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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

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Prof. Didier Raoult

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SOMMAIRE

RÉSUMÉ

Ce travail s'articule sur trois axes ; le premier est une contribution à l'étude du répertoire des bactéries associées aux arthropodes vecteurs (tique et puces) en Afrique du nord (Algérie) et en Afrique Sub-saharienne (Bénin, Tanzanie et République Démocratique du Congo). Nous avons pu ainsi détecter par biologie moléculaire (qPCRs, PCR standard et séquençage) et pour la première fois au Bénin, *Rickettsia typhi* (l'agent du typhus murin), et *Bartonella* sp dans des puces collectées sur des rongeurs à Cotonou. Dans ce travail, nous avons également détecté *Yersinia pestis,* l'agent de la peste et *R. felis* (responsable de la fièvre boutonneuse) dans des puces de la RD du Congo. En Tanzanie, nous avons mis en évidence la présence de *R. felis* et *R. typhi* dans des puces de rongeurs. En Algérie, nous avons décrit pour la première fois la présence d'agent de borréliose de Lyme (*Borrelia garinii*) dans les tiques. Nous avons confirmé la présence de *R. massiliae*, *R. monacensis* *R. aeschlimannii*, *R. slovaca* et *R. felis* et nous avons également détecté pour la première fois en Algérie, *Bartonella tamiae,* une bactérie dont la pathogénicité est peu connue *et Coxiella burnetii*, l'agent de la fièvre Q.

Dans la deuxième partie de notre travail, nous nous sommes intéressés à l'évaluation des compétences vectorielles des puces de chat (*Ctenocephalides felis*) et punaises de lit (*Cimex lectularius*) dans la transmission de l'agent de la fièvre des tranchées (*Bartonella quintana*) dont le vecteur connu est le pou de corps. Trois approches ont été utilisées : la qPCR, la culture et l'immunohistochimie. Nous avons constaté que les puces et les punaises acquièrent la bactérie et excrètent des micro-organismes vivants dans leurs fèces. Ces insectes peuvent être considérés comme vecteurs potentiels de *Bartonella quintana*.

La troisième partie de ce travail s'est intéressée à l'application d'outil d'identification précis des vecteurs et des pathogènes associés. Les méthodes d'identification des arthropodes existantes (morphologie, biologie moléculaire..) présentent des limites. L'émergence d'une technologie protéomique (MALDI-TOF) est actuellement utilisée en routine pour l'identification des bactéries et depuis peu appliquée à l'entomologie. Ainsi dans ce projet nous avons évalué l'utilisation du MALDI-TOF MS pour l'identification des puces ainsi que pour la détection des pathogènes associés notamment sur les punaises de lit infectées expérimentalement par *B. quintana*.

ABSTRACT

This work focuses on three areas; the first is a contribution to the study of the repertoire of bacteria associated with arthropod vectors (tick and flea) in North Africa (Algeria) and in Sub-Saharan Africa (Benin, Tanzania and the Democratic Republic of Congo). We could thus detected by molecular tools (qPCRs, standard PCR and sequencing) and for the first time in Benin, *Rickettsia typhi* (the agent of murine typhus) and *Bartonella sp* in fleas collected from rodents in Cotonou. In this work, we have also associated the agent of plague (*Yersinia pestis*), and for the first time in fleas of DR of Congo, and we detected also *R. felis* (the causative agent of spotted fever). In Tanzania, we have highlighted the presence of *R. typhi and R. felis* fleas on rodents. In Algeria, we described for the first time the presence of Lyme disease agent (*Borrelia garinii*) in hard ticks. We confirmed the presence of *R. massiliae*, *R. monacensis,* R. *aeschlimannii*, *R.* slovaca and *R. felis*, we also detected for the first time *Bartonella tamiae* and *Coxiella burnetii* associated with bat ticks in Algeria.

Regarding the second part we was interested in the evaluation of vector competence of cat fleas (*Ctenocephalides felis*) and bed bugs (*Cimex lectularius*) in the transmission of trench fever

agent (*Bartonella quintana*) that is known to be transmitted by lice. Three approaches have been tested; qPCR, culture and immunohistochemistry. We found that fleas and bed bugs acquire the bacteria and excrete living microorganisms in their feces; this allows them to consider them as potential vector of this bacterium.

The third part of this work has focused on the application of specific tool for the identification of vectors and associated pathogens. Arthropods identification methods (morphology, molecular biology...) and detection of microorganisms associated (discoloration, molecular biology...) existing has several limitations. The emergence of proteomics technology (MALDI-TOF) some years ago and is now routinely used for the identification of bacteria and started to be applied to entomology. Thus in this project we evaluated the use of MALDI-TOF MS to identify fleas and for the detection of associated pathogens including bed bugs experimentally infected with *B. quintana*.

I - INTRODUCTION

Depuis plusieurs décennies, nous assistons à l'émergence et la réémergence de plusieurs pathologies zoonotiques [1], constituant une menace pour la santé humaine et animale, surtout celles à transmission vectorielle [2]. Cette émergence constitue la réponse évolutive des agents pathogènes eux même, ou à la modification de leur environnement. En effet, ces maladies induites étaient connues essentiellement dans les zones tropicales et subtropicales [3], mais certaines d'entre elles ont déjà fait des incursions dans les régions tempérées.

Un vecteur par définition est un arthropode hématophage qui assure la transmission biologique (ou mécanique) active d'un agent infectieux de vertébré à un autre vertébré. Les tiques sont des arthropodes hématophages considérées comme les deuxièmes vecteurs (après les moustiques) d'agents de maladies humaines et les plus importants vecteurs chez les animaux [4]. Elles peuvent transmettre de nombreux agents pathogènes, virus, protozoaires et bactéries [5]. De même, les puces sont également capables de transmettre plusieurs agents de maladies infectieuses [6]. La transmission de ces agents zoonotiques à l'homme et aux animaux se fait principalement par piqûre ou par contact de leurs excréments infectés avec des lésions de grattage et des muqueuses [7]. L'implication des punaises de lit, dans la

transmissions de pathogènes à l'homme reste, en revanche controversée [8].

Les rickettsioses, bartonelloses, la peste, les borrélioses et la fièvre Q sont des maladies transmises par l'intermédiaire de vecteurs objets de cette étude. Les rickettsies sont des bactéries à Gram négatif intracellulaires [9]. En Algérie, 11 espèces de rickettsies ont été détectés dans les tiques, les puces, les poux et dans des cas humains, y compris *R. conorii conorii*, *R. aeschlimannii, R. sibirica mongolitimonae, R. massiliae, R. slovaca, R. helvetica, R. africae, R. Monacensis, R. felis, R. typhi* et *R. prowazekii* [10]. De même, les bartonelloses sont des maladies causées par les bactéries fastidieuses, hémotropes du genre *Bartonella* [11], qui parasitent une gamme d'hôtes de mammifères, y compris les humains, les rongeurs et les chiroptères [12-14]. *Coxiella burnetii*, l'agent responsable de la fièvre Q, est une bactérie intracellulaire zoonotique hautement infectieuse qui peut affecter différentes espèces de mammifères sauvages et domestiques, elle peut aussi infecter les arthropodes et les oiseaux [15]. En Afrique y compris l'Algérie seulement quelques cas humains de fièvre Q ont été documentés [16]. La peste est une maladie infectieuse ré-émergentes la plus célèbre, transmise par les puces et constitue un problème majeur de santé publique en Afrique [7]. Elle sévit dans des foyers endémiques

principalement en Afrique et en Amérique du Sud [17,18]. Dans la dernière décennie, la peste a réapparu dans des anciens foyers en Algérie [19], Tanzanie [20], Libye [21], ainsi elle a causé des épidémies buboniques et pneumoniques à Madagascar [22] et en République Démocratique du Congo [23]. Concernant la borréliose de Lyme (LB), quelques études basées sur les caractéristiques cliniques et sérologiques, ont soupçonné sa présence en Afrique du Nord [24] [25].

Un des objectifs de notre travail était de compléter le répertoire des bactéries associées aux arthropodes, ainsi que chez les animaux domestiques et sauvages. Par une approche de PCR standard, PCR en temps réel et séquençage, nous avons testé principalement les tiques et les puces collectées sur des animaux domestiques (Bovins, ovins, caprins et chiens) et sauvages [chauves-souris (*Chiroptera spp*), sangliers (*Sus scrofa algira*), mangoustes (*Echinomon herpestis*), porc-épic, hérissons et chacals (*Canis aureus*)] et rongeurs en Algérie, Bénin, Tanzanie et République Démocratique du Congo. Cette partie contribue à l'actualisation et l'enrichissement du répertoire des micro-organismes associés aux arthropodes en Afrique (***Articles N° 2, 3, 4 et 5***).

Dans la deuxième partie nous avons évaluer les compétences vectorielles des puces de chat (*Ctenocephalides felis*) et des

punaises de lit (*Cimex lectularius*) dans la transmission de l'agent responsable de la maladie de la fièvre des tranchées (*Bartonella quintana*) qui est connue pour être transmise par les poux de corps (*Pediculus humanus corporis*). Ces modèles expérimentaux d'infection viennent compléter la première partie, dans laquelle la seule détection de l'ADN du pathogène par outils moléculaire chez l'arthropode ne peut être une preuve de transmission vectorielle. Ces modèles expérimentaux avaient pour objectifs de répondre aux questions de la possibilité des puces et des punaises des lits de transmettre *B. quintana* (***Articles N° 6 et 7***). En effet des travaux antérieurs ont montré la présence de l'ADN de *B. quintana* dans les puces [26,27] et les punaises de lit [28]. Ces questions concernant le pouvoir d'acquisition et la transmission de *B. quintana* par les puces ont été déjà évoquées suite aux rapports de deux cas cliniques d'adénopathies chroniques à *B. quintana,* dont le seul facteur de risque épidémiologique identifié était la présence de puces [29,30]. Pour réaliser ces modèles expérimentaux d'infections nous avons mis au point un élevage artificiel des puces et punaises de lit au laboratoire en premier lieu afin de les exposer par la suite à *B. quintana* via un repas sanguin infecté.

La mise au point des modèles expérimentaux a nécessité de faire le point sur les méthodes d'élevage des arthropodes par des

moyens artificiels sans avoir recours aux animaux de laboratoire. En effet, les animaux de laboratoire comme les serpents, souris, lapins, poulets, porcs et les hamsters ont été fréquemment utilisés comme fournisseurs de sang à ces arthropodes hématophages [31]. L'utilisation d'animaux vivants comporte de nombreuses contraintes, techniques et/ou administratives [32,33]. De ce fait l'élevage des arthropodes hématophages en utilisant des moyens artificiels [34] se présente comme étant la méthode la plus préconisée pour réduire la souffrance impliquée aux animaux dans la recherche biomédicale et ainsi comprendre par la suite plusieurs aspects d'entomologie et de microbiologie. La synthèse de cette recherche a fait l'objet d'une revue soumise à publication (***Article N° 1***).

Une troisième partie a investigué l'application du MALD-TOF à l'entomologie. A la fin des années 1990 l'approche protéomique MALDI-TOF MS a été développée pour identifier des micro-organismes tels que les bactéries, champignons et virus. Grâce à sa sensibilité, sa rapidité et le moindre coût des consommables cette approche est utilisée actuellement en routine dans de nombreux laboratoires de microbiologie clinique [35]. Plus récemment, le MALDI-TOF MS a été évalué pour identifier les organismes multicellulaires notamment les arthropodes [36,37] à partir des protéines extraites d'un organe (généralement les

pattes) ou du corps entier du spécimen [38]. Le MALDI-TOF a été et également utilisé pour déterminer le statut infectieux et identifier les micro-organismes associés aux arthropodes. Dans cette partie, nous exposons l'identification des puces par cet outil (***Article N° 8***) et un travail préliminaire pour l'application de l'outil MALDI-TOF à identification des punaises de lit infectées expérimentalement par *B. quintana*.

REVUE DE LITTÉRATURE

The use of artificial device in the colonization and mass rearing of arthropods.

Hamza LEULMI, Jean Michel Bérenger, Idir Bitam and Philippe PAROLA.

Les agents pathogènes responsables de maladies à transmission vectorielle sont transmis à l'homme et aux animaux par le biais d'arthropodes vecteurs hématophages. L'élevage en laboratoire de ces arthropodes est un facteur important dans le développement des stratégies novatrices pour lutter contre ces vecteurs. Toutefois, cette maintenance au laboratoire a besoin de sang pour rendre ces invertébrés capables de compléter leur cycle de vie. Bien que les animaux vivants soit fréquemment utilisés pour cette procédure d'alimentation, mais certain dispositif artificiel sont disponibles et se présente comme des alternatives potentielles. Depuis peu on assiste à l'émergence d'élevage artificiel basé sur l'appareil pionnier de Rutledge. De nombreux articles ont abordé la faisabilité des élevages artificiels d'arthropodes et une grande proportion d'entre eux ont développé des adaptations créatives pour promouvoir la construction de différents moyens artificiels de nourrissage des arthropodes hématophages et avec des matériaux facilement accessibles [39]. La plupart de ces dispositifs d'alimentation sont constitués d'un élément de chauffage (pour le réchauffement du sang), d'un réservoir pour le sang et d'une membrane artificielle simulant la peau du vertébré. Ces dispositifs révèlent la possibilité du remplacement des animaux vivants comme source de sang pour les vecteurs arthropodes [40] et ainsi ouvre une voie

à la faisabilité de plusieurs modèles expérimentations d'infection. Le but de cette revue est de discuter l'utilisation des méthodes d'alimentation artificielle dans l'élevage des tiques, puces et punaises de lit.

Title: The use of artificial device in the colonization and mass rearing of arthropods.

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Conflict of interests

The authors declared that they have no competing interests.

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ABSTRACT

Pathogens responsible of vector-borne diseases are transmitted to humans by hematophagous arthropods and these blood-sucking organisms are target to researches worldwide.

The laboratory colonization of these species is an important tool for the development of innovative strategies in vector control. However, this maintenance requires blood to make these invertebrates able to complete their life cycle. Although live vertebrate animals are frequently used for this feeding procedure but artificial device are available and increasingly used as potential alternatives to live animals. This review discusses the use of artificial feeding methods for the rearing of the main arthropods vector ticks, fleas and currently and emerging human pest, the bed bugs.

The scientific community focused on vector-borne diseases studies needs to strongly consider these artificial feeding options as a bioethical alternative to maintain blood feeding arthropods in laboratory.

BACKGROUND

In recent years, the scope and importance of emergent vector-borne diseases have increased dramatically (Parola et al., 2013). Pathogens responsible of vector-borne diseases are transmitted to the hosts by hematophagous arthropods which are of great importance in medicine (Parola and Raoult, 2001) and target to researches worldwide (Steen et al., 2004). This explains the growing interest in the breeding of these vector in laboratory to facilitate research under controlled conditions (Costa-da-Silva et al., 2014). Researches benefit tremendously when vector's species can be rearing in laboratory, there by facilitating studies of vector control and associated vector borne diseases (Bailey et al., 1980; Costa-da-Silva et al., 2014).

To complete their life cycle, these arthropods necessarily require a blood meal (Costa-da-Silva et al., 2014). Initially, laboratory animals like snakes, mice, rabbits, chickens, pigs and guinea pigs were frequently used as blood suppliers (Slowik and Lane, 2009). Obviously, the use of live animals for this purpose is now increasingly tedious and only authorized after bioethical certification by animal care committees under frequently revised protocols (Balls, 2015; Burch, 1995), also over the years, there has been a long standing and influential movement within the

scientific community both in Europe and North America to reduce the suffering involved in biomedical research (Burch, 2009). Moreover, studies using natural animal hosts can be impeded by many factors as problem for locate laboratory animal without antibodies presents, immunity reaction developed by animal which induced experimental errors, rapid immunization of the host without sign of illness (Abbassy et al., 1994). To overcome the obstacles associated with animal as blood suppliers, artificial breeding techniques have increased. Over the years, the techniques for feeding arthropods colonies have undergone a several evolutionary changes (Thomas et al., 1985). Main evolution of material relates to feeders and the type of membrane. The first model of feeders are basic and hand-made by astute researchers (Loomis, 1961) and are low cost effective, based on the pioneering apparatus, a Rutledge feeder for the mosquito's maintenance (Rutledge et al., 1964), many articles have addressed this issue and a large proportion of them developed a creative adaptations to promote the construction of different feeders for blood-sucking invertebrate organisms with easily accessible materials. The first artificial feeding of a blood-sucking arthropod (*Glossina palpalis*) accomplished by Rodhain in 1912 and was the membrane technique (Tarshis, 1985). The apparatus may be a simple floating cage (Tarshis, 1985), a food

cup with a membrane floor (Collins et al., 1964) or more sophisticated models with machined parts and a self-contained water bath or incubator (Bar-Zeev and Smith, 1959). Currently, material dedicated exclusively for arthropods feeding are marketed.

In this review each device and method used to maintain ticks, fleas and bed bugs colonies in laboratory will be critically discussed.

EXPOSURE OF THE REVIEW

We have chosen ticks and fleas because they are the major causes of health problems in both humans and animals worldwide (Bitam et al., 2010; Parola et al., 2013) and bed bugs because they are a reemergent human pest since last decade (Delaunay et al., 2011). Ticks, fleas and bed bugs are found in every part of the world and infest every class of wild and domestic animals and also humans .They are important for the maintenance and transmission of many pathogens including several species of bacteria, viruses, protozoa and helminthes causing diseases (Billeter et al., 2008). Ticks are considered to be the second vectors (after mosquitoes) of disease-causing pathogens of

human and the most important vectors of disease-causing pathogens in animals (Mediannikov and Fenollar, 2014; Parola et al., 2013). Likewise, fleas are able to transmit several agents of infectious diseases (Bitam et al., 2010). Researches on bed bugs are increasing in scope (Eddy and Jones, 2011a; Eddy and Jones, 2011b) because this pest have undergone a major resurgence in frequency and in geographic distribution leading to clinical problems (Doggett et al., 2012a).

THE REVIEW

TICKS

Generalities

Ticks are divided in two large families, the Argasidae (soft ticks) and the Ixodidae (hard ticks), with a total of about 850 species (Guglielmone et al., 2010). The two families have a different life cycle (Estrada-Pena and de la Fuente, 2014). Importance of ticks in public health and veterinary medicine explains that ticks are the subject of numerous laboratory studies about their biology, morphology and ability to transmit pathogenic organisms (Estrada-Pena et al., 2013).

General condition of maintenance

The increasing demand for laboratory-reared Argasid and Ixodid ticks for research and control purposes makes it necessary to develop effective and standardized tick feeding methods without using live animals as hosts (Schwan et al., 1991). Each family and species of ticks needs specific method of rearing. Indeed, the life histories of different species of ticks contain so many peculiarities that, although the basic design is similar, rearing procedures must usually be fitted to the species under study. These differences, together with the many subtleties that appear when the parasite meets the host, render tick rearing a challenging and interesting endeavor (Smith, 1966). It was well reviewed by Enigk and Grittner in the rearing of tick using animal models (Enigk and Grittner, 1953). Many of the procedures and conditions that are common to both hard and soft ticks rearing are also listed by (Audy and Lavoipierre, 1964). It necessary here to distinguish argasid ticks, which take a short blood meal on host, leave it and hide in a neighboring place and hard ticks that take a long blood meal of some days, fixed on their hosts (Young et al., 1996). In this review, we focus only on hard ticks, the most difficult arthropods rearing.

In hard ticks, feeding is preceded by an attachment process, and each instars has to remain fully attached to the same feeding site for a period of 2 - 14 days. These biological demands made relatively difficult the development of viable methods where ixodid ticks could be fed without using a natural host (Young et al., 1996). Depending on the mode of host acquisition and feeding behavior patterns, Ixodidae ticks are classified into three categories (Waladde et al., 1996). The first category is based on different behavior for finding the host as active hunting for *Hyalomma* spp, or ambushing, as is the case in *Rhipicephalus* spp. (Waladde et al., 1996). The second category is based on the number of hosts used during the life cycle which includes three stages: larvae, nymph and adult. When there is the successive stages on the same host, it's a monophasic cycle; when larvae and nymphs have their life cycle successively on the same host, adults on another, it's a diphasic cycle; and when each stage have a life cycle independently on different host, it's a triphasic cycle. A third category is based on the range of acceptable hosts. Most ixodid genera show little specificity for particular animal groups (Estrada-Pena et al., 2013) and other close relationship with one animal. It depends on the ticks living environment as animal diversity found in the biotope, seasonal period of activity of these animals and climate of the country (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, there by 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. Many studies have demonstrated that diurnal rhythms and other environmental factors can 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).

Tick-feeding devices

The different feeding methods found in literature include semi-artificial method with capillary tubes feeding and artificial method with various membranes, natural as skins of host (rabbit, mice, bovine) or artificial as silicone membrane or other.

Capillary tubes 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 *Dermacentor andersoni* (Gregson, 1937). Feeding from diet-filled capillary tubes placed over the tick's mouthparts was first reported by (Chabaud, 1950), who used this method to feed *H. excavatum, H. dromedarii* and *R. snnguinens.* (Purnell and Joyner, 1967), used the same technique for *R. appendiculatus.* Later, the method has been used in a variety of studies to infect ticks; *I. ricinus*, *I. hexagonus* and *I. pacificus* ticks with *B. burgdorferi*, and also to introduce *Ehrlichia chaffeensis* into Ixodid ticks (Hu et al., 1992; Monin et al., 1989; Rechav et al., 1999). In this method, tick feeding by capillary methods presents the advantage of using the natural route via the mouthparts and the digestive tract mainly in infections models. However ticks had to be pre-fed on the natural host before putting them on the capillary tube. Ticks could only take in very small volumes of fluid from the capillary and therefore did not feed to repletion (Purnell and Joyner, 1967). This method is more adapted of experimental models and infection of ticks such as *Leptospira pomona* / *Amblyomma maculatum-Dermacentor andersoni* (Burgdorfer (Burgdorfer, 1957), *Theileria parva* / *Rhipicephalus*

appendiculatus (Purnell and Joyner, 1967; Walker et al., 1979), *B. burgdorferi* / *I. ricinus* (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). This technique permits also one to control the amount of fluid ingested by the tick and the titer of the pathogen that enters the tick in the case of infection models. 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.

Natural membranes

In 1956, Pierce and Pierce fed *Boophilus microplus* larvae through the air cell membrane of an embryonated hen egg (Pierce A.E. and Pierce, 1956). In 1975, Kemp et al. (Kemp et al., 1975) achieved >50% molt by engorged *B. microplus* larvae fed on tissue culture medium through skin slices of cattle (Doube and Kemp, 1979) 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* (Voigt et al., 1993; Young et al., 1996), calf mesentery rabbit skin for *A. variegatum 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), mouse skin for *D. andersoni* (Howarth and Hokama, 1983) and *I. scapularis* (Burkot et al., 2001), and gerbil skin for *I. ricinus* (Bonnet et al., 2007).

Artificial membranes

Following on from Waladde's (Waladde et al., 1991; Waladde et al., 1995) use of a biodegradable glue-impregnated Baudruche membrane, the introduction of silicone membranes for hard ticks in 1993 by Habedank and Hiepe led to the next advances (Krober

and Guerin, 2007a; Krober and Guerin, 2007b). These membranes permitted high tick attachment rates, engorgement, detachment, molting of different life stages and oviposition. It was on such a silicone-impregnated membrane that Kuhnert et al. completed the life cycle of *Amblyomma hebraeum* in vitro in 1995 (Kuhnert, 1996; Kuhnert et al., 1995). Subsequently this membrane was modified to accommodate ticks with shorter mouthparts (Krober and Guerin, 2007a; Krober and Guerin, 2007b).

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, 2007a; Krober and Guerin, 2007b; Waladde et al., 1996) modified Baudruche membranes for *R. microplus* (Kemp et al., 1975) and *R. appendiculatus* (Waladde et al., 1991; Young et al., 1996). 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, 2007a), *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. 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). 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), as was *R. appendiculatus* with the same pathogens and a modified Baudruche membrane (Young et al., 1996). In 1993, Wallade 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 successfully using rabbit skin membranes (Musyoki (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; Cotte et al., 2008; Voigt et al., 1993) or the bottom of the tick containment unit (Howarth and Hokama, 1983; Kuhnert et al., 1995; Paine et al., 1983). 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, 2007b), honey jars (Kuhnert et al., 1995) or glass feeders (Bonnet et al., 2007). 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). 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, also used cattle/tick washes and tick feces (Young et al., 1996). However, (De Moura et al., 1997) demonstrated that for *A. cajennense* silicone membranes treated with blood vestiges was more efficient than other tested phago-stimulants. Finally, adenosine triphosphate (ATP) and reduced glutathione have also been used as phago-stimulants (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.

FLEAS

Generalities

Fleas (order Siphonaptera) are holometabolous obligate blood-feeding ectoparasites of birds and mammals; the immature stages (eggs, larvae and pupa) are found in burrows or nests, whereas adult's fleas are usually found on animal hosts (Bitam et al., 2010; Dobler and Pfeffer, 2011; Traversa, 2013). Fleas are normally considered as ectoparasites of dogs and cats, however, they readily infest and feed on other species of domestic animals, including birds, and humans (Dobler and Pfeffer, 2011). Main genera of fleas don't need an attachment to the host; blood meals are short but frequent, a feature to take into account for a breeding… Fleas are important vectors and reservoirs of several pathogens that cause emerging or re-emerging infectious diseases such as *Yersinia pestis* (the agent of plague), *Rickettsia typhi* (the agent of murin typhus), *Rickettsia felis* (spotted fever), *Bartonella henselae* (cat scratch diseases) and other dangerous pathogens (Eisen and Gage, 2012). In general, rearing insects including fleas in the laboratory is recognized as the first

essential step to study the biology, toxicology and vector role. The rapid strides in the field of biological control of insect's vectors of diseases have necessitated studies of techniques for the mass rearing of fleas (Smith, 1966).

General condition of maintenance

The oriental rat flea (*Xenopsylla cheopis*) and the cat flea (*Ctenocephalides felis*) were the well suited for mass rearing (Felcetto et al., 2002; Schotthoefer et al., 2011). Before a simple and artificial technique suitable for this purpose is described, the experience of number of investigators who raised flea cultures in the past will be reviewed briefly to extract the general conditions of flea's maintenance. The first recorded attempt to breed fleas in the laboratory was in 1683 by Leeuwenhoek (Smith, 1966). Ever since this day, scientists have been attempting breeding of fleas in the laboratory. (Sikes, 1930) has briefly discussed the efforts of these naturalists. Bacot (Bacot, 1912; Bacot, 1914) has given detailed account of the studies he carried out regarding breeding of rat fleas and their bionomics. He raised colonies of *X. cheopis* in Poona, India, during the period 1900 to 1912, when he studied the biology of this species with special reference to factors such as temperature and humidity conditions influencing

rate of reproduction, length of life, variation in their rate of growth, and pupation.

Method of feeding

Methods for the laboratory rearing of fleas species, such as *C. f. felis*, *Xenopsylla cheopis*, *Xenopsylla astia* (Siphonaptera: Pulicidae) and *Leptopyslla segnis* (Siphonaptera: Leptopsyllidae), have already been described, but all of these methods rely on live hosts for maintenance, mainly cats, mice, rats, hamsters and guinea pigs (Hudson and Prince, 1958a; Hudson and Prince, 1958b). Over the last 50 years, efforts have been made to develop methods for the *in vitro* feeding and, ultimately, the mass rearing of medically and economically important species of flea, especially *C. f. felis* (Thomas et al., 2004). The introduction of an artificial rearing method by (Wade and Georgi, 1988), which represented an adaptation of the Rutledge insect blood feeder (Rutledge et al., 1964), removed the need to use live hosts for flea production. (Kartman, 1954) described an apparatus for the experimental feeding of fleas in relation to studies on *Pasteurella pestis*. The apparatus is based essentially upon the principle of transferring controlled thermal gradients from a water bath to a metal dish containing a liquid upon which fleas are allowed to feed through an animal skin

membrane, which is in direct contact with the fluid. The feeding is accomplished in a "thermostabilizer," made of brass parts, which transfers the heat to the liquid food, maintains a constant thermal level, and allows the skin membrane to contact the food while preventing it from being upset (Kartman, 1954). The thickness of the skin membrane of this system is probably a limiting factor in the ability of fleas to feed. The age of the blood and the effects of freezing it had little influence on flea feeding responses provided that a favorable skin membrane was used. It is suggested that the apparatus may be used in an experimental approach to basic problems of flea physiology, nutrition, and disease transmission.

Machine of Wade and Georgie 1988

The so-called 'artificial dog' is a feeding system commonly used to breed cat fleas (Wade (Wade and Georgi, 1988) . Wade and Georgi described the artificial feeding system for adult cat fleas, *Ctenocephalides felis* (Bouché). Survival and reproductive success of artificially fed fleas were attributed to continuous provision of blood at 37°C and a substrate that allowed the fleas to walk but not to jump. Fleas were confined for artificial feeding in three different kinds of cages, all of which were cylinders of

clear plastic with nylon mesh cemented across both ends. The cages differed in dimensions and in the inclusion or lack of hair. The upper end of the cage was opposed to a Rutledge insect blood feeder supplied with a Parafilm membrane through which the fleas were able to feed on cattle or dog blood. Artificially fed fleas yielded equivalent survival rates and 13–19% the reproductive output of fleas fed on cats. This system in conjunction with standard larval and pupal rearing techniques has resulted in a colony that has completed 14 artificially reared generations at the time of writing (Wade and Georgi, 1988).

BED BUGS

Cimex lectularius and *C. hemipterus* (Cimicidae: Hemiptera), commonly called bed bugs, continue to increase in scope (Doggett et al., 2012a; Eddy and Jones, 2011a). In recent years, these hematophagous arthropods have undergone a major resurgence in frequency and in geographic distribution leading to clinical problems. An increasing number of infestations have been reported in Europe (Doggett et al., 2012a; Doggett et al., 2012b; Masetti and Bruschi, 2007; Paul and Bates, 2000), America (Hwang et al., 2005), Australia (Doggett et al., 2012a), Asia (How and Lee, 2010; Lee et al., 2008) and Africa (Omudu

and Kuse, 2010). A bite causing cutaneous lesions is the most common clinical consequence of bed bugs on public health. In addition, mental health can be affected by knowledge of a bed bug infestation in one's living environment (Doggett et al., 2012a). Bed bugs are suspected of transmitting infectious agents; however there is little evidence that such transmission has ever occurred. More than 45 pathogens associated with human infection and disease have been suspected to be transmitted by bed bugs (Delaunay et al., 2011). These hematophagous Heteroptera, feeding on human blood, have been the subject of significant medical investigation. In particular, they have been colonized under laboratory conditions to study their medical relevance (Cannet et al., 2015).

General condition of maintenance

The bed bug resurgence has led to an increase in both basic and applied research directed at biology and control of this pest. The increased research efforts have created a subsequent need for producing sufficient numbers of consistent quality biological specimens for empirical tests (Smith, 1966). Bed bugs are obligate, blood-feeding insects. Each of five nymphal stages requires a blood meal to molt and reach the next nymphal or the adult stage, and adults require periodic blood meals to reproduce

(Delaunay et al., 2011). Therefore, the blood-feeding of laboratory colonies is a critical component of any laboratory research program designed around bed bugs. Previous studies have demonstrated the adaptability of bed bugs to a wide range of environmental factors (Cannet et al., 2015). Laboratory colonies should experimentally reproduce the natural living conditions in a controlled environment to obtain results in scientific research (Cannet et al., 2015). That is because necessary to find a balance between the natural environment and laboratory condition for the rearing of bed bugs over long term (Cannet et al., 2015).

Method of feeding

In the past decades, and as with all hematophagous arthropods, bed bugs were reared in vivo on animals, chickens (Pfiester et al., 2008), rabbits (Reinhardt et al., 2003; Stutt and Siva-Jothy, 2001), mice and pigeons,(Araujo et al., 2009) and on humans (Moore and Miller, 2006; Wintle and Reinhardt, 2008). In vitro feeding has also utilized, where small glass vials stoppered with cotton or covered with bolting cloth have been used as rearing chambers for bed (Girault, 1910). Later different blood or blood products were used, blood of cattle (Montes et al., 2002),

chickens (Montes et al., 2002; Romero et al., 2009), Rabbits (Romero et al., 2009), sheep (Montes et al., 2002) and humans (Moore and Miller, 2006). Most Current in vitro feeding techniques are derived from methods developed by Montes et al 2002 (Montes et al., 2002), or modifications of methods originally developed for head lice or mosquitoes using Hemotek and membranes (Leulmi et al., 2015; Takano-Lee et al., 2003a; Takano-Lee et al., 2003b). Other in vitro methods have been used prior to this time though most were designed for either feeding individual bugs or for specific feeding experiments and not for colony maintenance (Rivnay, 1930). During the in vitro feeding, the blood meal is warmed to 37°C by circulating heated water through a jacketed feeding vessel (Montes et al., 2002) or by bloc electrically heated the blood as in Hemotek (Leulmi et al., 2015).The importance of warming the blood source to stimulate bed bug feeding has been previously noted (Montes et al., 2002). We focused on the details of two artificial devices to feed bed bugs, the Montes's machine and the Hemotek.

The Montes's machine

The system consisted of a water bath, glass feeder (10 cm height x 6 cm diameter) with a stretched parafilm M (American National Can, Chicago, IL) membrane placed at the bottom and

a pump with pipe to circulate the warm water. Blood meal was maintained at 37°C and monitored regularly using infrared thermometer (Montes et al., 2002).

Montes et al (Montes et al., 2002) described by this machine the maintenance technique which has been used successfully to rear *Cimex lectularius* (L.) by feeding for more than 2 years rall nymphal stages and adults through paraphilm "M" sealing on different types of blood. Using this feeding technique, the subsequent egg production of female bed bugs was remarkably high. The blood was maintained at 37°C to enhance the attachment of the bugs. The effect of anticoagulation methods for the blood meal was also investigated, and the heparinized blood was found the most suitable for feeding bed bugs. All stages of the bugs fed weekly on blood in the artificial feeding system remained attached for up to 0.5h -1.0 h, until completion of their blood meals, and all reached engorged weights. More than 90% of the bugs fed artificially on whole blood, and they molted or laid eggs successfully (Montes et al., 2002).

Hemotek

This machine was firstly invited to the mass colonies of mosquitoes mainly Aedes and Anopheles mosquito's genera (Damiens et al., 2013). Many laboratories that are currently

authorized to use animals for feeding insects are switching to the Hemotek system as a preferred method using either blood or formulated meal. The durable, plastic body of the Feeder houses an accurate, electronic temperature control and heater and is fitted with 1 meter of cable which plugs into the front panel of the power unit (**figure 1**). The feeder has a meal reservoir which is removable for easy cleaning and preparation. The meal reservoir can be autoclaved if necessary with facilities (Cosgrove et al., 1994).The operating temperature of each feeder can be adjusted to suit the preferences of different insect species; this precise control is an important feature to many experimental procedures. Synthetic or natural membranes can be used with the feeders. The choice will depend on species preference and research criteria (Waka et al., 2004). The collagen membrane provided with the system is easy to prepare and is ideal for certain species. Hemotek was used in routine in the mass rearing of bed bugs (Leulmi et al., 2015; Sheele et al., 2013). Since 2012, bed bugs (*Cimex lectularius*) have been maintained in a laboratory insectarium at the WHO collaborative center for rickettsioses and other arthropod borne bacterial diseases in Marseille, France (Leulmi et al., 2015). They were maintained in containers kept in incubator at 60% humidity and 22°C (figure 2). The bed bugs were fed once a week using citrated human

blood. Two mL of blood was placed in a Hemotek artificial feeder machine (Hemotek 5W1; Discovery Workshops, Accrington, UK) covered by an artificial membrane of Parafilm M (Sigma-Aldrich, Saint-Louis, Missouri, USA) that was stretched to the twice of its length and width (Leulmi et al., 2015).

CONCLUSION

Recent developments in entomological research have augmented the importance of arthropods hematophagous colonies to basic research and practical control. Students in such field as arthropod physiology, behavior, and genetics need plentiful supplies of specimens of standard quality, such as can be obtained only by rearing strains of known ancestry under controlled regimens of nutrition, temperature, and photoperiod. Control methods now in use, or foreseen for the future, such as the release of sterile males, the extensive distribution of insects pathogens, the practical use of pheromones, and the genetic manipulation of natural populations, will require the production of millions of arthropods per week for the control of certain economically important species.

Within recent years great strides have been made in the culture of some of the important species, whereas other equally important groups cannot yet be maintained in even small self-perpetuating laboratory colonies. Initially, laboratory animals such as snake, mice, rabbits, chickens, pigs and hamsters were frequently used as blood suppliers to these blood-sucking arthropods. The use of live animals for this purpose requires permission and bioethics certification, making more and more difficult the use of animals for experimental purposes. Thus the breeding of bloodsucking arthropods using artificial device purports to be the most preferred method to reduce the suffering of animals involved in biomedical research and thus understand later the aspects of entomology, microbiology and vector-borne diseases.

In this review, we selected ticks, fleas and bed bugs to discuss their mass colonies using artificial devices. Some of the *in vitro* feeding tick's methods tried including the use of capillary tubes feeding, feeding ticks through various membranes such as slices of bovine skin, and silicone membrane and other membranes. Where the dog machine is the best artificial device used to rear fleas, finally the Hemotek machine was shown to be useful in the colonies of bed bugs using artificial devices.

This set up has the intention to bring awareness for the feasibility of artificial feeders utilization in entomology laboratories and finally the research community could start to change the way they perform their experiments over time.

References

1. Abbassy, M.M., Stein, K.J., Osman, M., 1994. New artificial feeding technique for experimental infection of Argas ticks (Acari: Argasidae). J. Med. Entomol. 31, 202-205.

2. Araujo, R.N., Costa, F.S., Gontijo, N.F., Goncalves, T.C., Pereira, M.H., 2009. The feeding process of Cimex lectularius (Linnaeus 1758) and Cimex hemipterus (Fabricius 1803) on different bloodmeal sources. J Insect Physiol 55, 1151-1157.

3. Audy, J.R., LAVOIPIERRE, M.M., 1964. The laboratory rearing of parasitic acarina. A general review. Bull. World Health Organ 31, 583-586.

4. Bacot, A.N., 1912. Observation on flea breeding in Poona. J Hyg (Lond) 12, 300-325.

5. Bacot, A.N., 1914. Study of bionomics of common rat fleas. J. Hyg. 14, 447-634.

6. Bailey, D.L., Lowe, R.E., Dame, D.A., Seawright, J.A., 1980. Mass rearing the genetically altered MACHO strain of Anopheles albimanus Wiedemann. Am. J. Trop. Med. Hyg. 29, 141-149.

7. Balls, M., 2015. To be credible, success in "reducing the use of animals in scientific research" must involve the use of fewer animals. Altern. Lab Anim 43, 75-77.

8. Bar-Zeev, M., Smith, C.N., 1959. Action of repellents on mosquitoes feeding through treated membranes or on treated blood. J Econ Ent 52, 263-267.

9. Bianchi, M.W., Barre, N., 2003. Factors affecting the detachment rhythm of engorged Boophilus microplus female ticks (Acari: Ixodidae) from Charolais steers in New Caledonia. Vet. Parasitol. 112, 325-336.

10. Billeter, S.A., Levy, M.G., Chomel, B.B., Breitschwerdt, E.B., 2008. Vector transmission of Bartonella species with emphasis on the potential for tick transmission. Med. Vet. Entomol. 22, 1-15.

11. Bitam, I., Dittmar, K., Parola, P., Whiting, M.F., Raoult, D., 2010. Fleas and flea-borne diseases. Int. J. Infect. Dis. 14, e667-e676.

12. Bonnet, S., Jouglin, M., Malandrin, L., Becker, C., Agoulon, A., L'Hostis, M., Chauvin, A., 2007. Transstadial and transovarial persistence of Babesia divergens DNA in Ixodes ricinus ticks fed on infected blood in a new skin-feeding technique. Parasitology 134, 197-207.

13. Booth, T.F., Steele, G.M., Marriott, A.C., Nuttall, P.A., 1991. Dissemination, replication, and trans-stadial persistence of Dugbe virus (Nairovirus, Bunyaviridae) in the tick vector Amblyomma variegatum. Am. J Trop. Med. Hyg. 45, 146-157.

14. Broadwater, A.H., Sonenshine, D.E., Hynes, W.L., Ceraul, S., De, S.A., 2002. Glass capillary tube feeding: a method for infecting nymphal Ixodes scapularis (Acari: Ixodidae) with the lyme disease spirochete Borrelia burgdorferi. J Med. Entomol. 39, 285-292.

15. Burch, R.L., 1995. The progress of humane experimental technique since 1959: a personal view. Altern. Lab Anim 23, 776-783.

16. Burch, R.L., 2009. The progress of humane experimental technique since 1959: a personal view. Altern. Lab Anim 37, 269-275.

17. Burgdorfer, W., 1957. Artificial feeding of ixodid ticks for studies on the transmission of disease agents. J Infect. Dis. 100, 212-214.

18. Burkot, T.R., Happ, C.M., Dolan, M.C., Maupin, G.O., 2001. Infection of Ixodes scapularis (Acari: Ixodidae) with Borrelia burgdorferi using a new artificial feeding technique. J Med. Entomol. 38, 167-171.

19. Cannet, A., Akhoundi, M., Berenger, J.M., Michel, G., Marty, P., Delaunay, P., 2015. A review of data on laboratory colonies of bed bugs (Cimicidae), an insect of emerging medical relevance. Parasite 22, 21.

20. Chabaud, A.G., 1950. Sur la nutrition artificielle des tiques. Ann. Parasitol . Hum. Comp 25, 142-147.

21. Collins, W.E., Harisson, A.J., Skinner, J.C., 1964. The use of membrane feeding technique to determine infection and transmission threshold of Semliki Forest virus to *Anopheles quadrimaculatus* and *Anopheles albimanus*. Mosq News 24, 25-27.

22. Cosgrove, J.B., Wood, R.J., Petric, D., Evans, D.T., Abbott, R.H., 1994. A convenient mosquito membrane feeding system. J Am. Mosq Control Assoc. 10, 434-436.

23. Costa-da-Silva, A.L., Carvalho, D.O., Burini Kojin, B., Capurro, M.L., 2014. Implementation of the artificial feeders in hematophagous arthropod research cooperates to the vertebrate animal use replacement, reduction and refinement (3Rsprinciple). J Clin Res Bioeth 5, 1-3.

24. Cotte, V., Bonnet, S., Le, R.D., Le, N.E., Chauvin, A., Boulouis, H.J., Lecuelle, B., Lilin, T., Vayssier-Taussat, M., 2008. Transmission of Bartonella henselae by Ixodes ricinus. Emerg. Infect. Dis. 14, 1074-1080.

25. Damiens, D., Soliban, S.M., Balestrino, F., Alsir, R., Vreysen, M.J., Gilles, J.R., 2013. Different blood and sugar feeding regimes affect the productivity of Anopheles arabiensis colonies (Diptera: Culicidae). J Med. Entomol. 50, 336-343.

26. De Moura, S.T., da Fonseca, A.H., Fernandes, C.G., Butler, J.F., 1997. Artificial feeding of Amblyomma cajennense (Fabricius, 1787) (Acari:Ixodidae) through silicone membrane. Mem. Inst. Oswaldo Cruz 92, 545-548.

27. Delaunay, P., Blanc, V., del, G.P., Levy-Bencheton, A., Chosidow, O., Marty, P., Brouqui, P., 2011. Bedbugs and infectious diseases. Clin. Infect. Dis. 52, 200-210.

28. Dobler, G., Pfeffer, M., 2011. Fleas as parasites of the family Canidae. Parasit. Vectors. 4, 139.

29. Doggett, S.L., Dwyer, D.E., Penas, P.F., Russell, R.C., 2012a. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25, 164-192.

30. Doggett, S.L., Dwyer, D.E., Penas, P.F., Russell, R.C., 2012b. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25, 164-192.

31. Doube, B.M., Kemp, D.H., 1979. The influence of temperature, relative humidity and host factors on the attachment and survival of Boophilus microplus (Canestrini) larvae to skin slices. Int. J. Parasitol. 9, 449-454.

32. Eddy, C., Jones, S.C., 2011a. Bed bugs, public health, and social justice: Part 1, A call to action. J. Environ. Health 73, 8-14.

33. Eddy, C., Jones, S.C., 2011b. Bed bugs, public health, and social justice: Part 2, An opinion survey. J. Environ. Health 73, 15-17.

34. Eisen, R.J., Gage, K.L., 2012. Transmission of flea-borne zoonotic agents. Annu. Rev. Entomol. 57, 61-82.

35. Enigk, K., GRITTNER, I., 1953. [The rearing and biology of ticks]. Z. Parasitenkd. 16, 56-83.

36. Estrada-Pena, A., de la Fuente, J., 2014. The ecology of ticks and epidemiology of tick-borne viral diseases. Antiviral Res. 108, 104-128.

37. Estrada-Pena, A., Gray, J.S., Kahl, O., Lane, R.S., Nijhof, A.M., 2013. Research on the ecology of ticks and tick-borne pathogens--methodological principles and caveats. Front Cell Infect. Microbiol. 3, 29.

38. Felcetto, T., Ondeyka, J., Colletti, S.L., Meinke, P.T., Shoop, W.L., 2002. Comparison of nodulisporic acid analogs in a

Lucilia sericata in vitro assay and a Ctenocephalides felis membrane feeding system. J Parasitol 88, 223-226.

39. Girault, A.A., 1910. Preliminary studieson on the biology of the bed bugs, Cimex lectularius Linn. J. Econ. Biol 5, 88-91.

40. Gregson, J.D., 1937. Notes on some phenomenal feeding of ticks. B. C. Entotnological Society 34, 8-12.

41. Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Horak, I.G., Barker, S.C., 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa, 1-28.

42. How, Y.F., Lee, C.Y., 2010. Survey of bed bugs in infested premises in Malaysia and Singapore. J. Vector. Ecol. 35, 89-94.

43. Howarth, J.A., Hokama, Y., 1983. Artificial feeding of adult and nymphal Dermacentor andersoni (Acari: Ixodidae) during studies on bovine anaplasmosis. J Med. Entomol. 20, 248-256.

44. Hu, C.M., Gern, L., Aeschlimann, A., 1992. Changes in the protein profile and antigenicity of different Borrelia burgdorferi strains after reintroduction to Ixodes ricinus ticks. Parasite Immunol. 14, 415-427.

45. Hudson, B.W., Prince, F.M., 1958a. A method for large-scale rearing of the cat flea, Ctenocephalides felis felis (Bouche). Bull. World Health Organ 19, 1126-1129.

46. Hudson, B.W., Prince, F.M., 1958b. Culture methods for the fleas Pulex irritans (L.) and Pulex simulans (Baker). Bull. World Health Organ 19, 1129-1133.

47. Hwang, S.W., Svoboda, T.J., De Jong, I.J., Kabasele, K.J., Gogosis, E., 2005. Bed bug infestations in an urban environment. Emerg. Infect. Dis. 11, 533-538.

48. Kartman, L., 1954. Studies on Pasteurella pestis in fleas. 1. An apparatus for the experimental feeding of fleas. Exp. Parasitol 3, 525-537.

49. Kemp, D.H., Koudstaal, D., Roberts, J.A., Kerr, J.D., 1975. Feeding of *Boophilus microplus* larvae on a partially defined medium through thin slices of cattle skin. Parasitology 70, 243-254.

50. Kocan, K.M., Yoshioka, J., Sonenshine, D.E., de la Fuente, J., Ceraul, S.M., Blouin, E.F., Almazan, C., 2005. Capillary tube feeding system for studying tick-pathogen interactions of Dermacentor variabilis (Acari: Ixodidae) and Anaplasma marginale (Rickettsiales: Anaplasmataceae). J Med. Entomol. 42, 864-874.

51. Korshus, J.B., Munderloh, U.G., Bey, R.F., Kurtti, T.J., 2004. Experimental infection of dogs with Borrelia burgdorferi sensu stricto using Ixodes scapularis ticks artificially infected by capillary feeding. Med. Microbiol. Immunol. 193, 27-34.

52. Krober, T., Guerin, P., 2007a. The tick blood meal: from a living animal or from a silicone membrane? ALTEX. 24 Spec No, 39-41.

53. Krober, T., Guerin, P.M., 2007b. In vitro feeding assays for hard ticks. Trends Parasitol. 23, 445-449.

54. Kuhnert, F., 1996. Feeding of Hard Ticks In Vitro: New Perspectives for Rearing and for the Identification of Systemic Acaricides. ALTEX. 13, 76-87.

55. Kuhnert, F., Diehl, P.A., Guerin, P.M., 1995. The life-cycle of the bont tick Amblyomma hebraeum in vitro. Int. J. Parasitol. 25, 887-896.

56. Lee, I.Y., Ree, H.I., An, S.J., Linton, J.A., Yong, T.S., 2008. Reemergence of the bedbug *Cimex lectularius* in Seoul, Korea. Korean J. Parasitol. 46, 269-271.

57. Leulmi, H., Bitam, I., Berenger, J.M., Lepidi, H., Rolain, J.M., Almeras, L., Raoult, D., Parola, P., 2015. Competence of *Cimex lectularius* Bed Bugs for the Transmission of *Bartonella quintana*, the Agent of Trench Fever. PLoS. Negl. Trop. Dis. 9, e0003789.

58. Loomis, E.C., 1961. Life histories of ticks under laboratory conditions (Acarina: Ixodidae and Argasidae). J. Parasitol. 47, 91-99.

59. Macaluso, K.R., Sonenshine, D.E., Ceraul, S.M., Azad, A.F., 2001. Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* ticks acquired by artificial feeding. Vector. Borne. Zoonotic. Dis. 1, 45-53.

60. Masetti, M., Bruschi, F., 2007. Bed bug infestations recorded in Central Italy. Parasitol. Int. 56, 81-83.

61. Mediannikov, O., Fenollar, F., 2014. Looking in ticks for human bacterial pathogens. Microb. Pathog. 77, 142-148.

62. Monin, R., Gern, L., Aeschlimann, A., 1989. A study of the different modes of transmission of *Borrelia burgdorferi* by *Ixodes ricinus*. Zentbl Bakteriol Suppl 18, 14-20.

63. Montes, C., Cuadrillero, C., Vilella, D., 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera: Cimicidae) using an artificial feeding technique. J Med. Entomol. 39, 675-679.

64. Moore, D.J., Miller, D.M., 2006. Laboratory evaluations of insecticide product efficacy for control of *Cimex lectularius*. J Econ Entomol. 99, 2080-2086.

65. Musyoki, J.M., Osir, E.O., Kiara, H.K., Kokwaro, E.D., 2004. Comparative studies on the infectivity of *Theileria parva* in ticks fed in vitro and those fed on cattle. Exp. Appl. Acarol. 32, 51-67.

66. Omudu, E.A., Kuse, C.N., 2010. Bed bug infestation and its control practices in Gbajimba: a rural settlement in Benue state, Nigeria. J. Vector. Borne. Dis. 47, 222-227.

67. Paine, S.H., Kemp, D.H., Allen, J.R., 1983. In vitro feeding of *Dermacentor andersoni* (Stiles): effects of histamine and other mediators. Parasitology 86 (Pt 3), 419-428.

68. Parola, P., Paddock, C.D., Socolovschi, C., Labruna, M.B., Mediannikov, O., Kernif, T., Abdad, M.Y., Stenos, J., Bitam, I., Fournier, P.E., Raoult, D., 2013. Update on tick-borne rickettsioses around the world: a geographic approach. Clin. Microbiol. Rev. 26, 657-702.

69. Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32, 897-928.

70. Paul, J., Bates, J., 2000. Is infestation with the common bedbug increasing? BMJ 320, 1141.

71. Pfiester, M., Koehler, P.G., Pereira, R.M., 2008. Ability of bed bug-detecting canines to locate live bed bugs and viable bed bug eggs. J Econ Entomol. 101, 1389-1396.

72. Pierce A.E., Pierce, M.H., 1956. note on the cultivation of *Boophilus microplus* (Canestrini, 1887) (Ixodidae:Acarina) on the embryonated hen egg. Aust. Vet. J. 32, 144-146.

73. Purnell, R.E., Joyner, L.P., 1967. Artificial feeding technique for *Rhipicephalus appendiculatus* and the transmisison of *Theileria parva* from the salivary secretion. Nature 216, 484-485.

74. Rechav, Y., Zyzak, M., Fielden, L.J., Childs, J.E., 1999. Comparison of methods for introducing and producing artificial infection of ixodid ticks (Acari: Ixodidae) with Ehrlichia chaffeensis. J Med. Entomol. 36, 414-419.

75. Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. Proc. Biol. Sci. 270, 2371-2375.

76. Rivnay, E., 1930. Techniques in artificial feeding of the bed bug, *Cimex lectularius L.* Parasitol. 16, 246-249.

77. Romero, A., Potter, M.F., Haynes, K.F., 2009. Behavioral responses of the bed bug to insecticide residues. J Med. Entomol. 46, 51-57.

78. Rutledge, L.C., Ward, R.A., Gould, D.J., 1964. Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. Mosq News 24, 407-419.

79. Schotthoefer, A.M., Bearden, S.W., Holmes, J.L., Vetter, S.M., Montenieri, J.A., Williams, S.K., Graham, C.B., Woods, M.E., Eisen, R.J., Gage, K.L., 2011. Effects of temperature on the transmission of *Yersinia Pestis* by the flea, *Xenopsylla Cheopis*, in the late phase period. Parasit. Vectors. 4, 191.

80. Schwan, E.V., Hutton, D., Shields, K.J., Townson, S., 1991. Artificial feeding and successful reproduction in Ornithodoros moubata moubata (Murray, 1877) (Acarina: Argasidae). Exp. Appl. Acarol. 13, 107-115.

81. Sheele, J.M., Anderson, J.F., Tran, T.D., Teng, Y.A., Byers, P.A., Ravi, B.S., Sonenshine, D.E., 2013. Ivermectin causes *Cimex lectularius* (bed bug) morbidity and mortality. J Emerg. Med. 45, 433-440.

82. Sikes, E.K., 1930. Larvae of *Ceratophyllus wickami* and other species of fleas. Parasitology 22, 242-259.

83. Slowik, T.J., Lane, R.S., 2009. Feeding preferences of the immature stages of three western north American ixodid ticks (Acari) for avian, reptilian, or rodent hosts. J. Med. Entomol. 46, 115-122.

84. Smith, C.N., 1966. Insect Colonization and Mass Production. Edited by Carroll N. Smith. [With Illustrations.]. Academic Press.

85. Steen, C.J., Carbonaro, P.A., Schwartz, R.A., 2004. Arthropods in dermatology. J. Am. Acad. Dermatol. 50, 819-42, quiz.

86. Stone, B.F., Commins, M.A., Kemp, D.H., 1983. Artificial feeding of the Australian paralysis tick, *Ixodes holocyclus* and collection of paralysing toxin. Int. J Parasitol 13, 447-454.

87. Stutt, A.D., Siva-Jothy, M.T., 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. Proc. Natl. Acad. Sci. U. S. A 98, 5683-5687.

88. Tajeri, S., Razmi, G.R., 2011. *Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* (Acari: Ixodidae) imbibe bovine blood in vitro by utilizing an artificial feeding system. Vet. Parasitol 180, 332-335.

89. Takano-Lee, M., Velten, R.K., Edman, J.D., Mullens, B.A., Clark, J.M., 2003a. An automated feeding apparatus for in vitro maintenance of the human head louse, *Pediculus capitis* (Anoplura: Pediculidae). J Med. Entomol. 40, 795-799.

90. Takano-Lee, M., Yoon, K.S., Edman, J.D., Mullens, B.A., Clark, J.M., 2003b. In vivo and in vitro rearing of *Pediculus humanus capitis* (Anoplura: Pediculidae). J Med. Entomol. 40, 628-635.

91. Tarshis, B., 1985. Feeding techniques for bloodsucking arthropods. In: Montreal, pp. 767-783.

92. Thomas, J.A., Bailey, D.L., Dame, D.A., 1985. Maintenance of *Anopheles albimanus* on frozen blood. J. Am. Mosq. Control Assoc. 1, 538-540.

93. Thomas, R.E., Macaluso, K.R., Azad, A.F., 2004. Maintenance, and experimental infection of fleas (Siphonaptera). In: Marquardt, M.W. (Ed.). Dreibelbis,D.

94. Traversa, D., 2013. Fleas infesting pets in the era of emerging extra-intestinal nematodes. Parasit. Vectors. 6, 59.

95. Voigt, W.P., Young, A.S., Mwaura, S.N., Nyaga, S.G., Njihia, G.M., Mwakima, F.N., Morzaria, S.P., 1993. In vitro feeding of instars of the ixodid tick *Amblyomma variegatum* on skin membranes and its application to the transmission of Theileria mutans and Cowdria ruminatium. Parasitology 107 (Pt 3), 257-263.

96. Wade, S.E., Georgi, J.R., 1988. Survival and reproduction of artificially fed cat fleas, Ctenocephalides felis Bouche (Siphonaptera: Pulicidae). J Med. Entomol. 25, 186-190.

97. Waka, M., Hopkins, R.J., Curtis, C., 2004. Ethnobotanical survey and testing of plants traditionally used against hematophagous insects in Eritrea. J Ethnopharmacol. 95, 95-101.

98. Waladde, S.M., Ochieng', S.A., Gichuhi, P.M., 1991. Artificial-membrane feeding of the ixodid tick, *Rhipicephalus appendiculatus*, to repletion. Exp. Appl. Acarol. 11, 297-306.

99. Waladde, S.M., Young, A.S., Morzaria, S.P., 1996. Artificial feeding of ixodid ticks. Parasitol. Today 12, 272-278.

100. Waladde, S.M., Young, A.S., Mwaura, S.N., Njihia, G.N., Mwakima, F.N., 1995. Optimization of the in vitro feeding of *Rhipicephalus appendiculatus* nymphae for the transmission of Theileria parva. Parasitology 111 (Pt 4), 463-468.

101. Waladde, S.M., Young, A.S., Ochieng, S.A., Mwaura, S.N., Mwakima, F.N., 1993. Transmission of *Theileria parva* to cattle by *Rhipicephalus appendiculatus* adults fed as nymphae in vitro on infected blood through an artificial membrane. Parasitology 107 (Pt 3), 249-256.

102. Walker, A.R., Brown, C.G., Bell, L.J., McKellar, S.B., 1979. Artificial infection of the tick *Rhipicephalus appendiculatus* with *Theileria parva*. Res Vet. Sci. 26, 264-265.

103. Wintle, K., Reinhardt, K., 2008. Temporary feeding inhibition caused by artificial abdominal distension in the bedbug, *Cimex lectularius*. J Insect Physiol 54, 1200-1204.

104. Young, A.S., Waladde, S.M., Morzaria, S.P., 1996. Artificial feeding systems for ixodid ticks as a tool for study of pathogen transmission. Ann. N. Y. Acad. Sci. 791, 211-218.

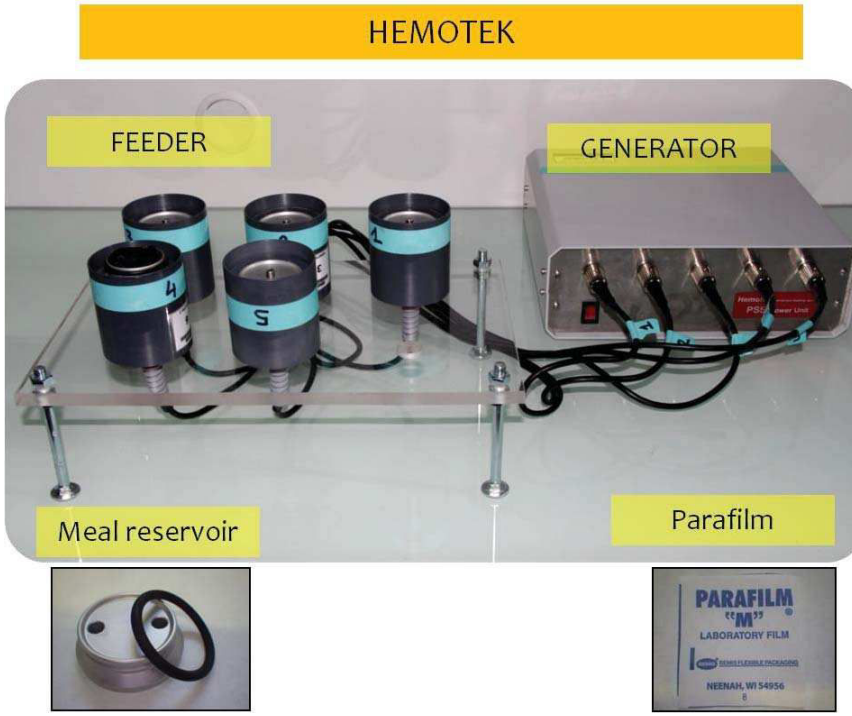

Figure1: The composition of the machine Hemotek

Figure 2: Complete feeding *of Cimex lectularius* using the Hemotek machine

II - ÉPIDEMIOLOGIE MOLÉCULAIRE DES BACTÉRIES TRANSMISES PAR LES ARTHROPODES.

Les puces (Insecta, Siphonaptera) sont des insectes de petite taille, aptères et aplatis latéralement. Plus de 2574 espèces appartenant à 16 familles et 238 genres sont décrites à ce jour [41]. Les puces sont d'une importance médicale et économique considérable en tant que vecteurs d'agents de plusieurs maladies humaines. La peste, causée par *Yersinia pestis*, est la plus connue et la plus redoutée [42]. Les puces sont également associées à d'autres maladies comme le typhus murin à *Rickettsia typhi*. Ces dernières années, la fièvre boutonneuse à puces, causée par *Rickettsia felis* a émergé et se retrouve quasiment dans le monde entier [41]. Enfin, les puces peuvent transmettre des Bartonella, à savoir *Bartonella henselae*, *B. quintana*, *Bartonella* sp et autres.

Les tiques sont des arthropodes hématophages considérées comme les plus importants vecteurs d'agents de maladies humaines et animales. Elles peuvent transmettre un large éventail d'agents pathogènes, notamment des virus, protozoaires et bactéries [5].

*** Investigations en Afrique Sub-saharienne (Bénin, Tanzanie et République Démocratique du Congo)**

Un premier travail (***Article N° 2)*** a été réalisé sur 550 puces collectées sur des rongeurs par nos collaborateurs au Bénin (Cotonou : Dr Houemenou G), en Tanzanie (Lushoto : Dr Laudisoit A) et en République Démocratique du Congo (RDC) [Ituri : (Dr Laudisoit A) et Kinshasa : (Dr Davoust B)]. L'utilisation des outils moléculaires de PCR en temps réel, PCR standard et séquençage nous a permis de détecter l'agent du typhus murin (*Rickettsia typhi*) et *Bartonella sp* au Bénin, l'agent de la peste (*Yersinia pestis*), *Rickettsia felis* (l'agent de la fièvre boutonneuse) et *Bartonella* sp au RDC ainsi que *Rickettsia typhi* et *Rickettsia felis* en Tanzanie. Tous ces résultats sont originaux et il s'agit d'une première mise en évidence directe de ces pathogènes dans les zones étudiées.

*** Investigations en Algérie**

En utilisant la PCR en temps réel et le séquençage, nous avons détecté pour la première fois à l'échelle maghrébine et par des outils moléculaire et des méthodes valides, *Borrelia garinii* dans 2% (4/80) d'*Ixodes ricinus* collectées dans les sites d'El Ghora,

El Tarf, Algérie, et *Rickettsia monacensis* dans 8,75% (7/80) des tiques *Ixodes ricinus* dans l'Extrême Nord est algérien (***Article N° 3***).

Par la suite nous avons travaillé sur des tiques d'animaux domestiques (bovins, ovins, caprins et chiens) de la région d'El Tarf et les tiques, puces et les rates d'animaux sauvages capturés à Souk Ahras (Algérie). En utilisant les outils moléculaires, nous avons détecté différentes rickettsiae associées à leurs vecteurs connus à savoir : *Rickettsia felis, R. aeschlimannii, R. massiliae et R. slovaca.* L'ADN de *Bartonella tamiae,* une bactérie récemment classée comme pathogène et *Coxiella burnetii* (l'agent de la fièvre Q) ont été identifiées pour la première fois en Algérie (***Article N° 4***).

Une dernière investigation (***Article N° 5***) a porté sur la détection d'*Erhlichia canis, Coxiella burnetii* et *Bartonella henselae* dans des rates de chiens et chats capturés dans la fourrière canine d'Alger. Nous avons également collecté les ectoparasites (tiques et puces) de ces chiens et chats où nous avons détecté et confirmé la présence de *R. massiliae, R. conorii.* De même *E. canis* a été dans les tiques de l'espèce *Rhipicephalus sanguineus*.Nous avons identifié pour la première fois *Bartonella vinsonii subs. berkhoffii* associée aux *Xenopsylla cheopis* collectées sur des

chiens et nous avons également identifié *R. felis*, *B. henselae et B. clarridgeaia* sur des *Ctenocephalides felis* collectées sur des chats.

L'ensemble des investigations en Algérie et en Afrique Sub-saharienne a enrichi le répertoire des maladies vectorielles en Afrique

ARTICLE 2

Article N.2. **Leulmi H**, Socolovschi C, Laudisoit A, Houemenou G, Davoust B, Bitam I, Raoult D and Parola P. **Detection of *Rickettsia felis, Rickettsia typhi, Bartonella* species and *Yersinia pestis* in Fleas (Siphonaptera) from Africa**. PLoS Negl Trop Dis. 2014 Oct 9;8(10):e3152. doi:10.1371/journal.pntd. 0003152.eCollection 2014.4.

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PLOS | NEGLECTED TROPICAL DISEASES

Detection of *Rickettsia felis*, *Rickettsia typhi*, *Bartonella* Species and *Yersinia pestis* in Fleas (Siphonaptera) from Africa

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Abstract

Little is known about the presence/absence and prevalence of *Rickettsia* spp, *Bartonella* spp. and *Yersinia pestis* in domestic and urban flea populations in tropical and subtropical African countries.

Methodology/Principal findings: Fleas collected in Benin, the United Republic of Tanzania and the Democratic Republic of the Congo were investigated for the presence and identity of *Rickettsia* spp., *Bartonella* spp. and *Yersinia pestis* using two qPCR systems or qPCR and standard PCR. In *Xenopsylla cheopis* fleas collected from Cotonou (Benin), *Rickettsia typhi* was detected in 1% (2/199), and an uncultured *Bartonella sp*. was detected in 34.7% (69/199). In the Lushoto district (United Republic of Tanzania), *R. typhi* DNA was detected in 10% (2/20) of *Xenopsylla brasiliensis*, and *Rickettsia felis* was detected in 65% (13/20) of *Ctenocephalides felis strongylus*, 71.4% (5/7) of *Ctenocephalides canis* and 25% (5/20) of *Ctenophthalmus calceatus calceatus*. In the Democratic Republic of the Congo, *R. felis* was detected in 56.5% (13/23) of *Ct. f. felis* from Kinshasa, in 26.3% (10/38) of *Ct. f. felis* and 9% (1/11) of *Leptopsylla aethiopica aethiopica* from Ituri district and in 19.2% (5/26) of *Ct. f. strongylus* and 4.7% (1/21) of *Echidnophaga gallinacea*. *Bartonella sp*. was also detected in 36.3% (4/11) of *L. a. aethiopica*. Finally, in Ituri, *Y. pestis* DNA was detected in 3.8% (1/26) of *Ct. f. strongylus* and 10% (3/30) of *Pulex irritans* from the villages of Wanyale and Zaa.

Conclusion: Most flea-borne infections are neglected diseases which should be monitored systematically in domestic rural and urban human populations to assess their epidemiological and clinical relevance. Finally, the presence of *Y. pestis* DNA in fleas captured in households was unexpected and raises a series of questions regarding the role of free fleas in the transmission of plague in rural Africa, especially in remote areas where the flea density in houses is high.

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Introduction

The importance of fleas in human and animal health is largely related to their ability to transmit agents of infectious diseases [1]. The transmission of these zoonotic agents to human occurs mainly through bites or inoculation of feces into pruritic bite lesions [2,3]. Plague is the most notorious flea-borne disease known to man and is a reemerging public health issue mainly in Africa and South America [3]. The etiological agent of plague, *Yersinia pestis*, is a facultative gram-negative bacterium restricted nowadays to well defined endemic foci [4,5]. In the last decade, plague reemerged in old quiescent foci of Algeria [6], the United Republic of Tanzania [7] and Libya [8] and caused remarkable bubonic and pneumonic outbreaks in known endemic foci in Madagascar [9] and in the Democratic Republic of the Congo [10]. Fleas are also associated with other bacterial diseases such as bartonelloses and rickettsioses. *Rickettsia* spp., the etiological agents of rickettsioses, are intracellular gram-negative bacteria that represent an emergent global threat, particularly in the tropics [11]. *R. felis*, an emerging pathogen, and *R. typhi*, the agent of murine typhus (MT), are the main rickettsial pathogens associated with fleas [1], belonging to the spotted fever group (SFG) [12] and typhus group rickettsiae, respectively [13]. Although these two flea-borne rickettsiae are distributed worldwide, *R. typhi* appears to be more endemic in tropical regions, coastal areas and ports, where its transmission cycles between rats (*Rattus* spp.) and oriental rat fleas (*X. cheopis*) [14]. Also, several closely related rickettsiae, referred as Rickettsia felis–like organisms (RFLO), identified in fleas and other

Author Summary

Fleas are associated with many bacterial diseases such as rickettsioses, bartonelloses and plague. These diseases may be severe, and little is known about their prevalence. Accordingly, we believe that our data shed light on the problem of unexplained fevers in tropical and subtropical African areas. Using molecular tools, we surveyed and studied selected flea-borne agents, namely *Rickettsia* spp. (*R. felis* and *R. typhi*), *Bartonella* spp. and *Y. pestis*, in fleas collected in Ituri (Linga and Rethy health zone) and Kinshasa in the Democratic Republic of the Congo, the Lushoto district in the United Republic of Tanzania and in Cotonou in Benin. We found that these bacteria are present in the studied regions. *R. typhi* and an unidentified *Bartonella sp.* were detected in fleas from Cotonou (Benin). *R. felis* and *R. typhi* were also detected in the Lushoto district (United Republic of Tanzania). Finally, we detected *R. felis, Bartonella sp.* and *Y. pestis* in the Democratic Republic of the Congo. As these emerging zoonotic diseases have a global distribution and affect public health, the implementation of vector control strategies is urgent.

arthropods around the world [15]. Likewise, bartonelloses are diseases caused by the fastidious, hemotropic bacteria of the genus *Bartonella*, especially in debilitated and immunocompromised individuals [16]. Importantly, the list of host species harboring *Bartonella* spp. includes a large number of mammals, mostly rodents, some of which are kept as pets [17].

An increasing number of papers have reported the occurrence of fleas and human flea-borne infections, especially in relation to wildlife and zoonotic risk. However, the identity and distribution of flea-borne agents in urban, domestic or peridomestic settings have been poorly documented in Sub-Saharan African countries such as the Democratic Republic of the Congo, the United Republic of Tanzania and Benin. Historical data about human infection with *Rickettsia* and *Bartonella* species are fragmentary, and virtually nothing is known about the current distribution of these flea-borne zoonotic agents in potential vectors and reservoir hosts in these countries.

In the Democratic Republic of the Congo, recent small-scale surveys have reported serological evidence for *Bartonella* infection in human patients [18] and molecular data in rodents [19] and fleas [20], suggesting a global underreporting at the country scale. Rickettsioses in humans are mentioned in historical reports; however, their notification remains anecdotal, and the species identification is likely erroneous. Recently though, among febrile military patients in Kisangani, Democratic Republic of the Congo, one patient tested positive in 1999, for the *R. typhi* antigen using serological tools. In addition, *R. felis* has been found to circulate in arthropod vectors in Kinshasa [21]. As a general trend, flea-borne agents in fleas are underreported, whereas in the United Republic of Tanzania, a growing number of publications confirm their presence and wide distribution in humans [22] exposed to their bites and in infested rodents [19].

In recent years, our laboratory (Unité de Recherche sur les Maladies Infectieuses et Tropicales, the WHO Collaborative Centre for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases in Marseille, France) initiated collaboration with correspondents and universities in the United Republic of Tanzania, the Democratic Republic of the Congo and Benin.

The present survey pursued the objectives of detecting the presence and identity of *Rickettsia* spp., *Bartonella* spp. and *Y. pestis* in flea specimens collected from domestic and peridomestic areas in the Democratic Republic of the Congo, the United Republic of Tanzania and Benin within the context of entomological studies.

Methods

Ethical considerations

Risk assessment was submitted to and approved by the ethical committee and decision board of each institution involved in small mammals trappings, and involved informed consent of the domestic animal owners; ethical approval are available from original publications on mammal hosts on which flea were collected [19,23,24]. The Ethical commitee of the University of Antwerp, Belgium and the Sokoine University of Agriculture Morogoro under the project RATZOOMAN granted by the European Commission Framework 5 Programme on International Cooperation, project contract number ICA4 CT 2002 10056, approved the experiment in the South-eastern Africa.

See here technical annex: http://projects.nri.org/ratzooman/docs/technical%20annex.pdf.

Sites of study and flea collection

The material analyzed consisted of fleas (Siphonaptera) collected in domestic and peridomestic areas in Benin, the United Republic of Tanzania and the Democratic Republic of the Congo (Figure 1). A portion of the collected fleas was used for the present study. A convenient sample was selected according to a good representation of species, host and localities.

In 37 sites in the capital city of Benin, Cotonou (6°21′36″N; 2°26′24″E), rodent fleas were collected from rodents trapped monthly inside human residences and peridomestic areas between November 2009 to July 2010, as described previously [24]. In the United Republic of Tanzania, 17 sites in the Lushoto district (04°40′00″S 38°19′00″E) located in the Tanga Region were surveyed [23,25]. Lushoto district is a mountainous area where plague was reported from the first time in 1981; this endemic plague focus has however been quiescent since 2004. Between May 2005 and November 2008, fleas were collected – as in Benin – from small mammals in domestic and peridomestic habitats during the dry and rainy seasons. Further details on the rodent measurements and flea collection have been published elsewhere [23,25].

Finally, in March and April 2007, rodent fleas and free domestic fleas were collected from 4 villages (15 capture sites) in the Linga and Rethy health zones, Ituri district, Orientale Province, the Democratic Republic of the Congo; off-host fleas were collected in 4 villages during an investigation following a plague outbreak that occurred in the third trimester of 2006 [26]. Our investigation area was limited to Djalusene (2°12′10″5 N 30°88′02″7 E) and Kpandruma (2°05′90″1 N 30°88′70″4 E), which had confirmed plague patients, and Wanyale (2°10′11″8 N 30°80′60″5 E) and Zaa (2°14′03″2 N 30°85′65″9 E), which had several suspect cases but were considered control areas at the time of the study. We collected fleas in 40 houses (bedroom) in each village, for 3 nights in a row, using a kerosene lamp hung above a 45-cm diameter tray containing water as described in [27]. In April 2010 and July 2012, additional flea samples were collected from the Ituri district in Rethy village (1°50′N 29°30′E) and in Kinshasa (4°19′19″S 15°19′16″E) by means of light traps in human residences (bedroom) and rodent burrows, and flat tweezers on dogs.

All fleas collected in Benin, the United Republic of Tanzania and the Democratic Republic of the Congo were stored in 70% ethanol and identified morphologically using classical entomologic

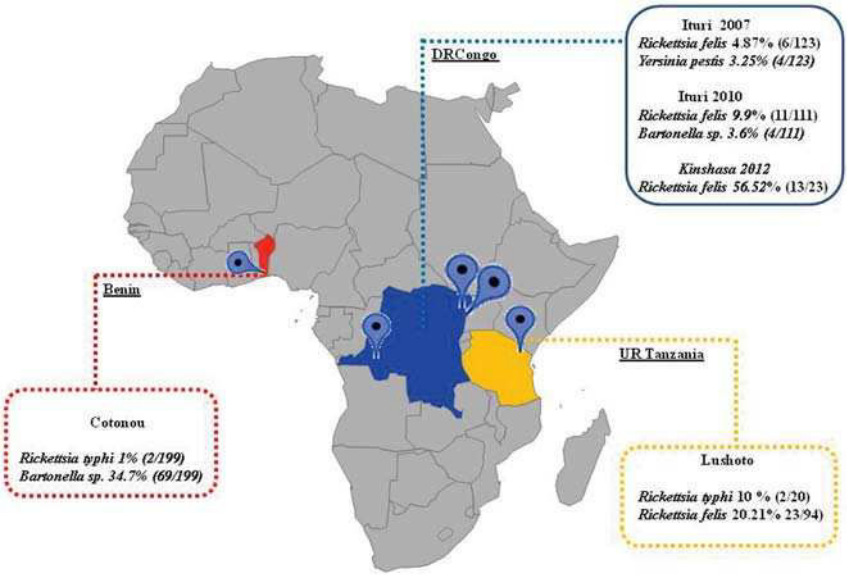

Figure 1. Sites of the study.
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taxonomic keys. The samples were later processed in the WHO Collaborative Center for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases, in Marseille, France.

DNA extraction

Fleas were rinsed twice in distilled water for 10 minutes and dried on sterile filter paper; the handling was performed in a laminar flow biosafety hood. The fleas were individually crushed in sterile Eppendorf tubes, as described [28]. A total of 50 µl of DNA was extracted from one half of each flea using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) by QUIAGEN-BioRobot EZ1, according to the manufacturer's instructions. The genomic DNA was stored at −20°C under sterile conditions until used as the template in PCR assays. The remaining portion of each flea was kept at −80°C for an additional control.

Detection of *Rickettsia felis*

All samples were screened by quantitative real-time PCR (qPCR) targeting the biotin synthase (*bioB*) gene, as previously described [29]. Positive results were confirmed by another qPCR targeting a membrane phosphatase gene with primers (Rfel_phosp_MBF: 5′-GCAACCATCGGTGAAATTGA-3′ and Rfel_phosp_MBR: 5′-GCCACTGTGCTTCACAAACA-3′) and a probe (Rfel_phosp_MBP: 6FAM-CCGCTTCGTTATCCGTG-GGACC-TAMRA) designed in our laboratory. The final mixture of the qPCR reaction was composed of 15 µL of mix that contained 10 µL of master mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany), 0.5 µL (20 pmol) of each primer, 0.5 µL (62.5 nmol) of probe, 3.5 µL of RNase DNase-free water and 5 µL of DNA extracted from fleas. qPCR was performed as follows: 15 min at 95°C, followed by 40 cycles of 1 s at 95°C, 40 s at 60°C and 40 s at 45°C, as described [29]. The negative control consisted of DNA extracted from uninfected fleas from our laboratory colony and was used for all the PCR assays in this work. The positive control was DNA extracted from a diluted strain of *R. felis* from our laboratory in Marseille. Positive results were recorded if the cycle threshold (Ct) value obtained was lower than 36 using the 2 PCR systems.

Detection of *Rickettsia typhi*

Samples were screened by qPCR targeting a fragment of the Rpr 274P gene coding for a hypothetical protein, as described previously [30]. Positive results were confirmed by qPCR targeting the glycosyltransferase gene using a previously described Rpr 331 system [31]. qPCR was conducted using the same method as described for *R. felis* detection. The positive control was DNA extracted from a diluted strain of *R. typhi Wilmington* (ATCC VR-144) cultured in our laboratory in Marseille.

Detection of *Bartonella* spp.

DNA samples were screened by quantitative real-time PCR targeting the ITS region [32]. Positive samples with ITS primers were then confirmed by standard PCR performed with *Bartonella*-specific primers for the citrate synthase (*gltA*) gene, amplifying an approximately 334-bp fragment [33]. The positive control was *B. alsatica* strain IBS 382 (CIP 105477) DNA extracted from a strain and previously diluted to 10^{-6}.

The success of PCR amplification was verified by 2% agarose gel migration. The products were purified using NucleoFast 96 PCR plates (Machery-Nagel EURL, France) as recommended by

the manufacturer. The purified PCR products were sequenced with *gltA* primers using the BigDye version 1.1 cycle sequencing ready reaction mix (Applied Biosystems, Foster City, CA) with the ABI 31000 automated sequencer (Applied Biosystems). The sequences were assembled and analyzed with the ChromasPro program (version 1.5).

Detection of *Yersinia pestis*

DNA samples were screened by qPCR targeting the plasminogen activator gene (*Pla*) [6] using primers Yper_PLA_F (5′-ATG-GAG-CTT-ATA-CCG-GAA-AC-3′) and Yper_PLA_R (5′-GCG-ATA-CTG-GCC-TGC-AAG-3′) and probe Yper_PLA_P (6- FAM-TCC-CGA–AAG-GAG-TGC-GGG-TAA-TAGG-TAMRA). Positive results were confirmed with standard PCR targeting the glpD gene, as described [34], and then sequenced using the same method used for *Bartonella* spp. sequencing. The positive control was *Y. pestis* DNA extracted from the CSUR P 100 strain, and diluted to 10^{-6}.

Results

Benin

In Benin, 886 fleas were collected from 199 sexually mature small mammals of four species, namely, *Crocidura olivieri* (17/199, 8.5%), *Mastomys natalensis* (36/199, 18%), *Rattus norvegicus* (40/199, 20.1%) and *Rattus rattus* (109/199, 54.7%). Three flea species were collected from rodents, with the oriental rat flea *X. cheopis* being the most abundant (861/886, 97.1%), followed by *X. brasiliensis* (24/886, 2.7%) and *Ct. felis strongylus* (1/886, 0.1%). In the present study, a convenient sample of 199 *X. cheopis* (picked off *Rattus rattus*) individuals – 55.78% females and 44.2% males – were selected for an initial molecular screening (the remaining fleas were preserved for subsequent studies).

All fleas tested negative for *R. felis* and *Y. pestis*. qPCR performed for the detection of *R. typhi* revealed 2 positive *X. cheopis* (2/199, 1%), with a Ct of 32.6 and 34.5, from 2 sites (Bokossi Tokpa and Dédokpo). *Bartonella* spp. were detected in 69/199 (34.6%) of the fleas (Ct, 31.81, +/−2.97) (24≤Ct≤35) collected from all studied sites (Table 1). DNA sequence analyses of the PCR products of the gltA gene of 8 representative samples (with high Ct values) showed 100% similarity with the Uncultured *Bartonella* sp. clone Pd5700t (GenBank no. EJ851115.1, 334/334 bp) detected in *Praomys delectorum* rodents in Mbulu district, northern Tanzania [19]. More information about the Ct value and localization of each positive flea is reported in Supplementary data S1.

United Republic of Tanzania

A total of 3821 fleas (rodent fleas and free-roaming fleas present in the environment) were collected from localities of the Lushoto district (United Republic of Tanzania) and were distributed into 23 species. A total of 94 fleas belonging to six common species were screened (Supplementary data S2) (20 *Ct. f. strongylus*, 7 *Ct. canis*, 20 *Ctenophthalmus calceatus calceatus*, 20 *X. brasiliensis*, 20 *Pulex irritans* and 7 *Nosopsyllus incisus*. All tested fleas were negative for *Y. pestis* and *Bartonella* spp. DNA. However, *R. typhi* DNA was detected in 10% (2/20) of *X. brasiliensis* collected from 2 villages (Magamba and Manolo). *R. felis* DNA was also detected in 20.2% (23/94) of analyzed fleas, including 65% (13/20) of *Ct. f. strongylus*, 71.4% (5/7) of *Ct. canis* and 25% (5/20) of *Ct. ca. calceatus*.

Democratic Republic of the Congo

In 2007, in the Linga and Rethy health zones, Ituri district, 1190 fleas captured in households, belonging to 6 species (394 *P. irritans*, 153 *Tunga penetrans*, 280 *Ct. f. strongylus*, 89 *Echidnophaga gallinacea*, 88 *L. a. aethiopica* and 186 *X. brasiliensis*). A total of 123 fleas were conveniently selected for this work (Supplementary data S3). qPCR for *R. typhi* and *Bartonella* spp. was negative for all 123 fleas; however, 4.8% (6/123), namely 19.2% (5/26) of *Ct. f. strongylus* and 4.7% (1/21) of *E. gallinacea*, contained *R. felis* DNA (Table 1).

Y. pestis DNA was detected in 3.8% (1/26) of *Ct. f. strongylus* and 10% (3/30) of *P. irritans* from 2 villages (Wanyale and Zaa). DNA sequence analyses of the PCR products targeting the glpD gene showed 100% similarity with *Yersinia pestis Angola* isolated from Angola (GenBank accession no. CP000901.1, 321/333 bp).

In 2010, 111 fleas, belonging to 3 species, were collected in the same district, namely, *X. cheopis* (62/111, 55.8%), *Ct. f. felis* (38/111, 34.2%) and *L. a. aethiopica* (11/111, 9.9%) (Supplementary data S4). qPCR for *R. typhi* and *Y. pestis* detection was negative for all fleas (Table 1); however, 9.9% (11/111) of two flea species (*Ct. f. felis* and *L. a. aethiopica*) were positive for *R. felis*. A total of 10 *Ct. f. felis* from 38 tested (26.3%) and one of 11 *L. a. aethiopica* (9%) contained *R. felis*. *Bartonella* spp DNA was detected in 3.6% (4/111) of fleas, with 36.36% (4/11) from only *L. a. aethiopica*. Sequencing of the *gltA* gene fragment from these four *Bartonella*-positive samples showed 100% similarity with *Bartonella sp*. MNga6 (GenBank no. AJ583126.1, 320/334 bp) detected in fleas collected in South Africa.

Finally, in 2012, from the fleas collected in Kinshasa (Table 1), 56.5% (13/23) of *Ct. f. felis* collected from 3 dogs was positive for *R. felis* but negative for *R. typhi, Bartonella* spp. and *Y. pestis* by qPCR.

Discussion

We report the first direct evidence of *R. typhi* and *Bartonella* sp. in *X. cheopis* fleas in Benin (Cotonou). In Lushoto (United Republic of Tanzania), we detected for the first time the presence of *R. typhi* DNA in *X. brasiliensis* and *R. felis* DNA in *Ct. f. strongylus*, *Ct. canis* and *Ct. ca. calceatus*. Finally, in the Democratic Republic of the Congo, we confirmed the presence of *R. felis* in *Ct. felis* in Kinshasa and for the first time report the presence of *R. felis* and *Bartonella* DNA in *L. a. aethiopica* and, most importantly *Y. pestis* DNA in *P. irritans* and *Ct. felis* from Wanyale and Zaa villages in the Rethy health zone.

The robustness of our results and the detection of these pathogens in fleas on rodents are supported by the use of a validated method of real-time PCR and subsequent sequencing. The validity of the data that we report is based on strict laboratory procedures and controls that are commonly used in the WHO Center for Rickettsial Diseases, including rigorous positive and negative controls to validate the test. Each positive qPCR result was confirmed by another specific qPCR or confirmed with a successful DNA amplification and sequencing.

R. typhi was detected in *X. cheopis* collected from *Rattus rattus* in Bokossi Tokpa and Dédokpo sites (Cotonou, Benin) and in *X. brasiliensis* from the United Republic of Tanzania. *X. cheopis* is the primary vector of *R. typhi*, the etiological agent of murine typhus (MT), in most locations around the world, and *X. brasiliensis* appears to be an effective vector under experimental conditions [3]. MT is most often a relatively mild disease; yet *R. typhi* can cause acute febrile illness and death [35]. The diagnosis of MT may be missed or underreported due to its non-specific symptoms or the absence of epidemiological criteria [36,37] because laboratory tests and validated methods of diagnosis must be performed to confirm the diagnosis [30]. Before our study, *R. typhi* was never detected in Benin, and it is rarely directly reported

Table 1. Distribution of the positive fleas according to species and country.

Country		Fleas species	Total	Number of fleas positive to			
				R. felis	*R. typhi*	*Bartonella sp*	*Yersinia pestis*
BENIN		*X. cheopis*	199	-	**2**	**69**	-
UNITED REPUBLIC OF TANZANIA		*Ct. f. strongylus*	20	**13**	-	-	-
		Ct. canis	7	**5**	-	-	-
		Ct. ca. calceatus	20	**5**	-	-	-
		X. brasiliensis	20	-	**2**	-	-
		P. irritans	20	-	-	-	-
		N. incisus	7	-	-	-	-
DEMOCRATIC REPUBLIC OF THE CONGO	**ITURI (2007)**	*Ct. f. strongylus*	26	-	-	-	**1**
		E. gallinacea	21	-	-	-	-
		P. irritans	30	-	-	-	**3**
		X. brasiliensis	19	-	-	-	-
		L. ae. aethiopica	1	-	-	-	-
		T. penetrans	26	-	-	-	-
	ITURI (2010)	*Ct. f. felis*	38	**10**	-	-	-
		X. cheopis	62	-	-	-	-
		L. ae. aethiopica	11	**1**	-	**4**	-
	KINSHASA	*Ct. f. felis*	23	**13**	-	-	-

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in vectors and patients in Africa, specifically in sub-Saharan Africa. *R. typhi* in African fleas was only detected in *X. cheopis* fleas collected in Algeria [38]. Additionally, *R. typhi* has been reported in patients using serological methods in African countries [30]. Cases have been documented in international travelers returning from Tunisia, Morocco, Ivory Coast, Central African Republic, Madagascar, Reunion and Chad [30]. In the United Republic of Tanzania, a seroprevalence study among pregnant women from the port city of Dar es Salaam found a prevalence of 28% [39] and 0.5 to 9.3% in the town of Moshi and the Mbeya region, respectively [22,40].

R. felis is an emergent agent of infectious disease in humans, and this agent of spotted fever is known to be maintained in cat fleas (*Ct. felis*) [41,42]. To date, 12 species of fleas, 8 species of ticks and 3 species of mites have been found to be infected with *R. felis* [42]. This Rickettsiae has also recently been detected in several mosquito species in sub-Saharan Africa [29,43,44]. Interestingly, the *R. felis* genogroup seems large with recent organisms or genotypes related as *R. felis* like organisms (RFLO). Our 2 qPCR were specifically designed to amplify *R. felis* type strain (URRWXCal2). However, the biotin synthase and membrane phosphatase gene sequences of many RFLO are not known. We however know that at least our qPCR system targeting the biotin synthase (*bioB*) gene do not amplify some RFLO such as Rickettsia sp. RF2125 and Rickettsia sp. SGL01. Recently, a new qPCR assay has been proposed to address this issue by providing new qPCR primers and probe to specifically amplify *R. felis* OmpB gene fragments [15]. The clinical features of *R. felis* may include fever, fatigue, headache, generalized maculopapular rash and inoculation eschar(s) [42]. *R. felis* seems to be a frequent agent of unknown fever in Sub-Saharan Africa [44]. We detected *R. felis* in 5 species of fleas (*Ct. f. strongylus, Ct. canis, Ct. ca. calceatus*, *L. a. aethiopica* and *E. gallinacea*); some from the United Republic of Tanzania (Lushoto district), and other from the Democratic Republic of the Congo (Ituri District). *R. felis* had already been detected in the Ituri district [25], but not in *E. gallinacea*, the fowl flea, and has been previously shown to circulate in arthropod vectors (*Ctenocephalides felis*) in Kinshasa, the capital city of the country [21]. *E. gallinacea* is usually found on poultry, and can occurs on rodents (*Rattus* spp.) foraging in fowl shelters around houses [45]. While chicken DNA has been found in blood meal of fleas collected on rodents in the same area [46] other *Rickettsia* spp. antibodies have been found in poultry in Brazil [47], whether or not *R. felis* and *R. typhi* infects poultry or if poultry can act as a source of infection to human is unknown. Furthermore, no data on the potential vertical transmission of *R. felis* in *E. gallinacea*, or on the vectorial transmission of *R. felis* by *E. gallinacea* males (females are semi-sessile) between rodents and birds, are available. The questions raised by the findings of the present study in relation to *Rickettsia* in fleas are of real epidemiological significance and should be further investigated.

Molecular evidence of *Bartonella* sp. in fleas from the Democratic Republic of the Congo is supported by a recent serological survey in human patients in the Ituri who tested seropositive for *B. henselae, B. quintana* or *B. clarridgeiae* [18]. Gundi and collaborators also found that local rodents harbor *Bartonella* spp. closely related to *B. elizabethae* or *B. tribocorum* which shows that a wide variety of *Bartonella* species is present in the country, and differ according to host [19]. Bitam and collaborators [48] report that *B. elizabethae*, which causes endocarditis, and *B. tribocorum* are usually known to be transmitted by *X. cheopis* fleas. However, while in our study, we detected an Uncultured *Bartonella* sp., clone Pd5700t (GenBank no. FJ851115.1) in *X. cheopis* of Benin, we also detected *Bartonella* sp. MN-ga6 (GenBank no. AJ583126.1) in *L. a. aethiopica*, from Ituri. This *Bartonella sp.* had been previously found in the Democratic Republic of the Congo and the United Republic of Tanzania in rodents [19].

The detection of *Y. pestis* DNA in fleas collected in villages and houses where no current human plague cases had been reported for the last 6 months is puzzling. About 80 species and subspecies of Siphonaptera are known to be carriers and potentially vector of *Y. pestis* [49], via various transmission mechanisms [50]; in particular in fleas from the genus *Xenopsylla* (*X. cheopis*), which played a major role in historical plague pandemics [9]. In the present survey, DNA of *Y. pestis* was detected in the human flea, *P. irritans*, and the cat flea *Ct. felis* in a well known endemic focus of the Democratic Republic of the Congo [51]. In 2006, in the Rethy and Linga health zone more than 600 human cases were reported [52], which triggered the entomological investigation reported previously [25] and the collection of fleas analyzed herein. This survey occurred 6 months after the end of the epidemics, and at the time of the flea sampling, no confirmed human plague cases were reported to the Health centre of the villages (Zaa and Wanyale) or Rethy general Hospital. Several hypotheses can be proposed to explain this finding. A first hypothesis is that infected fleas from rodents, dogs or cats could have been imported in the infested houses, did not bite people and as such no human cases occurred, at the time of collection. A second hypothesis is that infected fleas containing *Y. pestis* DNA remained infected and alive without biting any potential host or that no human cases were reported to the health authorities which are unlikely due to the recent outbreak and constant surveillance. Other options are that *Y. pestis* DNA is reminiscent in the flea but the bacterium is either dead (degraded DNA) but the targeted sequences (gene fragment and gene flanking regions are still complete) or alive but in a quiescent form or VBNC state, possibly controlled by epigenetic mechanisms causing virulence gene repression. The human flea (*P. irritans*) may play an important role in spreading plague via human-to-human transmission as suggested in Lushoto district [27] and could possibly harbor *Y. pestis* without transmission for several months. Unfortunately no fleas were cultured in the field and the viability of the strain detected cannot be proven, but this finding calls for more research at times post outbreaks in order to answer this question. Similarly, cat fleas could play such a role both in northwest Uganda [53] and in Democratic Republic of the Congo (Laudisoit and al 2014, unpublished data), where *C. felis* spp. is the most common flea species collected in the domestic environment above a given altitude threshold.

In conclusion, we widened knowledge of the repertoire of flea-borne bacteria present in Sub-Saharan Africa. In our study, we also illustrate the role of fleas in the entomological survey of vector -borne disease, which allow clinicians to confirm the etiological cause for some of the unknown cause of fever in African patients. Future studies on rickettsioses, bartonelloses and other vector-borne diseases should be performed to assess their epidemiological and clinical relevance in tropical and subtropical areas, to estimate the real prevalence and to allow the establishment of antivectorial control plans.

Supporting Information

Data S1 Detection of flea borne diseases in Benin.
(XLS)

Data S2 Detection of flea borne diseases in United Republic of Tanzania.
(XLSX)

Data S3 Detection of flea borne diseases in Ituri, Democratic Republic of the Congo.
(XLSX)

Data S4 Detection of flea borne diseases in Kinshasa, Democratic Republic of the Congo.
(XLSX)

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Author Contributions

Conceived and designed the experiments: DR PP. Performed the experiments: HL CS. Analyzed the data: CS AL IB DR PP. Contributed reagents/materials/analysis tools: GH AL BD CS. Wrote the paper: HL CS AL GH BD IB DR PP. First drafted the paper: HL.

References

1. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D (2010) Fleas and flea-borne diseases. Int J Infect Dis 14: e667–e676. S1201-9712(10)00029-9 [pii];10.1016/j.ijid.2009.11.011 [doi].
2. Parola P, Davoust B, Raoult D (2005) Tick- and flea-borne rickettsial emerging zoonoses. Vet Res 36: 469–492. 10.1051/vetres:2005004 [doi];v4057 [pii].
3. Eisen RJ, Gage KL (2012) Transmission of flea-borne zoonotic agents. Annu Rev Entomol 57: 61–82. 10.1146/annurev-ento-120710-100717 [doi].
4. Drancourt M (2012) Plague in the genomic area. Clin Microbiol Infect 18: 224–230. 10.1111/j.1469-0691.2012.03774.x [doi].
5. Raoult D, Mouffok N, Bitam I, Piarroux R, Drancourt M (2013) Plague: history and contemporary analysis. J Infect 66: 18–26. S0163-4453(12)00277-0 [pii];10.1016/j.jinf.2012.09.010 [doi].
6. Bitam I, Ayyadurai S, Kernif T, Chetta M, Boulaghman N, Raoult D, Drancourt M (2010) New rural focus of plague, Algeria. Emerg Infect Dis 16: 1639–1640. 10.3201/eid1610.091854 [doi].
7. Neerinckx S, Bertherat E, Leirs H (2010) Human plague occurrences in Africa: an overview from 1877 to 2008. Trans R Soc Trop Med Hyg 104: 97–103. S0035-9203(09)00264-8 [pii];10.1016/j.trstmh.2009.07.028 [doi].
8. Cabanel N, Leclercq A, Chenal-Francisque V, Annajar B, Rajerison M, et al. (2013) Plague outbreak in Libya, 2009, unrelated to plague in Algeria. Emerg Infect Dis 19: 230–236. 10.3201/eid1902.121031 [doi].
9. Andrianaivoarimanana V, Kreppel K, Elissa N, Duplantier JM, Carniel E, et al. (2013) Understanding the persistence of plague foci in Madagascar. PLoS Negl Trop Dis 7: e2382. 10.1371/journal.pntd.0002382 [doi];PNTD-D-12-01523 [pii].
10. Bertherat E, Thullier P, Shako JC, England K, Kone ML, et al. (2011) Lessons learned about pneumonic plague diagnosis from two outbreaks, Democratic Republic of the Congo. Emerg Infect Dis 17: 778–784. 10.3201/eid1705.100029 [doi].
11. Parola P, Raoult D (2006) Tropical rickettsioses. Clin Dermatol 24: 191–200. S0738-081X(05)00169-0 [pii];10.1016/j.clindermatol.2005.11.007 [doi].
12. Merhej V, El KK, Raoult D (2009) Whole genome-based phylogenetic analysis of Rickettsiae. Clin Microbiol Infect 15 Suppl 2: 336–337. CLM2265 [pii];10.1111/j.1469-0691.2008.02265.x [doi].
13. Nogueras MM, Pons I, Ortuno A, Miret J, Pla J, et al. (2013) Molecular Detection of *Rickettsia typhi* in Cats and Fleas. PLoS One 8: e71386. 10.1371/journal.pone.0071386 [doi];PONE-D-13-20078 [pii].
14. Azad AF, Radulovic S, Higgins JA, Noden BH, Troyer JM (1997) Flea-borne rickettsioses: ecologic considerations. Emerg Infect Dis 3: 319–327. 10.3201/eid0303.970308 [doi].
15. Odhiambo AM, Maina AN, Taylor ML, Jiang J, Richards AL (2014) Development and Validation of a Quantitative Real-Time Polymerase Chain Reaction Assay Specific for the Detection of *Rickettsia felis* and Not *Rickettsia felis*-Like Organisms. Vector Borne Zoonotic Dis 14: 476–481. 10.1089/vbz.2013.1518 [doi].
16. Chomel BB, Boulouis HJ, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, et al. (2009) Ecological fitness and strategies of adaptation of *Bartonella species* to their hosts and vectors. Vet Res 40: 29. 10.1051/vetres/2009011 [doi];v09181 [pii].
17. Breitschwerdt EB, Maggi RG, Chomel BB, Lappin MR (2010) Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. J Vet Emerg Crit Care (San Antonio) 20: 8–30. VEC496 [pii];10.1111/j.1476-4431.2009.00496.x [doi].
18. Laudisoit A, Iverson J, Neerinckx S, Shako JC, Nsabimana JM, et al. (2011) Human seroreactivity against *Bartonella species* in the Democratic Republic of Congo. Asian Pac J Trop Med 4: 320–322. S1995-7645(11)60094-1 [pii];10.1016/S1995-7645(11)60094-1 [doi].
19. Gundi VA, Kosoy MY, Makundi RH, Laudisoit A (2012) Identification of diverse Bartonella genotypes among small mammals from Democratic Republic of Congo and Tanzania. Am J Trop Med Hyg 87: 319–326. 87/2/319 [pii];10.4269/ajtmh.2012.11-0555 [doi].
20. Laudisoit A, Falay D, Amundala N, Akaibe D, de Bellocq JG, et al. (2014) High Prevalence of *Rickettsia typhi* and *Bartonella species* in Rats and Fleas, Kisangani, Democratic Republic of the Congo. Am J Trop Med Hyg. ajtmh.13-0216 [pii];10.4269/ajtmh.13-0216 [doi].
21. Mediannikov O, Davoust B, Socolovschi C, Tshilolo L, Raoult D, et al. (2012) Spotted fever group rickettsiae in ticks and fleas from the Democratic Republic of the Congo. Ticks Tick Borne Dis 3: 371–373. S1877-959X(12)00100-8 [pii];10.1016/j.ttbdis.2012.10.015 [doi].
22. Dill T, Dobler G, Saathoff E, Clowes P, Kroidl I, et al. (2013) High seroprevalence for typhus group rickettsiae, southwestern Tanzania. Emerg Infect Dis 19: 317–320. 10.3201/eid1902.120601 [doi].
23. Laudisoit A, Leirs H, Makundi R, Krasnov BR (2009) Seasonal and habitat dependence of fleas parasitic on small mammals in Tanzania. Integr Zool 4: 196–212. 10.1111/j.1749-4877.2009.00150.x [doi].
24. Houemenou G, Ahmed A, Libois R, Hartskeerl R.A (2013) *Leptospira* spp. Prevalence in Small Mammal Populations in Cotonou, Benin. International Scholarly Research Notices 2013: 1–8.
25. Laudisoit A, Beaucournu JC (2009) [Siphonaptera from Tanzania. Description of two taxa and complementary description of *Ctenophthalmus cophurus Hemingwayi* Hubbard, 1963 (Siphonaptera: Ctenophthalmidae)]. Parasite 16: 275–281.
26. Sackal C, Laudisoit A, Kosoy M, Massung R, Eremeeva ME, et al. (2008) *Bartonella* spp. and *Rickettsia felis* in fleas, Democratic Republic of Congo. Emerg Infect Dis 14: 1972–1974. 10.3201/eid1412.080610 [doi].
27. Laudisoit A, Leirs H, Makundi RH, Van DS, Davis S, et al. (2007) Plague and the human flea, Tanzania. Emerg Infect Dis 13: 687–693. 10.3201/eid1305.061084 [doi].
28. Bitam I, Parola P, De La Cruz KD, Matsumoto K, Baziz B, et al. (2006) First molecular detection of *Rickettsia felis* in fleas from Algeria. Am J Trop Med Hyg 74: 532–535. 74/4/532 [pii].
29. Socolovschi C, Pages F, Raoult D (2012) *Rickettsia felis* in *Aedes albopictus* mosquitoes, Libreville, Gabon. Emerg Infect Dis 18: 1687–1689. 10.3201/eid1810.120178 [doi].
30. Walter G, Botelho-Nevers E, Socolovschi C, Raoult D, Parola P (2012) Murine typhus in returned travelers: a report of thirty-two cases. Am J Trop Med Hyg 86: 1049–1053. 86/6/1049 [pii];10.4269/ajtmh.2012.11-0794 [doi].
31. Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, et al. (2010) *Rickettsia felis*-associated uneruptive fever, Senegal. Emerg Infect Dis 16: 1140–1142. 10.3201/eid1607.100070 [doi].
32. Varagnol M, Parola P, Jouan R, Beaucournu JC, Rolain JM, et al. (2009) First detection of *Rickettsia felis* and *Bartonella clarridgeiae* in fleas from Laos. Clin Microbiol Infect 15 Suppl 2: 334–335. CLM2272 [pii];10.1111/j.1469-0691.2008.02272.x [doi].
33. Norman AF, Regnery R, Jameson P, Greene C, Krause DC (1995) Differentiation of *Bartonella-like* isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol 33: 1797–1803.
34. Drancourt M, Signoli M, Dang LV, Bizot B, Roux V, et al. (2007) *Yersinia pestis Orientalis* in remains of ancient plague patients. Emerg Infect Dis 13: 332–333. 10.3201/eid1302.060197 [doi].
35. Adjemian J, Parks S, McElroy K, Campbell J, Eremeeva ME, et al. (2010) Murine typhus in Austin, Texas, USA, 2008. Emerg Infect Dis 16: 412–417. 10.3201/eid1603.091028 [doi].
36. Nogueras MM, Cardenosa N, Sanfeliu I, Munoz T, Font B, et al. (2006) Evidence of infection in humans with *Rickettsia typhi* and *Rickettsia felis* in Catalonia in the Northeast of Spain. Ann N Y Acad Sci 1078: 159–161. 1078/1/159 [pii];10.1196/annals.1374.028 [doi].
37. Civen R, Ngo V (2008) Murine typhus: an unrecognized suburban vectorborne disease. Clin Infect Dis 46: 913–918. 10.1086/527443 [doi].
38. Bitam I, Baziz B, Kernif T, Harrat Z, Parola P, et al. (2009) Molecular detection of *Rickettsia typhi* and *Rickettsia felis* in fleas from Algeria. Clin Microbiol Infect 15 Suppl 2: 255–256. CLM2275 [pii];10.1111/j.1469-0691.2008.02275.x [doi].
39. Anstey NM, Tissot DH, Hahn CG, Mwaikambo ED, McDonald MI, et al. (1997) Seroepidemiology of *Rickettsia typhi*, spotted fever group rickettsiae, and *Coxiella burnetti* infection in pregnant women from urban Tanzania. Am J Trop Med Hyg 57: 187–189.
40. Prabhu M, Nicholson WL, Roche AJ, Kersh GJ, Fitzpatrick KA, et al. (2011) Q fever, spotted fever group, and typhus group rickettsioses among hospitalized febrile patients in northern Tanzania. Clin Infect Dis 53: e8–15. cir411 [pii];10.1093/cid/cir411 [doi].

41. La SB, Meconi S, Fenollar F, Rolain JM, Roux V, et al. (2002) Emended description of *Rickettsia felis* (Bouyer et al. 2001), a temperature-dependent cultured bacterium. Int J Syst Evol Microbiol 52: 2035–2041.
42. Parola P (2011) *Rickettsia felis:* from a rare disease in the USA to a common cause of fever in sub-Saharan Africa. Clin Microbiol Infect 17: 996–1000. 10.1111/j.1469-0691.2011.03516.x [doi].
43. Keita AK, Socolovschi C, Ahuka-Mundeke S, Ratmanov P, Butel C, et al. (2013) Molecular evidence for the presence of *Rickettsia felis* in the feces of wild-living African apes. PLoS One 8: e54679. 10.1371/journal.pone.0054679 [doi];PONE-D-12-33654 [pii].
44. Mediannikov O, Socolovschi C, Edouard S, Fenollar F, Mouffok N, et al. (2013) Common epidemiology of *Rickettsia felis* infection and malaria, Africa. Emerg Infect Dis 19: 1775–1783. 10.3201/eid1911.130361 [doi].
45. Kapoor R, Elston DM (2012) What's eating you? The sticktight flea (*Echidnophaga gallinacea*). Cutis 89: 157–158.
46. Woods ME, Montenieri JA, Eisen RJ, Zeidner NS, Borchert JN, et al. (2009) Identification of flea blood meals using multiplexed real-time polymerase chain reaction targeting mitochondrial gene fragments. Am J Trop Med Hyg 80: 998–1003. 80/6/998 [pii].
47. Maciel JF, Krawczak FdS, Oliveira CSd, Moraes-Filho J, Labruna MB, et al. (2013) Detection of anti: *Rickettsia* spp. antibodies in domestic chickens of extensive breeding in an endemic area for spotted fever in the state of Rio Grande do Sul, Brazil. Ci_Öncia Rural 43: 2037–2041.
48. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D (2010) Fleas and flea-borne diseases. Int J Infect Dis 14: e667–e676. S1201-9712(10)00029-9 [pii];10.1016/j.ijid.2009.11.011 [doi].
49. Hinnebusch BJ (2005) The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol 7: 197–212.
50. Eisen RJ, Bearden SW, Wilder AP, Montenieri JA, Antolin MF, et al. (2006) Early-phase transmission of *Yersinia pestis* by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. Proc Natl Acad Sci U S A 103: 15380–15385. 0606831103 [pii];10.1073/pnas.0606831103 [doi].
51. Janssens PG, Pattyn SR (1994) [Plague in Zaire]. Verh K Acad Geneeskd Belg 56: 281–360.
52. WORLD HEALTH ORGANIZATION Geneva (2006) Plague, Democratic Republic of the Congo. Weekly epidemiological record 25: 241–248.
53. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, et al. (2008) Plague: past, present, and future. PLoS Med 5: e3. 06-PLME-ND-0545 [pii];10.1371/journal.pmed.0050003 [doi].

ARTICLE 3

*Article N.*3. Benredjem W*, **Leulmi H***, Bitam I, Raoult D, Parola P. ***Borrelia garinii* and *Rickettsia monacensis* in *Ixodes ricinus* ticks, Algeria**. 2014 Oct;20(10):1776-7. doi: 10.3201/eid2010.140265.

light the country's potential role in the global circulation of DENV.

Our findings are corroborated by a recently reported case of dengue in a traveler from Portugal that was acquired in Luanda concomitantly with our cases and also found to be caused by DENV-4 (*10*). In light of the apparent introduction of DENV-4 to Angola, probably from Brazil, health authorities should be encouraged to enhance surveillance and vector control efforts. In addition, health practitioners treating travelers returning from Angola should be aware of the risk for DENV infection.

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References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7. http://dx.doi.org/10.1038/nature12060
2. Were F. The dengue situation in Africa. Paediatr Int Child Health. 2012;32 Suppl 1:18–21. http://dx.doi.org/10.1179/20469047 12Z.00000000048
3. Schwartz E, Meltzer E, Mendelson M, Tooke A, Steiner F, Gautret P, et al. Detection on four continents of dengue fever cases related to an ongoing outbreak in Luanda, Angola, March to May 2013. Euro Surveill. 2013;18:pii:20488.
4. Ongoing dengue epidemic—Angola, June 2013. MMWR Morb Mortal Wkly Rep. 2013;62:504–7.
5. Sessions OM, Khan K, Hou Y, Meltzer E, Quam M, Schwartz E, et al. Exploring the origin and potential for spread of the 2013 dengue outbreak in Luanda, Angola. Glob Health Action. 2013;6:21822.
6. ProMed-mail. Dengue—Angola (06), update [cited 2014 Mar 20]. http://www.promedmail.org, article no. 1824865.
7. Nunes MR, Faria NR, Vasconcelos HB, Medeiros DB, Silva de Lima CP, Carvalho VL, et al. Phylogeography of dengue virus serotype 4, Brazil, 2010–2011. Emerg Infect Dis. 2012;18:1858–64. http://dx.doi.org/10.3201/eid1811.120217
8. Saluzzo JF, Cornet M, Castagnet P, Rey C, Digoutte JP. Isolation of dengue 2 and dengue 4 viruses from patients in Senegal. Trans R Soc Trop Med Hyg. 1986;80:5. http://dx.doi.org/10.1016/0035-9203(86)90182-3
9. Teixeira MG, Siqueira JB Jr, Ferreira GL, Bricks L, Joint G. Epidemiological trends of dengue disease in Brazil (2000–2010): a systematic literature search and analysis. PLoS Negl Trop Dis. 2013;7:e2520. http://dx.doi.org/10.1371/journal.pntd.0002520
10. Parreira R, Centeno-Lima S, Lopes A, Portugal-Calisto D, Constantino A, Nina J. Dengue virus serotype 4 and chikungunya virus coinfection in a traveller returning from Luanda, Angola, January 2014. Euro Surveill. 2014;19:pii:20730.

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Borrelia garinii and *Rickettsia monacensis* in *Ixodes ricinus* Ticks, Algeria

To the Editor: Lyme disease (Lyme borreliosis) is caused by a group of related spirochetes (*Borrelia burgdorferi* sensu lato) that include ≥11 species (*1*). In northern Africa, the main vector of Lyme disease in Europe (*Ixodes ricinus* ticks) is also present, and this disease has been suspected to be present in this region of Africa (*2*). Twenty-one cases of Lyme disease were reported in Algiers, Algeria, during 1996–1999 (*3*). However, these cases were diagnosed by detection of only serum antibodies against *B. burgdorferi* by ELISA without confirmation by Western blotting.

I. ricinus ticks are also known to harbor spotted fever group rickettsiae, including *Rickettsia monacensis*, which was detected in Algeria in 2009 (*4*). This rickettsia has been recently identified as a human pathogen in Spain and Italy (*5*).

To investigate Lyme disease and tickborne rickettsioses transmitted by *I. ricinus* ticks in northeastern Algeria, we collected ticks by using the flag method in El Ghora (Bougous, El Tarf) (36°39′34″N, 8°22′10″E). Ectoparasites were collected in March 2012 and identified to genus and species by using taxonomic morphologic keys (*6*).

Total genomic DNA was isolated by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) and BioRobot EZ1 (QIAGEN) as described by the manufacturer. DNA was used as template for quantitative real-time PCR. We used the RKND03 system, which is specific for the *gltA* gene of *Rickettsia* spp. (*7*), and the Bor16S system, which is specific for the *rrs* gene of *Borrelia* spp. (*8*). Real-time PCRs were performed by using the CFX96 Real Time System C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Singapore).

Positive results were confirmed by using a standard PCR specific for the *ompA* gene of *Rickettsia* spp. and the 16S rRNA and *flaB* genes of *Borrelia* spp. (*8*). We used bacteria-free DNA of *Rhipicephalus sanguineus* ticks reared in our laboratory colonies as a negative control and DNA of *B. crocidurae* and *R. montanensis*, which are not known to be associated with *I. ricinus* ticks, as a positive control.

PCR amplification was verified by electrophoresis of products on 2% agarose gels. Products were purified by using a NucleoFast 96 PCR plate (Macherey-Nagel EURL, Hoerdt, France) as recommended by the manufacturer. Purified PCR products were sequenced by using the same primers as for a standard PCR and the BigDye version 1–1 Cycle Ready Reaction Sequencing Mixture (Applied Biosystems, Foster City, CA, USA) in the ABI 31000 automated sequencer (Applied Biosystems). Sequences were assembled and analyzed by using ChromasPro version 1.34 software (Technelysium Pty. Ltd., Tewantin, Queensland, Australia).

Ninety-four ticks were collected by using the dragging method; these ticks belonged to 2 species: 85.1% (80/174) were *I. ricinus* ricks (43 females, 22 males, and 15 nymphs) and 14.9% (14/94) were *Rh. sanguineus* adult ticks (11 females and 3 males). We screened only the 80 *I. ricinus* ricks. *Rh. sanguineus* ticks were kept alive to establish laboratory colonies for other experiments. Overall, 5.0% (4/80) of *I. ricinus* ticks were positive for *Borrelia* spp. and 8.75% (7/80) were positive for *Rickettsia* spp.

Using a standard PCR specific for the *flaB* gene, we identified *B. garinii* in all ticks positive by quantitative real-time PCR (100% similarity, 736/736 bp) (GenBank accession no. CP003151.1). Using a standard PCR specific for the *ompA* gene of *Rickettsia* spp., we identified *R. monacensis* (100% similarity 760/760 bp) (GenBank accession no. FJ919640.1).

We have detected *B. garinii,* a cause of Lyme disease, in Algeria in *I. ricinus* ticks by using a standard PCR and sequencing methods. We also confirmed the presence of *R. monacensis* in this country.

Borrelia spp. have been detected in *I. ricinus* ticks in Tunisia and Morocco (*2*,*9*), and *B. lusitaniae* was found to be predominant (97% of *Borrelia* spp. in Tunisia and 93% in Morocco). However, *B. garinii* was also present (*2*,*9*,*10*). In Tunisia, 1/16 *I. ricinus* ticks were positive for *B. garinii* (*2*,*9*). In Morocco, 3 (3.6%) of 82 were positive for *B. burgdorferi* sensu stricto and 3 (3.6%) of 82 were positive for *B. garinii* (*9*). However, in these studies, *Borrelia* spp.were identified by using restriction fragment length polymorphism analysis (*2*,*9*).

B. garinii is the most neurotropic of the genospecies of *B. burgdorferi* sensu lato; it causes meningopolyneuritis and, rarely, encephalomyelitis (*1*). Clinicians need to be aware of the prevalence of this bacterium in Algeria. Our results help clarify the epidemiology of *B. garinii* in Algeria. *R. monacensis* is an agent of tickborne diseases that was detected in Algeria in 2009 (*4*). The few cases that have been described were characterized by influenza-like symptoms, fever, an inoculation eschar, and a generalized rash (*5*).

In northern Africa, the risk areas for Lyme disease and infection with *R. monacensis* include cool and humid areas in the Atlas Mountains. In this region, humans can come in contact with *I. ricinus* ticks, and these ticks might play a major role in transmission of *B. garinii* and *R. monacensis*.

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References

1. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. Lancet. 2012;379:461–73. http://dx.doi.org/10.1016/S0140-6736(11)60103-7
2. Bouattour A, Ghorbel A, Chabchoub A, Postic D. Lyme borreliosis situation in north Africa [in French]. Arch Inst Pasteur Tunis. 2004;81:13–20.
3. Alem A, Hadji N. Clinico-serologic study of Lyme disease in Algeria (1996–1999) [in French]. Arch Inst Pasteur Alger. 1999;63:49–58.
4. Dib L, Bitam I, Bensouilah M, Parola P, Raoult D. First description of *Rickettsia monacensis* in *Ixodes ricinus* in Algeria. Clin Microbiol Infect. 2009;15(Suppl 2):261–2. http://dx.doi.org/10.1111/j.1469- 0691.2008.02277.x
5. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, et al. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev. 2013;26:657–702. http://dx.doi.org/10.1128/CMR.00032-13
6. Estrada-Pena A, Bouattour A, Camicas JL, Walker AR. Ticks of domestic animals in the Mediterranean region, a guide to identification of species. Zaragoza (Spain); University of Zaragoza; 2004.
7. Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, Bassene H, et al. *Rickettsia felis*–associated uneruptive fever, Senegal. Emerg Infect Dis. 2010;16:1140–2. http://dx.doi.org/10.3201/eid1607.100070
8. Socolovschi C, Kernif T, Raoult D, Parola P. *Borrelia*, *Rickettsia*, and *Ehrlichia* species in bat ticks, France, 2010. Emerg Infect Dis. 2012;18:1966–75. http://dx.doi.org/10.3201/eid1812.111237
9. Sarih M, Jouda F, Gern L, Postic D. First isolation of *Borrelia burgdorferi* sensu lato from *Ixodes ricinus* ticks in Morocco. Vector Borne Zoonotic Dis. 2003;3:133–9. http://dx.doi.org/10.1089/153036603768395834
10. Younsi H, Postic D, Baranton G, Bouattour A. High prevalence of *Borrelia lusitaniae* in *Ixodes ricinus* ticks in Tunisia. Eur J Epidemiol. 2001;17:53–6. http://dx.doi.org/10.1023/A:1010928731281

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ARTICLE 4

Article N.4. **Leulmi H,** Aouadi A, Bitam I, Bessas A, Benakhla A, Raoult D, Parola P. **Detection of *Bartonella tamiae*, *Coxiella burnetii* and rickettsiae in arthropods and tissues from wild and domestic animals in northeastern Algeria.** Submitted to Parasites and vectors, June, 25th 2015), PARV-D-15-00634.

Detection of *Bartonella tamiae, Coxiella burnetii* and rickettsiae in arthropods and tissues from wild and domestic animals in northeastern Algeria.

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ABSTRACT

Background

In recent years, the scope and importance of emergent vector-borne diseases have increased dramatically. In Algeria, only limited information is currently available concerning the presence and prevalence of these zoonotic diseases. For this reason, we conducted a survey of hematophagous ectoparasites of domestic mammals and/or spleens of wild animals in El Tarf and Souk Ahras, Algeria.

Methods

Using real-time PCR, standard PCR and sequencing, the presence of *Bartonella* spp, *Rickettsia* spp, *Borrelia* spp and *Coxiella burnetii* was evaluated in 268/1626 ticks, 136 fleas, 11 *Nycteribiidae* flies and 16 spleens of domestic and/or wild animals from El Tarf and Souk Ahras areas.

Results

For the first time in Algeria, *Bartonella tamiae* was detected in (12/19, 63.2%) *Ixodes vespertilionis* ticks, (8/11, 72.7%) *Nycteribiidae* spp flies and in (6/10, 60%) spleens of bats (*Chiroptera* spp). DNA of *Coxiella burnetii*, the agent of Q fever, was also identified in (3/19, 15.8%) *I. vespertilionis* of

bats. *Rickettsia slovaca*, the agent of tick-borne lymphadenopathy, was detected in (1/1, 100%) *Haemaphysalis punctata* and (2/3, 66.7%) *Dermacentor marginatus* ticks collected from 2 boars (*Sus scrofa algira*) respectively. *Ri. massiliae*, an agent of spotted fever was detected in (38/94, 40.4%) *Rhipicephalus sanguineus* collected from cattle, sheep, dogs, boars and jackals. DNA of *Ri. aeschlimannii* was detected in (6/20, 30%) *Hyalomma anatolicum excavatum* and (6/20, 30%) *Hy. detritum detritum* of cattle. Finally *Ri. felis*, an emerging rickettsial pathogen, was detected in (80/110, 72.7%) *Archaeopsylla erinacei* and (2/2, 100%) *Ctenocephalides felis* of hedgehogs (*Atelerix algirus*).

Conclusion

In this study we expanded knowledge about the repertoire of ticks and flea-borne bacteria present in ectoparasites and/or tissues of domestic and wild animals in Algeria.

Keywords: *Bartonella tamiae*, *Rickettsia*, *Coxiella burnetii*, ticks, fleas, Algeria.

BACKGROUND

Since the beginning of the 20th century, ticks (Acarina), fleas (Siphonaptera) and other hematophagous arthropods have been implicated as vectors, reservoirs, and/or amplifiers of agents of human zoonoses [1].Ticks are hematophagous arthropods considered to be the second vectors (after mosquitoes) of human disease and the most important vectors of disease-causing pathogens in animals [2,3]. Ticks can transmit a broad range of pathogens, including viruses, protozoa and bacteria [4]. Likewise, fleas are also able to transmit several agents of infectious diseases [5]. The transmission of these zoonotic agents to humans occurs mainly through their bites or inoculation of their infected feces into pruritic bite lesions [6,7].

Rickettsioses, bartonelloses and Q fever are vector-borne diseases that may be severe and have a widespread geographical distribution.

Rickettsia spp., the etiological agent of rickettsioses, are intracellular Gram-negative bacteria that represent an emergent global threat [8]. *Ri. felis,* an emerging pathogen, and *Ri. typhi,* the agent of murine typhus (MT), are the main rickettsial pathogens associated with fleas [9], belonging to the spotted fever group (SFG) [10] and typhus group of rickettsiae, respectively [11]. Most of the SFG are transmitted by ticks [12]

that are widely distributed in northern Africa [13,14]. In Algeria, 11 rickettsial pathogens have been detected in ticks, fleas, lice and humans, including *Ri. conorii* subspecies *conorii*, *Ri. aeschlimannii*, *Ri. sibirica mongolitimonae*, *Ri. massiliae*, *Ri. slovaca*, *Ri. helvetica*, *Ri. africae, Ri. monacensis, Ri. felis, Ri. typhi* and *Ri. prowazekii* [15].

Likewise, bartonelloses are diseases caused by the fastidious, hemotropic bacteria of the genus *Bartonella* [16] which parasitize erythrocytes or epithelial cells across a range of mammalian hosts, including humans, rodents and chiroptera [17-19]. In Algeria, few investigations into the diversity of *Bartonella* spp. from animals and vectors have been conducted. Namely *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and *B. elizabethae* were detected infecting domestic dogs [20,21] and fleas collected from hedgehogs [22], *B. henselae* was isolated from stray cats [23] and *B. rochalimae* was detected in fleas collected from brown rats (*Rattus norvegicus*) [22].

Also, *Coxiella burnetii*, the causative agent of Q fever, is a highly infectious zoonotic intracellular bacterium which can affect different species of wild and domestic mammals, it can also infect arthropods and birds [24]. In Algeria few human cases of Q fever have been documented, with only 2 human cases reported in Oran [25].

The goal of this investigation was to assess the presence of emerging zoonotic bacteria in ectoparasites and tissues sampled from wild and domestic animals present in northeastern Algeria.

METHODS

Study areas

The first part of the study was conducted in May 2012 in El Ghora (Bougous, El Tarf) (36° 39′ 34″ N 8° 22′ 10″ E) in the far Northeast of Algeria. El Ghora is a humid bioclimatic zone. It was the highest site of the study area, corresponding to "forest" at an altitude of 1200 meters. Its coverage includes 96% vegetable and 2% herbaceous with the presence of *Asphodélus microcarpus*, *Chamaerops humilis*, 18% shrub stratum characterized by the presence of *Calycotome scistus*, *Rubusulmifolius*, *Erica arborea* and 90% of a tree layer composed of zeen oak (*Quercus canariensis*) and especially cork oak (*Quercus suber*).

The second part of this work was performed in July 2013, in Cheabat El Balout (Ouled Driss, Souk Ahras) in Northeastern Algeria near to El Tarf, (36° 22' 01.30" N 08° 07' 27.48" E). This study area is mountainous and is located at 1000 m above sea

level, representing an extension of the Telli Atlas. It has a semi-humid climate characterized by a hot summer and a cold and wet winter with a rainfall averaging 800 mm per year.

Collection of ectoparasites and tissue sampling

The investigation in El Ghora (Bougous, El Tarf) was conducted on domestic animals (cattle, sheep, goats and dogs). Ectoparasites were collected with the permissions of the animals' owners. All arthropods were collected using blunted clockmakers' forceps and immediately placed in 70% ethanol inside tubes labeled with the identification number and the date of collection. A portion of the collected ectoparasites was used for the present study. The field sampling in Cheabat El Balout (Ouled Driss, Souk Ahras), was conducted on wild mammals [two boars (*Sus scrofa algira*), two jackals (*Canis aureus*) one mongoose (*Echinomon herpestis*), ten bats (*Chiroptera* spp), one porcupine (*Hystrix cristata*) and four hedgehogs (*Atelerix algirus*)]. Ectoparasites and tissues (spleens) were sampled. Hedgehogs were captured with the aid of spotlights during nightly walks through parts of the study regions near to a poultry slaughterhouse. Hedgehogs were anesthetized using ketamine and released into their natural habitat after full recovery of

ectoparasites. This study on hedgehogs was authorized by the local ethics committee and by national legislation (le journal officiel n° 47 du 19 juillet 2006, http://www.iucnredlist.org/apps /redlist/details). Two boars, two jackals, one mongoose and one porcupine were found freshly dead following road accidents and were also inspected for ectoparasites, their spleens were sampled using adapted scalpels and stored in tubes containing 70% ethanol. Finally, using hunting nets, we caught ten bats. The nets (identical to those used by ornithologists to capture and band birds) are very fine with mesh like fishing net. They are stretched between two poles, and placed in the passageway of the bats, input of the cave. Bats were recovered the next day, and were dead. Once detached from the nets, we looked for ectoparasites and took spleen samples.

All biological materials were forwarded thereafter to Marseille, France, for morphological identification of ectoparasites at the species level using morphological criteria within standard taxonomic keys. Molecular analyses of ectoparasites and tissue samples were performed to detect *Rickettsia* spp, *Bartonella* spp, *Coxiella burnetii* and *Borrelia* spp.

DNA extraction

Prior to DNA extraction, a convenient sample was selected according to a good representation of species and hosts in El Ghora, and all collected ectoparasites and biological materials of Cheabat el Balout were used to extract their DNA.

Arthropods and spleens were rinsed twice in distilled water for 10 minutes and dried on sterile filter paper; handling was performed in a laminar flow biosafety cabinet. Ectoparasites and a portion of the spleen samples were individually crushed in sterile eppendorf tubes. A total of 200 µl of DNA was extracted from one half of each ectoparasite and a portion of spleen using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) by Qiagen-BioRobot EZ1, according to the manufacturer's instructions. Genomic DNA was stored at -22°C under sterile conditions.

Detection of bacteria

Once DNA had been extracted, it was used in qPCR template assays to detect *Bartonella* spp, *Rickettsia* spp, *Coxiella burnetii* and *Borrelia* spp. The final qPCR reaction mixture consisted of 5 µl of DNA and 15 µl of mix from the Takyon PCR Kit (Qiagen, Hilden, Germany) as described [26]. Negative controls were used in each qPCR and consisted of DNA extracted from uninfected ticks from our laboratory colony. Positive controls

included DNA extracted from a dilution of cultured strains of *B. elizabethae* (detection of *Bartonella* spp), *Ri. montanensis* (for the detection of *Rickettsia* spp), *Coxiella burnetii* (for the detection of *Coxiella burnetii*) and *Borrelia crocidurae* (for the detection of *Borrelia* spp). Results were deemed positive if the Cycle threshold (Ct) value obtained by CFX96^{Tm} was lower than 36. All positive results were confirmed with a second qPCR system and /or sequence reaction.

Detection of *Bartonella* spp.

DNA samples were screened by qPCR targeting the ITS for the detection of *Bartonella* spp [27]. The positive samples with ITS primers were then confirmed by standard PCR performed with *Bartonella*-specific primers of the intergenic spacer region between the 16S and 23S rRNA genes [28]. PCR amplification success was verified by migration in 2% Agarose gel, followed by purification using the NucleoFast 96 PCR plate (Machery-Nagel EURL, France), as recommended by the manufacturer. The purified PCR products were sequenced using Urb1 and Urb2 primers and using BigDye version 1.1 cycle sequencing ready reaction mix (Applied Biosystems, Foster City, CA). Data were collected with an ABI Prism 3130xl Genetic Analyzer capillary sequencer (ABI PRISM, PE Applied Biosystems, USA). Sequences were edited and assembled using Chromas Pro 1.34

(Technelysium Pty. Ltd., Tewantin, Australia). BLAST searches were performed to identify the obtained sequences.

Detection of *Rickettsia* spp

Rickettsial DNA was detected using a *Rickettsia genus*-specific qPCR with a 25-bp probe targeting the partial sequence of the citrate synthase gene (gltA) [29]. All samples identified as positive by qPCR were confirmed by a different standard PCR and sequencing for fragments of the gltA and OmpA genes [29]. DNA sequencing reactions were performed on positive samples amplified by standard PCR, as described above. The sequencing results were confirmed with qPCR specific to the species detected (*Ri. slovaca*, *Ri. massiliae*, *Ri. aeschlimannii* and *Ri. felis*). **Table 1** summarizes the probes and primers used to confirm and identify rickettsiae in samples.

Detection of *Borrelia* spp

qPCR targeting the 16S rRNA gene was used, as described [29], to screen DNA samples for all *Borrelia* spp.

Detection of *Coxiella burnetii*

Coxiella burnetii bacterial DNA was initially detected by qPCR with *C. burne*tii–specific primers and a probe designed to amplify the IS1111 gene [30]. QPCR with primers and a probe designed for the amplification of IS30a spacers were used to confirm *C. burnetii*–positive results [25].

Results

Sample collection

In El Ghora, a total of 1549 ticks were collected, including eight species. 565 ticks were sampled from 123 cattle; *Rhipicephalus sanguineus* (316/565), *R. bursa* (50/565), *Hyalomma detritum detritum* (94/565) and *Hy. anatolicum excavatum* (105/565). 529 ticks belonging to five species; *R. sanguineus* (454/529), *R. bursa* (72/529), *Hy. lusitanicum* (1/529), *Hy. d. detritum* (1/529) and *Ixodes ricinus* (1/529) were collected from 250 sheep. Four species; *R. sanguineus* (104/130), *R. bursa* (19/130), *Hy. lusitanicum* (3/130) and *Hy. marginatum marginatum* (4/130) were collected from 125 goats, Also 325 ticks were sampled from 50 dogs and belonging to two species ; *R. sanguineus* (323/325) and *I. hexagonus* (2/325).

For the investigation of wild animals and their ectoparasites in Cheabat El Balout, 77 ticks, 136 fleas, 11 *Nycteribiidae* and 16 spleens were sampled. First, on two boars, we sampled two spleens and 13 ticks [*R. sanguineus* (9/13), *Dermacentor marginatus* (3/13) and *Haemaphysalis punctata* (1/13)], also the spleen, two *R. sanguineus* and three *I. ricinus* (2 adults and 1 nymph) were collected from one mongoose. Ectoparasites and spleens were collected from one porcupine and two jackals, while we idenfied 21 fleas *Pariodontis riggenbachi* on the porcupine and 23 *R. sanguineus* on the two jackals. Using the nets we trapped ten bats which were examined for ectoparasites: we idenfied *I. vespertilionis* (19) (8 larva and 11 adults females), *Ischnopsyllus intermedius* fleas (3), *Nycteribiidae* spp (11), we also sampled the spleen of each bat. Finally we caught 4 hedgehogs and identified 112 fleas [*Archaeopsylla erinacei* (110/112) and *Ctenocephalides felis* (2/112)] and 19 ticks [*R. sanguineus* (10/19) and *I. hexagonus* (9/19)].

Detection of *Bartonella* spp

In El Ghora, over the 1549 ticks collected from domestic mammals, 191 ticks were conveniently selected and tested with PCR for the entire set of pathogens detected in this work (**Table 2**). All the 191 ticks tested negative for *Bartonella* spp.

However overall ticks, fleas, *Nycteribiidae* and spleen portions tested with qPCR, 26 samples collected on bat were positive, including (12/19, 63.2%) of *I. vespertilionis* ticks, (8/11, 72.7%) *Nycteribiidae* flies and (6/10, 60%) spleens. Using standard PCR, we analyzed and sequenced the PCR products of a representative sample (with Ct < 30), results showed that all sequences of *Bartonella* spp detected in ectoparasites and spleens of bats were similar to the sequence of *B. tamiae* (100% similarity with the *Bartonella tamiae* strain Th339 16S-23S ribosomal RNA intergenic spacer, partial sequence, GenBank no EF605284.1, 451/451 bp).

Detection of *Rickettsia* spp

In El Ghora, (29/191, 15.2%) of ticks tested positive for *Rickettsia* spp by qPCR. These included four species of ticks, including *R. sanguineus* (15/29, 51.7%), (2/29, 6.9%) of *R. bursa,* (6/29, 20.7%) of *Hy. d. detritum* and in (6/29, 20.7%) of *Hy. a. excavatum*. Concerning the (15/29) of *R. sanguineus*, (3/15) were collected from cattle, (11/15) were sampled from sheep and 1/20 were trapped on dogs. The (2/29, 6.89%) of *R. bursa* were all sampled from cattle, also the (6/29, 20.7%) of *Hy. d. detritum* were collected from cattle and finally the (6/29, 20.7%) of *Hy. a. excavatum* were sampled from goats. DNA

sequence analyses of the PCR products targeting *OmpA* on the *R. sanguineus* and *R. bursa* ticks, whatever the host's tick type, showed 100% similarity with *Rickettsia massiliae* (GenBank accession no. U43793.1). Also sequencing of the *OmpA* gene fragment from the positive *Hy. d. detritum* and *Hy. a. excavatum* showed 99% similarity with *Rickettsia aeschlimannii* strain EgyRickHimp-El-Arish-17 outer membrane protein A (*OmpA*) gene, partial cds (GenBank accession no. HQ335158.1, 632/633 bp).

In Cheabat el Balout, the RKND03 qPCR system was used to test the 77 ticks, 136 fleas, 11 *Nycteribiidae* flies and 16 spleens sampled from wild animals. Overall we detected DNA from *Rickettsia* spp in three ticks of boars (2 *D. marginatus* and 1 *Hae. punctata*), in 80 *A. erinacei* and 2 *C. felis* fleas of hedgehogs. DNA sequence analyses of the PCR products targeting OmpA on the two *D. marginatus* and on the one *Hae. punctata* showed 99% similarity with *Rickettsia slovaca* strain WB2/Dm Pavullo outer membrane protein A (ompA) gene, partial cds GenBank accession no. HM161787.1, 618/633 bp). Concerning the 80 *A. erinacei* and 2 *C. felis* of hedgehogs positive for *Rickettsia* spp using RKND03, two qPCRs targeting the biotin synthase (*bioB)* and membrane phosphatase genes of *R. felis* [31] were

performed and all the 82 fleas were positive for *Ri. felis* (**Table2**).

Detection of *Coxiella burnetii*

In El Ghora, the 191 ticks were screened with qPCR targeting the *Is1111* of *C. burnetii*, and were negative, However in Cheabat el Balout, Souk Ahras we detected DNA of *C. burnetii* in 3 *I. vespertilionis* of bats. We obtained the same result using another qPCR system targeting *Is30a* (**Table2**).

Detection of *Borrelia* spp

All tested samples were negative for *Borrelia* spp using the 16S qPCR system of *Borrelia* genus.

Discussion

This investigation reports the first direct evidence of *B. tamiae* in *I. vespertilionis*, *Nycteribiidae* and spleens of bats in Algeria. The association between *Ri. slovaca* and *H. punctata* of boar and also between *Ri. massiliae* and *R. bursa* of cattle are reported for the first time in Algeria.. Other rickettsiae were detected in this field as previously detected in Algeria namely *Ri. massiliae* in *R. sanguineus*, *Ri. aeschlimannii* in *Hyalomma* spp ticks and *Ri. felis* in *A. erinacei* and in *C. felis* fleas. Using molecular tools *C. burnetii*, the agent of Q fever, was also detected in *I. vespertilionis* ticks of bat.

In this study, pathogens in ticks, fleas and the spleens of wild and domestic animals of Algeria were detected using a validated method consisting of double qPCR and/or subsequent sequencing. The robustness of the data that we report is based on strict laboratory procedures and controls, including rigorous positive and negative controls to validate each test.

Bartonella-associated illnesses occur worldwide, and they encompass a broad clinical spectrum, including fever, skin lesions, endocarditis, lymphadenopathy, and abnormalities of the central nervous system, eye, liver and bone tissues [32]. *Bartonella tamiae,* is a newly described bacterial species,

initially isolated from the blood of three hospitalized patients in Thailand [28]. These patients presented with headache, myalgia, anemia, and mild liver function abnormalities [33]. This novel *Bartonella* species has been newly recognized as a pathogen [28,34]. Throughout our investigation *B. tamiae* was detected for the first time in Algeria, and in ticks, *Nycteribiidae* and spleens of bats. Bats are the second species group of mammals after Rodents confirmed to carry *Bartonella* spp [35]. Renewed interest in *Bartonella* research in mammals has confirmed the presence of *Bartonella* spp in bats (Chiroptera) in Guatemala, Kenya [36] and the United Kingdom [37].

C. burnetii was also detected in this field; this pathogen agent of Q fever is associated with many acute and chronic manifestations [24,38]. Q fever is typically an acute febrile illness with nonspecific clinical signs in humans, but isolated fever, hepatitis and/or atypical pneumonia are the most commonly described manifestations. A small proportion of infected people develop life-threatening valvular endocarditis [24,38]. Q fever has been described worldwide in outbreaks involving sheep, goats, cats, dogs and wild animals, while reservoirs are extensive but only partially known and include mammals, birds, and arthropods, mainly ticks [39]. In Algeria few human cases have been reported, one in 2005, where 2 patients were found to be

seropositive for *C. burnetii* (one was confirmed positive by nested PCR) [40]. In 2012, through 268 qPCR-tested samples from Oran, Western Algeria, only one patient was positive for *C. burnetii* [25].

We detected *Ri. slovaca* in ticks of boars, namely in *H. punctata* and *D. marginatus*. Our detection of *Ri. slovaca* in *H. punctata* ticks may be due to a co-feeding with infected *D. marginatus*, which is a recognized vector and reservoir of the bacteria. *Ri. slovaca* is associated with a syndrome characterized by scalp eschars and neck lymphadenopathy following tick bites [41]. This syndrome was named TIBOLA (tick-borne lymphadenopathy) or DEBONEL (*Dermacentor*-borne necrotic erythema and lymphadenopathy)[1]. The term "SENLAT" (**S**calp **E**schar and **N**eck **L**ymph**A**denopathy after a **T**ick bite) has also been recently proposed [14]. In Algeria, *Ri. slovaca* was previously detected in *D. marginatus* ticks collected from the vegetation of the Blida region, in 2012 [42].

In Algeria, *Ri. massiliae* was detected in *R. turanicus,* in 2006 [43]. Our results confirm the presence of *Ri. massiliae* in Algerian ticks, where we detected it on *R. sanguineus* of cattle, sheep, dogs and boars. Using qPCR, it was also detected in *R. bursa* of cattle. These SFG rickettsiae were described in 1992,

then subsequently detected in other *Rhipicephalus species*, including *R. bursa* in European countries [41].

Our results indicate the presence of *Ri. aeschlimannii* in *Hy.a. excavatum* and *Hy. d. detritum* ticks of cattle in the far North-East of Algeria. *Ri. aeschlimannii* is an emerging pathogen that causes symptoms similar to those of Mediterranean spotted fever [41]. It has been associated with ticks, particularly with *Hy. m. marginatum* and *Hy. m. rufipes* ticks, in southern Europe and Africa [41].

In Algeria, *Ri. aeschlimannii* was previously detected in *Hy. dromedarii* and *Hy. m. rufipes* of camels from southern Algeria [44] and *Hy. aegyptium* ticks of tortoises trapped near Algiers [45]. Our results confirm the presence of *Ri. aeschlimannii* in Algeria but also complete the geographical distribution of this pathogen from the south and center to the far north-east.

Finally we detected DNA of *Ri. felis* in hedgehog fleas (*Ct. felis and A. erinacei*). *Ri. felis* is an emergent agent of infectious disease in humans, and this SFG agent is known to be maintained in cat fleas (*Ct. felis*) [46,47]. To date, 12 flea species, 8 tick species and 3 mite species have been found to be infected with *Ri. felis* [47]. These rickettsiae have also recently been detected

in several mosquito species in sub-Saharan Africa [26,48,49]. Clinical features may include fever, fatigue, headache, generalized maculopapular rash and inoculation eschar(s) [47]. It is known to be a frequent agent of fever of unknown origin [50]. In Algeria *Ri. felis* was previously detected in *A. erinacei* fleas of hedgehogs of M'sila and Bordj-Bou-Arreridj, Algeria [50,51]. It was also detected in *Ct. canis* [52]. Here, we report for the first time the presence of *Ri. felis* in *Ct. felis* fleas from Algeria.

Conclusion

For the first time in Algeria, we detected *B. tamiae*, *Coxiella burnetii* and rickettsiae (*Ri. slovaca, Ri. massiliae, Ri. aeschlimannii* and *Ri. felis*) in two regions of the far north-east of Algeria. We expanded knowledge of the repertoire of ticks and flea-borne bacteria present in ectoparasites and/or tissues of domestic and wild animals in Algeria. We illustrated the role of ticks and fleas in the entomological survey of vector -borne disease, allowing clinicians to understand a portion of unknown fever in human and/or animal patients. Future studies on rickettsioses, bartonelloses and other vector-borne diseases should be performed to assess their epidemiological and clinical

relevance in Algeria, to estimate actual prevalence and to allow the establishment of anti-vector control plans.

Conflict of interests

The authors declared that they have no competing interests.

Authors' Contributions

HL contributed to arthropod collections (first part), performed DNA extractions, qPCRs, sequencing and first drafted the paper. AA contributed to arthropod collections (second part) and preparation of the manuscript. IB analyzed the data, coordinated the study and identification of the arthropods. ABES contributed to the preparation of the manuscript. ABEN contributed to conceiving, designing and coordinating the study. DR Contributed reagents/materials/analysis tools and analyzed the data. PP conceiving, designing and coordinating the experiment. All authors read and approved the final manuscript.

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References

1. Parola P, Paddock CD, Raoult D: **Tick-borne rickettsioses around the world: emerging diseases challenging old concepts.** *Clin Microbiol Rev* 2005, **18:**719-756.

2. Parola P, Raoult D: **Ticks and tickborne bacterial diseases in humans: an emerging infectious threat.** *Clin Infect Dis* 2001, **32:**897-928.

3. Mediannikov O, Fenollar F: **Looking in ticks for human bacterial pathogens.** *Microb Pathog* 2014, **77:**142-148.

4. Baneth G: **Tick-borne infections of animals and humans: a common ground.** *Int J Parasitol* 2014, **44:**591-596.

5. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D: **Fleas and flea-borne diseases.** *Int J Infect Dis* 2010, **14:**e667-e676.

6. Parola P, Davoust B, Raoult D: **Tick- and flea-borne rickettsial emerging zoonoses.** *Vet Res* 2005, **36:**469-492.

7. Eisen RJ, Gage KL: **Transmission of flea-borne zoonotic agents.** *Annu Rev Entomol* 2012, **57:**61-82.

8. Parola P, Raoult D: **Tropical rickettsioses.** *Clin Dermatol* 2006, **24:**191-200.

9. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D: **Fleas and flea-borne diseases.** *Int J Infect Dis* 2010, **14:**e667-e676.

10. Merhej V, El KK, Raoult D: **Whole genome-based phylogenetic analysis of Rickettsiae.** *Clin Microbiol Infect* 2009, **15 Suppl 2:**336-337.

11. Nogueras MM, Pons I, Ortuno A, Miret J, Pla J, Castella J, Segura F: **Molecular Detection of *Rickettsia typhi* in Cats and Fleas.** *PLoS One* 2013, **8:**e71386.

12. Bitam I: **Vectors of rickettsiae in Africa.** *Ticks Tick Borne Dis* 2012, **3:**382-386.

13. Mouffok N, Parola P, Lepidi H, Raoult D: **Mediterranean spotted fever in Algeria--new trends.** *Int J Infect Dis* 2009, **13:**227-235.

14. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE et al.: **Update on tick-borne rickettsioses around the world: a geographic approach.** *Clin Microbiol Rev* 2013, **26:**657-702.

15. Kernif T, Socolovschi C, Bitam I, Raoult D, Parola P: **Vector-borne rickettsioses in North Africa.** *Infect Dis Clin North Am* 2012, **26:**455-478.

16. Chomel BB, Boulouis HJ, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, Birtles RJ, Koehler JE, Dehio C: **Ecological fitness and strategies of adaptation of *Bartonella species* to their hosts and vectors.** *Vet Res* 2009, **40:**29.

17. Mogollon-Pasapera E, Otvos L, Jr., Giordano A, Cassone M: **Bartonella: emerging pathogen or emerging awareness?** *Int J Infect Dis* 2009, **13:**3-8.

18. Chomel BB, Kasten RW: **Bartonellosis, an increasingly recognized zoonosis.** *J Appl Microbiol* 2010, **109:**743-750.

19. Harms A, Dehio C: **Intruders below the radar: molecular pathogenesis of *Bartonella* spp.** *Clin Microbiol Rev* 2012, **25:**42-78.

20. Azzag N, Petit E, Gandoin C, Bouillin C, Ghalmi F, Haddad N, Boulouis HJ: **Prevalence of select vector-borne pathogens in stray and client-owned dogs from Algiers.** *Comp Immunol Microbiol Infect Dis* 2015, **38:**1-7.

21. Kernif T, Aissi M, Doumandji SE, Chomel BB, Raoult D, Bitam I: **Molecular evidence of Bartonella infection in domestic dogs from Algeria, North Africa, by polymerase chain reaction (PCR).** *Am J Trop Med Hyg* 2010, **83:**298-300.

22. Bitam I, Rolain JM, Nicolas V, Tsai YL, Parola P, Gundi VA, Chomel BB, Raoult D: **A multi-gene analysis of diversity of Bartonella detected in fleas from Algeria.** *Comp Immunol Microbiol Infect Dis* 2012, **35:**71-76.

23. Azzag N, Haddad N, Durand B, Petit E, Ammouche A, Chomel B, Boulouis HJ: **Population structure of *Bartonella henselae* in Algerian urban stray cats.** *PLoS One* 2012, **7:**e43621.

24. Maurin M, Raoult D: **Q fever.** *Clin Microbiol Rev* 1999, **12:**518-553.

25. Angelakis E, Mediannikov O, Socolovschi C, Mouffok N, Bassene H, Tall A, Niangaly H, Doumbo O, Znazen A,

Sarih M et al.: ***Coxiella burnetii*-positive PCR in febrile patients in rural and urban Africa.** *Int J Infect Dis* 2014, **28:**107-110.

26. Socolovschi C, Pages F, Raoult D: ***Rickettsia felis* in *Aedes albopictus* mosquitoes, Libreville, Gabon.** *Emerg Infect Dis* 2012, **18:**1687-1689.

27. Varagnol M, Parola P, Jouan R, Beaucournu JC, Rolain JM, Raoult D: **First detection of *Rickettsia felis* and *Bartonella clarridgeiae* in fleas from Laos.** *Clin Microbiol Infect* 2009, **15 Suppl 2:**334-335.

28. Kosoy M, Morway C, Sheff KW, Bai Y, Colborn J, Chalcraft L, Dowell SF, Peruski LF, Maloney SA, Baggett H et al.: ***Bartonella tamiae* sp. nov., a newly recognized pathogen isolated from three human patients from Thailand.** *J Clin Microbiol* 2008, **46:**772-775.

29. Socolovschi C, Kernif T, Raoult D, Parola P: ***Borrelia, Rickettsia, and Ehrlichia species* in bat ticks, France, 2010.** *Emerg Infect Dis* 2012, **18:**1966-1975.

30. Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, Sokhna C, Trape JF, Raoult D: ***Coxiella burnetii* in humans and ticks in rural Senegal.** *PLoS Negl Trop Dis* 2010, **4:**e654.

31. Leulmi H, Socolovschi C, Laudisoit A, Houemenou G, Davoust B, Bitam I, Raoult D, Parola P: **Detection of *Rickettsia felis*, *Rickettsia typhi, Bartonella Species* and *Yersinia pestis* in Fleas (Siphonaptera) from Africa.** *PLoS Negl Trop Dis* 2014, **8:**e3152.

32. Colton L, Zeidner N, Lynch T, Kosoy MY: **Human isolates of *Bartonella tamiae* induce pathology in experimentally inoculated immunocompetent mice.** *BMC Infect Dis* 2010, **10:**229.

33. Kabeya H, Colborn JM, Bai Y, Lerdthusnee K, Richardson JH, Maruyama S, Kosoy MY: **Detection of *Bartonella tamiae* DNA in ectoparasites from rodents in Thailand and their sequence similarity with bacterial cultures from Thai patients.** *Vector Borne Zoonotic Dis* 2010, **10:**429-434.

34. Anderson BE, Neuman MA: ***Bartonella* spp. as emerging human pathogens.** *Clin Microbiol Rev* 1997, **10:**203-219.

35. Jacomo V, Kelly PJ, Raoult D: **Natural history of Bartonella infections (an exception to Koch's postulate).** *Clin Diagn Lab Immunol* 2002, **9:**8-18.

36. Concannon R, Wynn-Owen K, Simpson VR, Birtles RJ: **Molecular characterization of haemoparasites infecting bats (Microchiroptera) in Cornwall, UK.** *Parasitology* 2005, **131:**489-496.

37. Kosoy M, Bai Y, Lynch T, Kuzmin IV, Niezgoda M, Franka R, Agwanda B, Breiman RF, Rupprecht CE: ***Bartonella* spp. in bats, Kenya.** *Emerg Infect Dis* 2010, **16:**1875-1881.

38. Bai Y, Kosoy M, Recuenco S, Alvarez D, Moran D, Turmelle A, Ellison J, Garcia DL, Estevez A, Lindblade K et al.: ***Bartonella* spp. in Bats, Guatemala.** *Emerg Infect Dis* 2011, **17:**1269-1272.

39. Kazar J: ***Coxiella burnetii*** **infection.** *Ann N Y Acad Sci* 2005, **1063:**105-114.

40. Angelakis E, Raoult D: **Q Fever.** *Vet Microbiol* 2010, **140:**297-309.

41. Benslimani A, Fenollar F, Lepidi H, Raoult D: **Bacterial zoonoses and infective endocarditis, Algeria.** *Emerg Infect Dis* 2005, **11:**216-224.

42. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE et al.: **Update on tick-borne rickettsioses around the world: a geographic approach.** *Clin Microbiol Rev* 2013, **26:**657-702.

43. Kernif T, Messaoudene D, Ouahioune S, Parola P, Raoult D, Bitam I: **Spotted fever group rickettsiae identified in *Dermacentor marginatus* and *Ixodes ricinus* ticks in Algeria.** *Ticks Tick Borne Dis* 2012, **3:**380-381.

44. Bitam I, Parola P, Matsumoto K, Rolain JM, Baziz B, Boubidi SC, Harrat Z, Belkaid M, Raoult D: **First molecular detection of *R. conorii, R. aeschlimannii,* and *R. massiliae* in ticks from Algeria.** *Ann N Y Acad Sci* 2006, **1078:**368-372.

45. Djerbouh A, Kernif T, Beneldjouzi A, Socolovschi C, Kechemir N, Parola P, Raoult D, Bitam I: **The first molecular detection of *Rickettsia aeschlimannii* in the ticks of camels from southern Algeria.** *Ticks Tick Borne Dis* 2012, **3:**374-376.

46. Bitam I, Kernif T, Harrat Z, Parola P, Raoult D: **First detection of *Rickettsia aeschlimannii* in *Hyalomma***

***aegyptium* from Algeria.** *Clin Microbiol Infect* 2009, **15 Suppl 2:**253-254.

47. La Scola B, Meconi S, Fenollar F, Rolain JM, Roux V, Raoult D: **Emended description of *Rickettsia felis* (Bouyer et al. 2001), a temperature-dependent cultured bacterium.** *Int J Syst Evol Microbiol* 2002, **52:**2035-2041.

48. Parola P: ***Rickettsia felis*: from a rare disease in the USA to a common cause of fever in sub-Saharan Africa.** *Clin Microbiol Infect* 2011, **17:**996-1000.

49. Keita AK, Socolovschi C, Ahuka-Mundeke S, Ratmanov P, Butel C, Ayouba A, Inogwabini BI, Muyembe-Tamfum JJ, Mpoudi-Ngole E, Delaporte E et al.: **Molecular evidence for the presence of *Rickettsia felis* in the feces of wild-living African apes.** *PLoS One* 2013, **8:**e54679.

50. Mediannikov O, Socolovschi C, Edouard S, Fenollar F, Mouffok N, Bassene H, Diatta G, Tall A, Niangaly H, Doumbo O et al.: **Common epidemiology of *Rickettsia felis* infection and malaria, Africa.** *Emerg Infect Dis* 2013, **19:**1775-1783.

51. Bitam I, Parola P, De La Cruz KD, Matsumoto K, Baziz B, Rolain JM, Belkaid M, Raoult D: **First molecular detection of *Rickettsia felis* in fleas from Algeria.** *Am J Trop Med Hyg* 2006, **74:**532-535.

52. Khaldi M, Socolovschi C, Benyettou M, Barech G, Biche M, Kernif T, Raoult D, Parola P: **Rickettsiae in arthropods collected from the North African Hedgehog (*Atelerix algirus*) and the desert hedgehog (*Paraechinus aethiopicus*) in Algeria.** *Comp Immunol Microbiol Infect Dis* 2012, **35:**117-122.

53. Bitam I, Baziz B, Kernif T, Harrat Z, Parola P, Raoult D: **Molecular detection of *Rickettsia typhi* and *Rickettsia felis* in fleas from Algeria.** *Clin Microbiol Infect* 2009, **15 Suppl 2:**255-256.

Table 1: Target sequences, primers and probes used to confirm the detection of rickettsiae by qPCR.

Table 2: detection of Rickettsiae, *Bartonella* spp, and *Coxiella burnetii* in arthropods

Table 1: Target sequences, primers and probes used to confirm the detection of rickettsiae by qPCR.

Quantitative real-time PCR designation and specificity	Target gene	Forward primer	Reverse primer	Probe
Rickettsia felis	Biotin synthase	ATG-TTC-GGG-CTTCCG-GTA-TG	CCG-ATT-CAG-CAGGTT-CTT-CAA	6-FAM- GCT-GCG-GCGGTA-TTT-TAG-GAA -TGGG-TAMRA
Rickettsia aeschlimannii	Scal	AAGCGGCACTTTAGGTAAAGAAA	CATGCTCTGCAAATGAACCA	6FAM-TGGGGAAATATGCCGTATACGCAAGC -TAMRA
Rickettsia massiliae	R.mass_9666	CCAACCTTTTGTTGTTGCAC	TTGGATCAGTGTGACGGACT	6FAM-CACGTGCTGCTTATACCAGCAAACA-TAMRA
Rickettsia slovaca	R.slov	GCAACGGTTTTTGGTATCGT	AATCGAATGCACCACCACTT	6FAM- TCCCGTCCCAGCCATTCGTC-TAMRA

Table 2: detection of Rickettsiae, *Bartonella* spp, and *Coxiella burnetii* in arthropods

Ectoparasite species	Localization	Animal (N)	No. of ectoparasites collected (m = male, f = female)	No. of ectoparasites tested by qPCR (m = male, f = female)	*Rickettsia spp*	*Bartonella spp*	*Coxiella burnetii*	*Borrelia spp*
Rhipicephalus sanguineus	El Ghora, El Tarf.	Cattle (123)	316 (104m, 212f)	20 (10m, 10f)	3/20 (1m, 2f) *Ri. massiliae*	-	-	-
		Sheep (250)	454 (217m, 237f)	20 (10m, 10f)	11/20 (9m, 2f) *Ri. massiliae*	-	-	-
		Goats (128)	104 (55m, 49f)	20 (10m, 10f)	-	-	-	-
		Dogs (50)	323 (222m, 101f)	20 (10m, 10f)	1/20 (1m) *Ri. massiliae*	-	-	-
	Cheabat El Balout, Souk Ahras	Boars (2)	9 (5m, 4f)	9 (5m, 4f)	8/9 (5m, 3f) *Ri. massiliae*	-	-	-
		Mangoose (1)	2 (1m, 1f)	2 (1m, 1f)	2/2 (1m, 1f) *Ri. massiliae*	-	-	-
		Jackals (2)	23 (15m, 8f)	23(15m, 8f)	13/23(8m, 5f) *Ri. massiliae*	-	-	-
		Hedgehogs (4)	10 (2m, 8f)	10(2m, 8f)	-	-	-	-
Rhipicephalus bursa	El Ghora, El Tarf.	Cattle (123)	50 (39m, 11f)	20 (10m, 10f)	2/20 (2f) *Ri. massiliae*	-	-	-
		Sheep (250)	72 (37m, 35f)	20 (10m, 10f)	-	-	-	-
		Goats (128)	19 (19m)	19 (19m)	-	-	-	-
Hyalomma lusitanicum	El Ghora, El Tarf.	Sheep (250)	1 (1m)	1 (1m)	-	-	-	-
		Goats (128)	3 (2m, 1f)	3 (2m, 1f)	-	-	-	-
Hyalomma detritum detritum	El Ghora, El Tarf.	Cattle (123)	94 (41m, 53f)	20 (10m, 10f)	6/20 (2m, 4f) *Ri. aeschlimannii*	-	-	-
		Sheep (250)	1 (1f)	1 (1f)	-	-	-	-
Hyalomma anatolicum excavatum	El Ghora, El Tarf.	Cattle (123)	105 (54m, 51f)	20 (10m, 10f)	6/20 (4m, 2f) *Ri. aeschlimannii*	-	-	-
Hyalomma marginatum marginatum	El Ghora, El Tarf.	Goats (128)	4 (4f)	4 (4f)	-	-	-	-
Ixodes ricinus	El Ghora, El Tarf.	Sheep (250)	1 (1m)	1 (1m)	-	-	-	-
	Cheabat El Balout, Souk Ahras	Mongoose (1)	1 (1m)	1 (1m)	-	-	-	-
Ixodes hexagonus	El Ghora, El Tarf.	Dogs (50)	2 (2f)	2 (2f)	-	-	-	-
	Cheabat El Balout, Souk Ahras	Hedgehogs (4)	9 (1m, 8f)	9 (1m, 8f)	-	-	-	-
Dermacentor marginatus	Cheabat El Balout, Souk Ahras	Boars (2)	3 (3f)	3 (3f)	2/3 (2f) *Ri. slovaca*	-	-	-
Haemaphysalis punctata	Cheabat El Balout, Souk Ahras	Boars (2)	1 (1f)	1 (1f)	1/1 (1f) *Ri. slovaca*	-	-	-
Ixodes vespertilionis	Cheabat El Balout, Souk Ahras	Bats (10)	19 (2f, 17 nymphs)	19 (2f, 17 nymphs)	-	12/19 (1f, 11 nymphs) *B. tamiae*	3/19 (2f, 1 nymph)	-
Ischnopsyllus intermedius	Cheabat El Balout, Souk Ahras	Bats (10)	3 (3f)	3 (3f)	-	-	-	-
Ctenocephalides felis	Cheabat El Balout, Souk Ahras	Hedgehogs (4)	2 (2f)	2 (2f)	2/2 (2f) *Ri. felis*	-	-	-
Pariodontis riggenbachi	Cheabat El Balout, Souk Ahras	Porcupine (1)	21 (3m, 18f)	21 (3m, 18f)	-	-	-	-
Nycteribudae	Cheabat El Balout, Souk Ahras	Bats (10)	11	11	-	8/11 *B. tamiae*	-	-
Archaeopsylla erinacei	Cheabat El Balout, Souk Ahras	Hedgehogs (4)	110 (39m, 71f)	110 (39m, 71f)	80/110 (19m, 61f) *Ri. felis*	-	-	-

ARTICLE 5

Article N.5. Bessas A, **Leulmi H**, Bitam I, Zaidi S, Parola P and Raoult D. **Molecular evidence of *Ehlichia canis, Coxiella burnetii* and *Bartonella henselae* in spleens of dog and cats in Algiers**. (Journal of Vector-borne and Zoonotic Diseases).

Molecular evidence of vector-borne pathogens in dogs and cats and their ectoparasites in Algeria.

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Conflict of interests

The authors declared that they have no competing interests.

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Abstract

Background: In recent years, emergent canine and feline vector-borne diseases have become a world heath concern. In Algeria, only limited information is currently available on the prevalence of these diseases in companion animals. The aim of the present work was to detect vector-associated bacteria in stray dogs and cats and their ectoparasites in Algiers, Algeria.

Methods/ findings: The presence of *Coxiella burnetii, Ehrlichia canis, Bartonella* spp., *Rickettsia* spp. and *Borrelia* spp. was detected by quatitative real-time PCR (qPCR) in spleen samples, ticks and fleas of dogs and cats trapped from animal shelters in Algiers. Bacteria were then identified by specific qPCR or regular PCR followed by sequencing.

Results: 18/117 (15.38 %) tested dogs were positive to at least one vector-borne agent, including 1/117 (0.85 %) co-infected with two agents. *C. burnetii* and *B. henselae* were identified in 1/117(0.85 %) dog individually. DNA of *E. canis* was detected in 17/117 (14.52 %) dogs. Out of the 107 cats sampled, 2/107 (1.87%) were positive to at least one vector-borne agent. 1/107

(0.93%) cat was positive to *C. burnetii* and another 1/107 (0.93%) to *B. henselae*.

DNA of *R. massiliae, R. conorii* and *E. canis* was detected in *Rhipicephalus sanguineus.* While cat fleas were infected with *R. felis, B. henselae* and *B. clarridgeiae. B. vinsonii* subsp. *berkhoffii* was identified in *Xenopsylla cheopis* collected from dogs.

Conclusions: To our knowledge, this is the first molecular detection of *C. burnetii* in dog and cat in Algeria. This is also the first report of *B. henselae* and *B. vinsonii* subsp. *Berkhoffii* in fleas and *E. canis* in ticks from Algeria.

Keywords: Dog, cat, tick, flea, *Coxiella burnetii*, *Ehrlichia canis*, *Bartonella* spp., *Rickettsia* spp. qPCR, Algeria.

INTRODUCTION

Vector-borne agents are increasingly recognized as important cause of morbidity and mortality in humans and domestic animals worldwide [1, 2]. Companion animals, such as dogs and cats, are potential victims, reservoirs and/or sentinels of different vector-borne pathogens [3]. They are exposed to several arthropods species which are incriminated in the transmission cycles of many pathogens [4, 5].

Coxiella burnetii is recognized as a worldwide zoonotic pathogen that causes Q fever [6]. Recently, many authors highlight the role of pets in the epidemiology of Q fever and indicate that the contact with infected dogs and cats represents a risk factor for acquiring the infection [6-9]. In Algeria, *C. burnetii* infection in humans has been poorly reported [10]. However no published data exist concerning the prevalence of *C. burnetii* in animals.

Ehrlichia canis is a bacteria belonging to the *Anaplasmataceae* family that causes canine monocytic ehrlichiosis [2]. The disease was firstly described in sick dogs from Algeria, in 1935 [11]. Newly two studies indicate the molecular presence of *E. canis* in dogs in this country [12, 13]. Recent researches demonstrate that domestic cats can be also efficient hosts of *E. canis* [14, 15].

Bartonella species are reemerging infectious organisms that have been recently documented in a large range of domestic and wild mammals. In Algeria, high prevalence of infective endocarditis is caused by *Bartonella quintana* in humans [16]. Previously, different species of *Bartonella* have been detected in fleas [17]. Investigation about the diversity of *Bartonella spp.* in reservoir animals from Algeria was conducted. To date, five *Bartonella* species (*B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, *B. elizabethae, B. rochalimae* and *B. henselae*) were detected infecting dogs [13, 18] and only one species (*B. henselae*) was described in cats [19]. Furthermore, *Bartonella* spp. was identified in hedgehogs and Rodents [20].

Rickettsioses are among the oldest known vector-borne diseases. Mediterranean Spotted Fever caused by *Rickettsia conorii conorii* is endemic in Algeria [21]. In the past ten years, particularly using entomological approach, other *Rickettsia spp,* including human pathogens have been detected in ticks and fleas from Algeria [22, 23]. Dogs have been considered as potential sentinel and reservoir of *R. conorii* [24]. Cats are also involved in the cycle of SFG rickettsiae and *R. typhi,* the agent of murine typhus [25, 26].

Borrelia burgdorferi sensu lato is a group of spirochete bacteria species, some of which cause lyme borreliosis especially in humans and dogs [27]. Cats were reported to be susceptible to the infection with this agent [28]. Recently, high seropositivity of *Borrelia burgdorferi s.l.* was found in dogs from Algeria [13].

To date, information about vector-borne diseases agents circulating in Algeria is still limited. Therefore the aim of the present study was to assess the presence of bacteria (*C. burnetii, E. canis, Bartonella* spp., *Rickettsia* spp. and *Borrelia* spp.) with veterinary and zoonotic importance in stray dogs and cats and their ectoparasites from Algiers using rapid specific molecular tests.

MATERIALS AND METHODS

2.1. Ethic statement

Risk assessment was submitted to and approved by the ethical committee and decision board of Hygiene Urbaine d'Alger (HURBAL). HURBAL is an institution affiliated to the Algerian Ministry of Interior, the Local Government and the Algerian Ministry of Agriculture and Rural Development. HURBAL with the decision of Ministry of the Interior and in the context of the National Program for Rabies Control, in which the authors of the paper are not involved, caught stray dogs and cats from Algiers.

Once stray animals captured, they are housed in cages, for being euthanized after expiration of the legal delay of guard (7 days, in order to permit owners to claim their pets).

To facilitate the fieldwork, collaborations were established with veterinary doctors and theirs assistants who work in this establishment.

2.2. Sample collection

Between October 2010 and September 2013, spleens were collected from stray dogs and cats living in the city of Algiers, Algeria. Sampling was conducted in a room dedicated to and equipped for veterinarian activities. A necropsy was performed immediately following euthanasia of the animals. Fragments of spleen were collected aseptically and stored in 70% ethanol.

An estimate age of each animal has been performed, based on dentition and physical aspect.

Information concerning sex, breed and the presence of ectoparasites were noted. Ticks, fleas and lice were collected and stored in 70% ethanol solution for later identification by genus and/or species using standard taxonomic morphological keys [1, 29, 30]. Dogs and cats were classified as apparently healthy or unwell based on their status at the time of sampling (Table 1).

All samples were later processed in the National Reference Center for Rickettsial Diseases in Marseille, France.

DNA extraction

The specimens (ectoparasites and spleens) which were conserved in ethanol were rinsed twice for 5 min in distilled water. All experiment was conducted in a laminar flow. Each sample was incised using an individually scalpel and crushed in sterile tubes (Eppendorf; Hamburg, Germany).

A total of 100 µl of DNA was extracted using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) by QUIAGEN-BioRobot EZ1, according to the manufacturer's instructions. The genomic DNA was stored at -20°C under sterile conditions until used as a template in PCR assays. The remaining piece of spleen and ectoparasites were kept at -80°C for additional control.

Detection of bacteria

DNA extracted was used in qPCR amplifications to detect *C. burnetii, E. canis, Bartonella* spp, *Rickettsia* spp. and *Borrelia* spp. The final qPCR reaction mixture composed by (5 µl) of DNA extracted with (15 µL) of mix from the Takyon PCR Kit (QIAGEN, Hilden, Germany) as previously described [31].

Negative controls for all PCR assays consisted in The DNA extracted from laboratory colonies of uninfected ticks. Positive controls incorporated DNA extracted from a diluted strain of *C. burnetii, E. canis, B. elizabethae, B. henselae, R. montanensis, R. typhi, R. conorii, R. massilae, R. felis* and *Borrelia burgdorferi* cultured in our laboratory in Marseille.

Results are recorded as positive when the cycle threshold (Ct) was lower than 36.

All samples were screened for *C. burnetii* DNA using the IS30a spacers [32]. *C. burnetii*-positive samples were then confirmed by another qPCR system targeting the IS1111 [32].

For *E. canis*, the DNA extracts from spleens and ticks were amplified using a qPCR primer and probe combination based on the Ecaj 0701 gene encoding the glutaredoxin-related protein for detection of *E. canis* as previously described [33].

Molecular detection and identification of *Bartonella* genus-specific qPCR was based on the 16S-23S rRNA intergenic transcribed spacer (ITS gene) [31]. Positive samples were subsequently analyzed by a second qPCR specific for *B. henselae* targeting the heme-binding protein gene, Pap31 [34]. Samples that were qPCR positive for *Bartonella* DNA by the ITS primers and negative for qPCR specific for *B. henselae* were then

confirmed by standard PCR performed with *Bartonella*-specific primers for the citrate synthase (*gltA*) gene [35].

PCR products were purified and sequenced with *gltA* primers as described previously [35]. All obtained sequences were assembled and edited using ChromasPro (version 1.7.7). The sequences were then analyzed by Basic Local Alignment Search Tool (BLAST) and compared with sequences available in the GenBank database.

Using qPCR, DNA samples were screened for all spotted fever group rickettsiae (SFG) by targeting a partial sequence of the citrate synthase gltA, RKND03 system [32].

R. massiliae and *R. conorii*-specific qPCR were conducted on positive ticks DNA samples as previously described [31, 36] .

The fleas which were positive for *Rickettsia* spp. at the first screening were subjected to *R. felis*-specific qPCR amplification of the membrane phosphatase gene [35]. Typhus group (TG) was also tested using specific primers and probe of *R. typhi* targeting the Rpr 274 gene [37].

All spleens were screened by using a *Borrelia* genus-specific qPCR targeting a fragment of the 16S rRNA gene as described [38].

RESULTS

Samples collection

From October 2010 to September 2013, a total of 117 dogs and 107 cats, from animal shelter in Algiers, were studied. These animals lived in urban and rural areas spend most of their time exclusively outdoors and don't receive any ticks and fleas control products. Of the 117 dogs included in this study, 59/117 (50.42 %) were males and 58/177 (49.57 %) were female. Among the 107 cats, 58/107 (54.20 %) were males and 49/107 (45.79 %) were female. The cats were described as belonging for the majority to mixed breeds and also to European and Siamois crosses breed. Most cats were estimated to be less than 3 years old. The canine population was predominantly crossbred dogs; the others belong to the German shepherd, shepherd crosses, American Staffordshire and Pit-bull mix breeds. