

LABORATORY ARTIFICIAL INFECTION OF HARD TICKS: A TOOL FOR THE ANALYSIS OF TICK-BORNE PATHOGEN TRANSMISSION

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(Received 12 March 2012; accepted 13 August 2012; published online 21 December 2012)

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ABSTRACT — Despite its importance, our knowledge of pathogen transmission by ticks is incomplete. Detailed studies on the transmission, maintenance, infectivity, virulence, and pathogenicity of tick-borne microparasites all require the use of large numbers of live ticks raised under controlled conditions and difficulties in rearing ticks in the laboratory could partly explain the current lack of data. The most complex part in maintaining tick colonies doubtlessly lies in their engorgement, as ticks are strict haematophagous arthropods. Indeed, relatively few research teams have worked on artificial feeding systems for ticks due to the long, complex, and poorly understood feeding patterns of these arthropods. It is nonetheless essential to investigate the mechanisms underlying tick infection and infectiousness in order to better understand parasite-host-vector relationships and elaborate new control strategies for transmitted pathogens. The various methods used to date to feed ticks and infect them with their associated pathogens are reviewed here and their advantages and inconveniences are discussed.

KEYWORDS — ticks; artificial feeding; in vitro infection

INTRODUCTION

Ticks are among the most important vectors of human and animal diseases and surpass all other arthropods in the variety of pathogenic organisms they can transmit: including fungi, viruses, bacteria and protozoa. To study the biology of ticks or their interactions with associated pathogens, it is indispensable to be able to maintain tick colonies under laboratory conditions and to have efficient techniques to artificially infect them. In addition, it is widely recognized that the dynamics, pathogenesis and symptoms of infection, as well as the subsequent immune response, strongly depend on the route of pathogen introduction into a suscepti-

ble vertebrate host (Crippa *et al.* 2002; de Souza *et al.* 1993; Gern *et al.* 1993; Massung *et al.* 2004; Motameni *et al.* 2005; Piesman 1993). During natural transmission, tick-borne pathogens are injected into the vertebrate host at the same time as tick saliva, which favors infection by interfering with host immunological responses (Nuttall 1999). This means that studying tick-borne pathogen transmission to vertebrate hosts requires that ticks be infected under laboratory conditions.

However, rearing ticks, and in particular hard ticks, is not easy due to their complex biological cycle. The problems encountered in the maintenance of productive laboratory colonies doubtlessly explain a significant proportion of the existing

gaps in our knowledge of tick vector competence and transmission pathways. The *Ixodidae* likely possess the most complex feeding biology of all hematophagous arthropods. Indeed, the fact that they only feed on blood, and do so for an extended period of time (3-12 days), greatly limits our ability to set up artificial feeding systems which can function over the required time intervals. Several methods have been developed and used to infect hard ticks with pathogens, including feeding ticks on infected animals, injecting pathogens through the cuticle, using capillary tubes filled with infectious suspensions to feed ticks, and feeding them on artificial or animal-derived membranes. In this review, we summarize each of these techniques, discuss their application to pathogen transmission, and present their strengths and weaknesses (summarized in the Table 1). However, we first start with a brief outline of the Ixodid tick life cycle and highlight the importance of considering tick biology and ecology when attempting artificial feeding and tick rearing in the laboratory.

Overview of the hard tick life cycle

The general life cycle for hard tick species can be found in Sonenshine (1991). Here, we summarise the main points that are important to consider for artificial tick infections. Hard ticks have larval, nymphal and adult forms, all of which require a blood meal. Adult ticks tend to be restricted to feed on large-bodied animal hosts, whereas larval and nymphal stages also exploit smaller animals. For many species, Humans can be incidental hosts to the three life stages. A three-host life cycle, which includes host-seeking, feeding and off-host moulting (or egg-laying) in each life stage, is the most common developmental pattern for the majority of hard ticks of medical and veterinary interest. After feeding on a rather substantial quantity of host blood, females drop from the hosts and commence oviposition in a sheltered microenvironment, laying up to several thousand eggs. During each life stage, ticks may enter diapause for a variable amount of time depending on environmental conditions. Under favorable conditions in the natural environment, the life cycle of three-host tick species, from

larval hatching to the hatching of the next larval generation, can be completed in less than one year, but is typically longer (2-3 years). Compared with other haematophagous arthropods, feeding ixodid ticks is therefore a slow and complex process, taking several days to several weeks for repletion and detachment alone. In addition, successful host attachment depends on the presence of an appropriate array of chemical and physical stimuli that entice ticks into feeding.

