine infectivity might be much more sensitive than the others. If differences in infection rates exist, it could reflect regional differences in the vector competency of individual Amblyomma sp., or some factor that results in different levels of exposure of C. ruminantium to ticks. Regional differences have been noted in the proportion of animals having antibodies to C. ruminantium and this is independent of the numbers of ticks infesting the animals (Camus & Barre, 1987). It was suggested that these regional variations might be due to differences in some unidentified environmental factor, resulting in differing infectivity of the ticks for ruminants.

In this study two laboratory colonies of A. variegatum ticks were compared for their ability to acquire and transmit infection with C. ruminantium. The two tick colonies were identified as being different during unpublished studies into the development of C. ruminantium in the salivary glands of A. variegatum ticks (Hahn, Kocan, Waghela, 1989, unpublished data) when it was observed with LM that colonies of C. ruminantium were present in lower numbers than had been described by other authors. The objective of this study was to confirm those observations and quantify infection rates in these two groups of ticks.

MATERIALS AND METHODS

Ticks

Ticks were from established colonies from two laboratories in Kenya. The first colony was from the National Veterinary Research Centre, Kenya Agricultural Research Institute (KARI), Kabete, where all stages were maintained on goats and in incubators at 28° C and 80% humidity. The second colony was from the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, where the ticks were maintained similarly, except that immatures (larvae and nymphs) were fed on rabbits. The ILRAD ticks were descended from specimens collected from Kiswani, Kilifi district, Coast Province, Kenya, in 1985. The KARI ticks were from Narok district, Kenya and had been in colony since 1985.

Dissection, preparation and microscopic examination.

The ticks were dissected in cold, phosphate-buffered saline. Guts were placed immediately in a cold mixture of 2.5% glutaraldehyde, 2.0% formaldehyde and 0.03% picric acid in 0.1M phosphate buffer, pH 7.4. The samples were post-fixed in 1.0% osmium tetroxide in 0.1M cacodylate buffer, pH 7.4, and stained en bloc in 1.0% uranyl acetate in maleate buffer, pH 5.2. The tissues were dehydrated in a graded series of ethanols and embedded in an Epon-Araldite mixture. No attempt was made to differentiate anatomic sections of tick guts. Thick sections (1 um) were cut on a microtome, stained with Mallory's stain (Richardson et al., 1960) and viewed with a light microscope. Sections were scored in one of three ways; no colonies seen, <10% infected (based on colonies/gut epithelial cell nucleus), or >10% infected.

Cowdria ruminantium acquisition and transmission.

Experimental animal were raised in tick-free conditions. Nymphs from both colonies were fed simultaneously on a goat that been inoculated with a blood stabilate of C. ruminantium from the Kilifi district of Kenya (Kocan et al., 1987b). Engorged nymphs that were replete from feeding on three consecutive days of the febrile response were maintained under identical conditions until molting, then used for the following procedures.

Four weeks after molting adults were treated in one of the following ways.

1. Eight pairs of both ILRAD and KARI ticks from the first of the three repletion days and five pairs from the second and third repletion day (total 36 ticks from each laboratory colony) were dissected and the guts prepared for microscopic examination.

2. Two exposed male ticks from each of the colonies and the same repletion day were placed on each of four goats in shaved areas contained within cloth patches. If males had not attached 5-7 days after placement in the patch two additional male ticks from the same repletion day were placed in the patch. Two females from the same colony and repletion day were placed in the patch 5-7 days after one or both of the males were seen to attach. The goats were monitored daily for rectal temperatures clinical signs of heartwater. Goats that did not die in the acute phase of the disease were euthanized when moribund or three days after the

peak of rectal temperature. Brain smears were made from all the goats, stained with Giemsa and examined with a light microscope for C. ruminantium.

Statistical analysis

The Chi-square test of homogeneity was used to determine group differences in the number of tick guts in which C. ruminantium were seen. The Fisher exact test was used to determine group differences in the proportion of ticks with greater or less than 10% infected gut epithelial cells and the attachment performance of male and female ticks.

RESULTS

The results of the microscopic examination of tick guts for infection with C. ruminantium are presented in Table 8. A significantly higher number of the ILRAD ticks were infected as compared to the KARI ticks ($P< 0.05$). The ILRAD ticks, when infected, were consistently >10% infected, whereas the KARI ticks were consistently <10% infected ($P< 0.05$).

The results of the transmission of heartwater to goats by infected ticks are presented in Table 9. No significant difference was seen in the ability of the two ticks colonies to transmit heartwater. The prepatent period was the same for both groups of ticks, although one goat receiving KARI ticks responded after 30 days. The performance of the two colonies of ticks differed in that males from the ILRAD colony attached earlier and in greater numbers than their KARI counterparts ($P<0.05$). The numbers of female ticks attached were too small for meaningful evaluation.

DISCUSSION

These results show a significant difference between the two colonies of laboratory raised A. variegatum ticks. Although group differences were seen in the numbers and degree of gut infection and in the feeding performance of the adult ticks, this did not reflect differences in the ability of the ticks to successfully transmit the disease. Single infected male ticks were capable of transmitting C. ruminantium in four of the goats. These results support those of other investigators which have demonstrated that one infected nymph (Neitz 1971), one infected adult female (Ilemobade & Leeflang, 1978) and single nymphs and adult males and females (Norval et al., 1990) are capable of transmitting C. ruminantium.

It can not be determined from these results if similar differences may exist in field collected ticks or if the observed differences are the result of different circumstances of colony maintenance between the two laboratories. Colony propagation may have resulted in selection that has changed one or both of these groups of ticks. Differences in colony-bred ticks have been noted by other authors. In an investigation into the competency of A. americanum ticks to transmit Borrelia burgdorferi one group of ticks was able to acquire the spirochete as nymphs whereas the other was not (neither was able to transmit as adults) (Piesman & Sinsky, 1988). Loss of performance of colony bred ticks has also been noted. Boophilus sp. ticks that had been in a colony over a number of years were compared to ticks of the same species which had been freshly collected from the field and were shown to have reduced vitallity as measured by maturation time, weight, fecundity and fertility. No explanation was given for these differences but it was suggested that they could be

attributed to selection pressures imposed by laboratory maintenance (Stewart et al., 1982).

A valuable follow-up study would be to collect ticks from the various regions of Africa where heartwater occurs and perform similar comparisons to determine if such differences can be found in field collected ticks. If differences exist it could explain differences in regional tick infection rates.

Yunker et al. (1987) suggested that infection rates of field ticks be determined by the examination of individual ticks by light microscopy. In these experiments ticks in which no organisms were seen were likely to be infected, as indicated by the high degree of competency in the transmission of disease by the ticks. These results therefore indicate that the direct observation of colonies of C. ruminantium is not an accurate way of determining tick infection rates. More sensitive and specific methods of detection of Cowdria, such as a DNA probe, are desirable to address the question of tick infection rates in heartwater endemic areas.

Table 8.

Infection of A. variegatum gut epithelial cells with C. ruminantium.

Laboratory	Repletion day	No. ticks examined	<10% infected*	>10% infected	none seen
KARI	3/1/89	13	3	0	10
KARI	3/2/89	10	2	0	8
KARI	3/3/89	10	4	0	6
total		36	9(25%)	0	27
ILRAD	3/1/89	13	0	7	6
ILRAD	3/2/89	10	0	7	3
ILRAD	3/3/89	10	0	3	7
total		36	0	17(47%)	19

* the percent infection is determined by comparing number of colonies per 100 gut epithelial cell nuclei.

Table 9.

Reaction of goats following attachment of A. variegatum ticks infected with C. ruminantium.

Lab	Goat #	Attachment date Males/Females	Days to >39.5 C Rectal Temp. Males/Females*	Males/ Females Attached	Number of male ticks placed on goat
ILRAD	4238	5-24/5-30	20/14	2/1	2
ILRAD	4272	5-24/6-7	17/3	2/1	2
ILRAD	4201	5-24/ **	14	2	2
ILRAD	4235	5-24/5/30	15/8	1/1	2 (8)
KARI	4295	6-1/ **	17	1	4
KARI	4236	6-6/ **	30	2	6
KARI	4284	5-24/5-30	19/3	1/1	2
KARI	2518	5-24/ **	16	1	2 (14)

* Brain smears were positive for Cowdria in all goats.

** Females had not attached by the time of the febrile response.

Conclusions

The three studies described in this dissertation contribute to the knowledge of the epidemiology heartwater and PHF. They illustrate that despite years of research on ehrlichial diseases, countless unsolved questions remain, ranging from the basic; identifying of the vector E. risticii, to the specific; why two groups of A. variegatum ticks differ in their ability to maintain infection with C. ruminantium.

The A. variegatum studies may have implications relating to PHF as well as other ehrlichial and unrelated tick-borne diseases. Vector-borne diseases are usually investigated using colonies of arthropods that have been in culture over a number of years. Often the arthropods are raised on animals which are not their preferred hosts. The results of the Cowdria experiment, which indicates different biological activity in two groups of laboratory raised ticks, might also be found in studies of vectors of other diseases.

The two colonies of A. variegatum ticks may also provide a model to investigate the mechanism of pathogen uptake by a vector. If a difference could be identified, for instance, in the gut membranes of these two groups of ticks it could give a clue as to why one tick is competent as vector for a given pathogen while another is not. In the context of these experiments it might provide an indication of why D. variabilis nymphs were able to ingest E. risticii but the organisms did not survive molting.

In this dissertation I have attempted to answer unresolved questions about how two closely related pathogens, E. risticii and C. ruminantium

are transmitted. I have documented that different laboratory colonies of an important cattle tick vary in their ability to ingest C. ruminantium when feeding on an infected host. I have added convincing evidence that the vector of PHF is not D. variabilis and provided additional data indicating that R. sanguineus, A. americanum and I. scapularis are also not vectors. These studies are a useful contribution to the general knowledge of the transmission of E. risticii and C. ruminantium.

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Vita

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