Laboratory-adapted conditions for tick feeding

Most species of non-nidicolous ticks (or exophilic ticks, *i.e.* that occupy open, exposed habitats) have a clear, well-defined seasonal period of activity, during which time they engage in questing, a behavior expressed by the willingness of the tick to crawl or climb to favorable locations where they may attach to passing hosts. This active period can vary within the zoogeographic range of the species and among life stages (Sonenshine, 1991). Under laboratory conditions, photoperiod and ambient temperature can be adapted to the specific tick species being studied in order to induce this active period, thereby stimulating the desire to eat and accelerating the biological cycle. In 1979, Doube and Kemp (1979) reported that environmental factors, *e.g.*, variation in temperature or relative humidity, influence tick attachment behavior and survival, but do not generally affect feeding duration once ticks are attached to warm-blooded vertebrates. However, since their study, it has been demonstrated that diurnal rhythms and other environmental factors can, in fact, affect engorgement and detachment patterns. For example, mated females of the southern cattle tick *Rhipicephalus (Boophilus) microplus* engorge most rapidly at night but do not drop-off until the animals begin leaving the cattle sheds, typically in early morning, facilitating the dispersal of the replete female ticks in the host's habitat (Bianchi and Barre 2003). Similarly, nidicolous ticks tend to concentrate their feeding activities during the period when the host is resting or sleeping in the nest or burrow (Olivier 1989). Recently, experiments performed on birds reported the capacity of *I. arboricola* to extend the duration of attachment when the

TABLE 1: Summary of the major strengths and weaknesses of techniques used to artificially infect ticks with pathogens. Only key models (ticks and pathogens) and associated references are mentioned here. More specific information can be found in the main text.

Infection method	Frequency of use	Tick species	Pathogens studied	Key references	Major strengths	Major weaknesses
Direct feeding on the host	Many studies	<i>I. ricinus</i>	<i>B. divergens</i>	Joyner <i>et al.</i> , 1963	Physiologically realistic; Relatively easy set-up; Ability to infect a large quantity of ticks	Expensive; Ethical considerations; Inability to quantify infective dose; Restricted use for wild hosts
		<i>D. andersoni</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 1986		
		<i>R. appendiculatus</i>	<i>T. parva</i>	Bailey, 1960		
		<i>A. variegatum</i>	<i>T. mutans</i>	Young <i>et al.</i> , 1996		
		<i>A. hebraeum</i>	<i>C. ruminantium</i>	Heyne <i>et al.</i> , 1987		
		<i>I. ricinus</i>	<i>B. birtlesii</i>	Reis <i>et al.</i> , 2011a		
Injection	Few studies	<i>R. appendiculatus</i>	<i>T. parva</i>	Jongejan <i>et al.</i> , 1980	Ability to quantify infective dose	Physiologically unrealistic; High tick mortality; Live animals needed (ethical and logistical considerations)
		<i>D. andersoni</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 1996		
		<i>I. scapularis</i>	<i>B. burgdorferi</i>	Kariu <i>et al.</i> , 2011		
Capillary	Many studies	<i>D. andersoni</i>	<i>L. pomona</i>	Burgdorfer, 1957	Natural infection route; Ability to quantify infective dose	Difficult set-up; Live animals needed (ethical and logistical considerations); Ingestion of blood and pathogen not simultaneous
		<i>R. appendiculatus</i>	<i>T. parva</i>	Purnel et Joyner, 1967		
		<i>I. ricinus</i>	<i>B. burgdorferi</i>	Monin <i>et al.</i> , 1989		
		<i>A. variegatum</i>	Dugbee virus	Booth <i>et al.</i> , 1991		
		<i>R. sanguineus</i>	<i>E. chaffeensis</i>	Rechav <i>et al.</i> , 1999		
		<i>D. variabilis</i>	<i>A. marginale</i>	Kocan <i>et al.</i> , 2005		
Membrane (animal skin or silicone membrane)	Many studies	<i>A. variegatum</i>	<i>T. mutans</i>	Voigt <i>et al.</i> , 1993	Natural infection route; Ingestion of blood and pathogen simultaneous; Ability to quantify infective dose; No need for live animals; Ability to infect a large quantity of ticks	Daily change of the blood (and risk of contamination); Membrane preparation required; Olfactory stimuli sometimes required (for non-animal membranes)
		<i>R. appendiculatus</i>	<i>B. ruminantium</i>	Young <i>et al.</i> , 1996		
		<i>R. appendiculatus</i>	<i>T. parva</i>	Wallade <i>et al.</i> , 1993		
		<i>I. ricinus</i>	<i>B. divergens</i>	Bonnet <i>et al.</i> , 2007		
		<i>I. ricinus</i>	<i>B. henselae</i>	Cotté <i>et al.</i> , 2008		

host bird did not return to a suitable environment for the tick, with no apparent costs of prolonged attachment (White *et al.* 2012).

Host-seeking ticks recognize a variety of stimuli from prospective hosts which, in turn, excites their host-finding behavior. Among these, odors are undoubtedly the most important and best-studied stimuli (Waladde and Rice 1982). Carbon dioxide represents one of the most important host-derived

odorants and can be used in the laboratory to stimulate tick activity. Radiant heat, such as host's body heat, also acts as a stimulus and acts synergistically with odors (Lees 1948). Other stimuli which ticks may potentially use in host-finding activities have received little attention, especially visual cues and vibrations. Finally, in some instances, tick-derived rather than host-derived stimuli are of critical importance in tick host-seeking behavior. For exam-

ple, *Amblyomma variegatum* and *A. hebraeum* are excited by the CO₂ produced by cattle but select tick-infested animals when they detect the aggregation-attachment pheromone emitted by attached, feeding ticks (Norval *et al.* 1989). Based on these studies, stimuli from hosts and pheromones produced by ticks can therefore be used in the laboratory to promote tick feeding.

Some tick species feed only on specific hosts, or on a narrow range of closely related hosts, whereas others may be categorized as opportunistic (Sonenshine 1975). Host specificity results from a process of selective host recognition and the ability of ticks to avoid host rejection (Ribeiro 1987) and can differ between life stages for a given tick species. To a certain extent, host choice is influenced by the height at which ticks seek hosts on the vegetation, but this is by no means the sole determinant of host specificity. Macro- and micro-habitat distribution also influences host selection by favoring encounters between ticks and their hosts. Host selection also requires tick recognition of specific host characteristics, such as host odors, for example. Unfortunately, our understanding of host selection in ticks and the functional basis of host specificity is extremely limited and can be a significant obstacle for tick rearing and study. As some hosts can not being maintained under laboratory conditions, the implementation of specifically-adapted artificial feeding systems may be necessary. For example, as mentioned above, odor from the appropriate host animal may be used to stimulate tick feeding on artificial membranes. Sweat and exfoliated skin collected from horses naturally infested with *A. cajennense*, has been used to stimulate feeding in this tick species (de Moura *et al.* 1997). In the same way, shredded bovine hair and hair extract (*i.e.*, lipid extracted from freshly shaven hair dissolved in dichloromethane) have been used, respectively, on silicone membranes in order to entice feeding in *A. hebraeum* and *I. ricinus*, tick species that readily exploit cattle (Krober and Guerin 2007a; Kuhnert *et al.* 1995).

In conclusion, the general environmental conditions that a tick is exposed to in its natural habitat, including temperature, humidity, diurnal rhythms, activity periods, questing behavior, sensory modal-

ities and stimuli, must all be considered carefully when setting up a laboratory system. In many cases, laboratory conditions will need to be adapted to the specific needs of the tick species of interest in order to increase the chances of successful tick feeding and colony establishment.

Systems for maintaining and/or infecting ticks

Feeding and infection directly on the host

Despite the constraints associated with host specificity, some tick species can be readily fed in the laboratory on easily handled animals. Indeed, rabbits are classically used to feed ticks in laboratory: immature stages of *Rhipicephalus evertsi evertsi* (Londt and Van der Bijl 1977), all life stages of *I. scapularis*, *I. pacificus*, *A. americanum*, *Dermacentor occidentalis*, *D. variabilis*, *Haemaphysalis leporispalustris* and *R. sanguineus* (Troughton and Levin 2007), *R. appendiculatus* (Bailey 1960), *A. variegatum* (Voigt *et al.* 1993), *D. andersoni* (Howarth and Hokama 1983), *A. hebraeum* (Heyne *et al.* 1987), *I. ricinus* (Bonnet *et al.* 2007). In these cases, the typical way to engorge ticks is to use feeding bags or capsules glued to clean-shaven skin on the back of the animal (Figure 1). Sometimes, larger animals have been used as blood sources, such as calves for *R. evertsi evertsi* (Londt and Van der Bijl 1977), *R. appendiculatus* (Musyoki *et al.* 2004), and *D. andersoni* (Kocan *et al.* 1986), or sheep for *A. hebraeum* (Heyne *et al.* 1987) and *I. ricinus* (Bonnet *et al.* 2007).

The use of natural hosts for tick feeding and methods of direct infection on infectious animals is the method of choice to obtain conditions that are closest to the physiological reality of transmission. However, acquisition, housing, and handling of animal hosts can be complicated, expensive and sometimes even impossible. In fact, in some cases and in particular for wildlife, maintaining the natural host of a specific tick-borne pathogen is impossible in laboratory. There are some examples where wild animals were used as the blood meal source, such as groundhogs for feeding *Ixodes cookei* (Farkas and Surgeoner, 1991) or tortoises for feeding *A. hebraeum* (Heyne *et al.*, 1987) but this practice remains exceptional. The most commonly used model of tick infection directly on animals involves pathogens in-

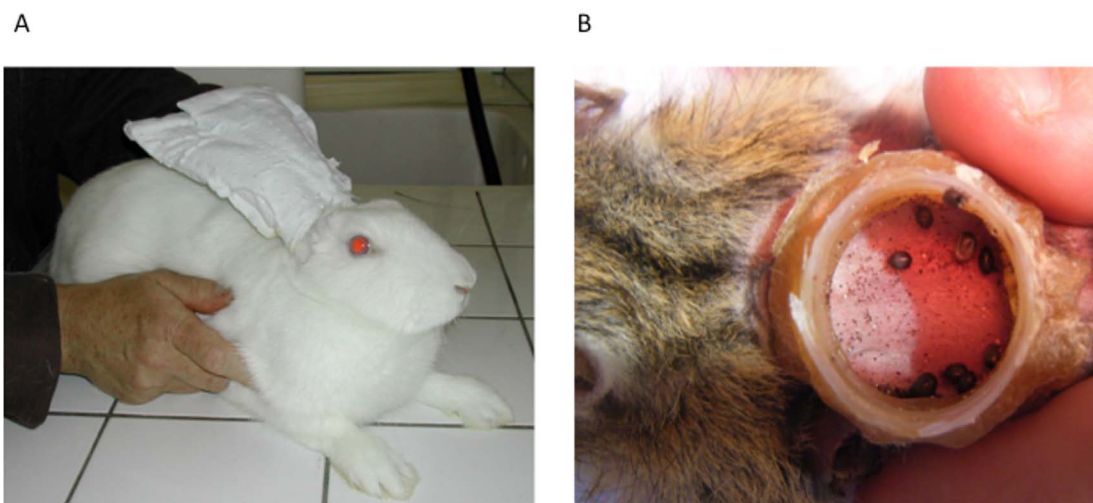


FIGURE 1: Views of *Ixodes ricinus* feeding in the laboratory on A – rabbit, and B – Siberian chipmunks (*Tamias sibiricus barberi*). On rabbits, ticks were put in an ear-bag which was placed on shaved ear skin and sealed with tape at the base. Ticks were checked daily until repletion, and were then collected and stored under standardized conditions. For chipmunks, animals were briefly anaesthetized with 3 % Isoflorane and a plastic cap, open at both ends, was glued onto their shaved back with wax. Hungry larvae and nymphs were placed in the cap, which was then sealed with tape. Ticks were allowed to feed until repletion for 5-6 days. At this time, the cap was opened, and the engorged ticks were collected and stored under standardized conditions.

fecting cattle, such as *Babesia divergens* transmitted by *I. ricinus* (Donnelly and Peirce 1975; Joyner *et al.* 1963; Lewis and Young 1980), *Anaplasma marginale* by *D. andersoni* (Kocan *et al.* 1986), *Theileria parva* by *Rhipicephalus appendiculatus* (Bailey 1960; Musyoki *et al.* 2004), or *T. mutans* and *Cowdria ruminantium* transmitted by *A. variegatum* (Young *et al.* 1996). Sheep were used to infect *A. hebraeum* with *C. ruminantium* (Heyne *et al.* 1987). Infectious gerbils have been used in order to infect *I. ricinus* with *B. divergens* (Lewis and Young 1980; Mackenstedt *et al.* 1990). Finally, laboratory mice have also been used for studying *Bartonella birtlesii* transmission by *I. ricinus* (Reis *et al.* 2011a), or *Borrelia burgdorferi* by *I. scapularis* (Burkot *et al.* 2001).

However, with the direct feeding technique, it is impossible to quantify the pathogen dose received by the tick during feeding and thus to standardize the experimental conditions. Even if a venous blood sample is simultaneously analyzed, it may have different pathogen concentrations than the tick's biting site and tick blood meals are too long to monitor temporal changes in pathogen concentrations with any precision. Likewise, for some pathogens, the parasitaemia, bacteraemia or viral peak in the host

can be short (1-4 days) and it may be difficult to synchronize it with tick feeding. Finally, for ethical considerations, it is always desirable to limit the use of laboratory animals and thus to find alternative artificial systems.

Infection by injection

In a few studies, ticks have been infected by direct injection of a suspension containing the pathogen through the cuticle. *R. appendiculatus* have been successfully infected after inoculation with fresh or cryopreserved blood containing *T. parva* (Jongejan *et al.* 1980; Walker *et al.* 1979), whereas attempts to infect ticks by inoculating cultured stages of *T. parva* failed (Jongejan *et al.* 1980). Another study reported that *D. andersoni* exposed percutaneously as nymphs to *Anaplasma marginale*, transmitted the pathogen to calves as feeding adults, even though no bacterial colonies were detected in gut tissues of the inoculated ticks (maybe because the infective dose was too low or because the bacteria developed in tissues other than gut) (Kocan *et al.*, 1986). In a study aimed at infecting *A. americanum*, *D. variabilis* and *R. sanguineus* with *Ehrlichia chaffensis*, Rechav *et al.* (1999) concluded that the inoculation technique by injec-

tion is not accurate or practical for routine infection of ticks with pathogens because of the low survival rate of inoculated ticks. Recently, a procedure for infecting *I. scapularis* with *B. burgdorferi* via a microinjection by the anal aperture was reported and seems more satisfactory in terms of tick survival (Kariu *et al.* 2011).

When using direct inoculations by injection, the exact assessment of the pathogen dose received by the tick is possible. However, in addition to the high tick mortality previously mentioned (Rechav *et al.* 1999), this technique does not enable the experimenter to avoid the use of animals for feeding ticks post-infection. Finally, and more importantly, it does not correspond to the normal infection pathway used by the pathogen to infect ticks, which naturally occurs via the mouthpart and the digestive tract during the blood meal. This difference can have important consequences for pathogen development, particularly when the parasite in question undergoes several developmental stages in the tick gut (Chauvin *et al.* 2009). It has also been demonstrated that bacteria, such as *Borrelia burgdorferi*, express different molecules depending on the engorgement status of the vector (Hovius *et al.* 2007). Consequently, the results obtained with direct inoculation systems may be difficult to extrapolate and apply to natural infections.

Infection by capillary feeding

The use of blood-filled capillary tubes placed over the mouthparts of the tick was first reported in 1938 by Gregson who used this technique to collect saliva from *D. andersoni* (Gregson 1938). Later, in 1950, Chabaud used it for engorging *Haemaphysalis excavatum*, *H. dromedarii* and *R. sanguineus* with different nutriment combinations as a means of studying tick nutrition (Chabaud 1950). In Chabaud's study, ticks were pre-fed on the host, removed, and the capillary tube containing various substrates was placed over the tick's mouthparts. Since these initial studies, capillary tubes filled with infectious suspensions have been used for feeding ticks in several biological models: *Leptospira pomona* / *A. maculatum*-*D. andersoni* (Burgdorfer 1957), *T. parva* / *R. appendiculatus* (Purnell and Joyner 1967; Walker *et al.* 1979),

B. burgdorferi / *I. ricinus* (Kurtenbach *et al.* 1994; Monin *et al.* 1989), *B. burgdorferi* / *I. scapularis* (Broadwater *et al.* 2002; Korshus *et al.* 2004), dugbee virus / *A. variegatum* (Booth *et al.* 1991), *E. chaffeensis* / *A. americanum*-*D. variabilis*-*R. sanguineus* (Rechav *et al.* 1999), *A. marginale* / *D. variabilis* (Kocan *et al.* 2005) or *R. montana*-*R. rhipicephali* / *D. variabilis* (Macaluso *et al.* 2001). In these studies, capillary feeding was performed either before or after feeding on the animal host, the animal host being necessary in order to feed ticks to repletion.

Tick infection by capillary feeding presents the advantage of using the natural infection route via the mouthparts and the digestive tract. It also permits one to control the amount of fluid ingested by the tick and the titer of the pathogen that enters the tick. However, tick manipulation during the pre- or post-feeding period on the natural host with a forced removal from the host is delicate in practice. Similarly, only very small amounts of fluid (0.01-0.03 ml) can be ingested by ticks with this technique (Burgdorfer 1957; Rechav *et al.* 1999) because ticks feed in an unnatural manner. Finally, and most importantly, natural transmission conditions are poorly replicated using this method, as the tick acquires the pathogen in large quantities and without blood. Normally, the pathogen is absorbed by the tick throughout the blood meal period during which time the tick has already begun digestion and the pathogen has started the next step its developmental cycle.

Membrane feeding systems

The membrane feeding technique consists in feeding ticks through a membrane on blood taken from animals or culture media. It is the most frequently used feeding technique for ticks as demonstrated by two previously published reviews on the subject (Krober and Guerin 2007b; Waladde *et al.* 1996). In 1956, Pierce and Pierce used air cell membranes from embryonated hen eggs in order to feed *R. microplus* larvae and nymphs (Pierce and Pierce 1956). Since then, several membranes from different animal origins have been used with variable success to engorge ticks, including pieces of cattle skin for *R. microplus* (Kemp *et al.* 1975) and *A. variegatum*



FIGURE 2: View of attached *I. ricinus* nymphs on a rabbit skin used in the membrane feeding system.

(Voigt *et al.* 1993; Young *et al.* 1996), calf mesentery and modified Baudruche membranes for *R. microplus* (Kemp *et al.* 1975; Waladde *et al.* 1979) and *R. appendiculatus* (Waladde *et al.* 1991; Young *et al.* 1996), rabbit skin for *A. variegatum* (Voigt *et al.* 1993; Young *et al.* 1996), *D. andersoni* (Howarth and Hokama 1983), *R. appendiculatus* (Musyoki *et al.* 2004) and *I. ricinus* (Bonnet *et al.* 2007) (Figure 2), mouse skin for *D. andersoni* (Howarth and Hokama 1983; Paine *et al.* 1983) and *I. scapularis* (Burkot *et al.* 2001), and gerbil skin for *I. ricinus* (Bonnet *et al.* 2007). Membranes of non-animal origin made from silicone have also been used with success, particularly for feeding the different instars of *A. hebraeum* (Kuhnert *et al.* 1995), *I. ricinus* females (Krober and Guerin 2007b), *A. cajennense* adults (de Moura *et al.* 1997), and recently *H. anatolicum anatolicum* and *H. dromedarii* (Tajeri and Razmi 2011). However, without the addition of specific stimuli, the use of such membranes has proved ineffective for ticks such as *A. variegatum* (Voigt *et al.* 1993). This is related to the fact that one of the greatest difficulties is to encourage the attachment of unfed ticks (see above). It is for this reason that attachment stimuli are always required with silicone membranes, and/or why some authors use these membranes after a pre-feeding step on live animals. This was the case, for example, for *I. holocyclus* where the authors wanted to collect tick-produced toxins to study tick paralysis (Stone *et al.* 1983). In addition to being logistically difficult, the major disadvantage of arti-

ficial membrane systems that employ pre-feeding is the low reattachment success on the membrane (Howarth and Hokama 1983).

However, regardless of the limitations associated with artificial membrane techniques, this method has proved successful in infecting feeding ticks. Howarth and Hokama (1983) were able to obtain infectious adults of *D. andersoni* when the preceding nymphal stages were infected with *Anaplasma marginale* via an animal skin membrane and after a pre-feeding step on a rabbit. An almost similar protocol was used by Burkot *et al.* (2001) for successfully infecting *I. scapularis* ticks with *B. burgdorferi*. Here, ticks were pre-fed on a mouse and the mouse skin was harvested with *I. scapularis* still attached. The skin was then fixed to a glass membrane feeder containing bacterial infected blood (Burkot *et al.* 2001). In other studies, animal skin membranes have been used with success and without the need of a pre-feeding step on a living animal. *A. variegatum* was infected with *T. mutans* and *Cowdria ruminantium* in this way (Voigt *et al.* 1993; Young *et al.* 1996), as was *R. appendiculatus* with the same pathogens and a modified Baudruche membrane (Young *et al.* 1996). In 1993, Waladde *et al.* succeeded in transmitting *T. parva* to susceptible cattle via adult *R. appendiculatus* infected as nymphs through a Baudruche membrane that was made attractive to ticks by the addition of a combination of tactile and olfactory stimuli (Waladde *et al.* 1993). The same experiment was then reproduced

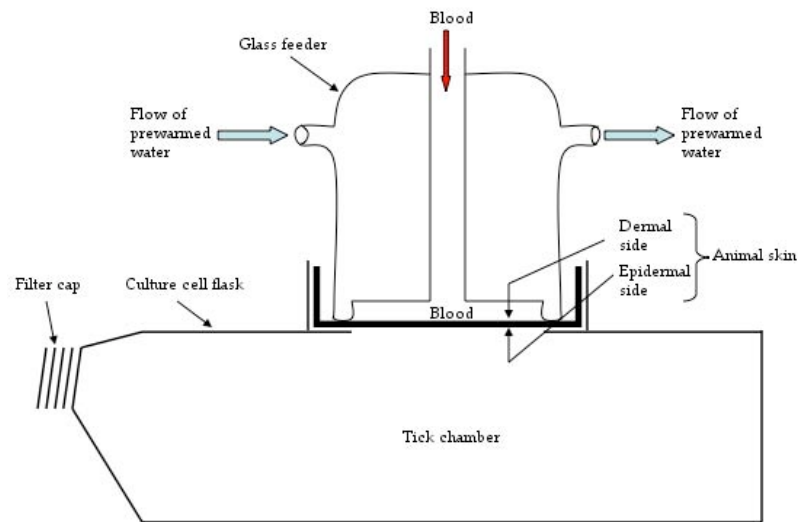


FIGURE 3: Diagram of the membrane feeding apparatus used in the experimental feeding of *Ixodes ricinus* ticks (adapted from Bonnet *et al.* 2007).

successfully using rabbit skin membranes (Musyoki *et al.* 2004). Finally, gerbil (for immature life stages) and rabbit (for adults) skin membranes have been used in order to infect *I. ricinus* with both *B. divergens* and *Bartonella henselae* without the need for additional stimuli (Bonnet *et al.* 2007; Cotte *et al.* 2008).

The membrane feeding apparatus consists of a blood container with a membrane placed either on the top (Bonnet *et al.* 2007; Burkot *et al.* 2001; Musyoki *et al.* 2004; Voigt *et al.* 1993; Young *et al.* 1996) or the bottom (Howarth and Hokama 1983; Kuhnert *et al.* 1995; Paine *et al.* 1983; Waladde *et al.* 1991) of the tick containment unit. Placing the blood above the membrane favors a continuous gravitational pressure on the membrane and is essential for infection with intraerythrocytic pathogens because of the rapid sedimentation of the red blood cells. Several tick-feeding devices with different blood containment units have been explored and tested, including plastic cylinders (Young *et al.* 1996), plate wells (Howarth and Hokama 1983; Krober and Guerin 2007a), honey jars (Kuhnert *et al.* 1995) or glass feeders (Bonnet *et al.* 2007) as represented on figure 3.

In order to mimic the host environment more closely, a temperature (35 – 39°C) adapted to the

tick species of interest should be applied either to the incubator, where the whole apparatus is placed, or just to the blood. As already mentioned, olfactory stimuli for attachment and feeding are sometimes required and are indispensable in the case of membranes from non-animal origins. The required stimuli could differ depending on the species and genera of ixodid ticks under study. A carbon dioxide atmosphere has been used as stimulant for tick attachment, between 5 and 10 % CO₂ for *A. variegatum* for example (Voigt *et al.* 1993; Young *et al.* 1996). Host hair, tick feces, animal fur extracts and synthetic aggregation-attachment pheromone mixtures have all been used for stimulating the attachment of *A. hebraeum* (Kuhnert *et al.* 1995). For stimulating *R. appendiculatus* feeding, Young *et al.* (1996) also used cattle/tick washes and tick feces. However, de Moura *et al.* (1997) demonstrated that for *A. cajennense* silicone membranes treated with blood vestiges was more efficient than other tested phagostimulants. Finally, adenosine triphosphate (ATP) and reduced glutathione have also been used as phagostimulants (Kuhnert *et al.* 1995; Paine *et al.* 1983). Finally, membrane thickness must be adapted to the size of the tick's mouthparts which can be short or long depending on the genera and the tick life stage.

Membrane feeding techniques mimic the natural conditions of tick infection more closely than other methods because pathogens are mixed in blood and are absorbed throughout the blood meal via the digestive tract. In most cases, using animal skin membranes has the important advantage that no tick pre-feeding is required for attachment and engorgement on the membrane, and no attachment stimuli are required. It is, on the other hand, necessary to sacrifice laboratory animals in order to obtain the skins and to carefully prepare them (Bonnet *et al.* 2007; Musyoki *et al.* 2004) to avoid any biodegradation and blood contamination. The final engorgement weight of membrane-fed ticks also tends to be lower (or equal) than that of animal fed ticks (Musyoki *et al.* 2004; Voigt *et al.* 1993; Young *et al.* 1996), even if molting and egg-laying success are generally comparable. Membrane feeding permits a direct assessment of pathogen concentration in the blood sample ingested by the ticks. Repeated assays with large tick numbers are also possible with this system. Finally, membrane-feeding techniques can allow one to evaluate the effects of drugs or transmission-blocking blood components, as well as helping to elucidate attachment stimuli, feeding stimuli and nutritional requirements of ticks. Feeding immature stages presents less difficulty than for adults because of their shorter feeding times. Indeed, the principal difficulty with this technique resides in maintaining a continuous bloodmeal without contamination by bacteria or fungi during the slow blood-feeding process and the required daily changes of the blood. In addition, the mouthparts and oral secretions of the ticks can also contaminate the blood in the feeding device because of the absence of host defense mechanisms. However, the addition of antibiotic and antifungal products to the circulating blood can prevent this problem. To favor feeding, anticoagulants should also be used and it was reported that heparinized blood was found to be the most suitable for tick feeding (Voigt *et al.* 1993; Waladde *et al.* 1993; Young *et al.* 1996). Consequently, within the framework of experimental pathogen transmission, it is necessary to test pathogen viability under the tick feeding conditions beforehand.

CONCLUSIONS

Ticks possess many unusual features that contribute to their remarkable success and vector potential. One of the most outstanding is their longevity and their reproductive potential (*i.e.*, ability to produce large numbers of eggs), which makes them substantial pathogen reservoirs in the field. Another is the fact that they are pool feeders (*i.e.*, sucking all the fluids and potential pathogens that are exuded into the wound generated by the bite). During feeding, they absorb a very large quantity of blood and over a relatively long period of time, thereby increasing the chance of ingesting a pathogen. It is this last parameter that makes them particularly difficult to study in the laboratory because these natural conditions are complicated to replicate. Nevertheless, there is no doubt that effective *in vitro* feeding systems for Ixodid ticks of medical and veterinary importance have major benefits. Even if feeding ticks on live experimental animals seems the simplest, it is not always practicable according to the biological model and may be considered as ethically debatable. Various methods have therefore been elaborated to feed and infect ticks artificially, among which the membrane feeding technique mimics reality more closely than the other techniques. However, each technique has strengths and weaknesses and the chosen method will depend on the question addressed. In all cases, infecting ticks under controlled conditions enables one to test a great spectrum of biological questions, including the ability to study the development of pathogens inside their vectors, to uncover transmission pathways, and to evaluate the influence of biologically active substances exchanged between host and vector. For now, these techniques all tend to have long and difficult set-up periods, giving sometimes unpredictable results. Efforts to standardize and simplify laboratory protocols, which would greatly improve our ability to exploit these methods, should now be the aim of future work.

ACKNOWLEDGEMENTS

Thanks are due to the "Tiques et Maladies à Tiques" working group (REID – Réseau Ecologie des In-

teractions Durables) for stimulating discussions. Part of the presented work was funded by EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext000 (<http://www.edenext.eu>). The contents of this publication are the sole responsibility of the author and do not necessarily reflect the views of the European Commission. Acknowledgements are also due to B. Allouche from the DSI of ENVA for use of photographs and to Dr K. McCoy and E. Fillol for their critical reading of the manuscript.

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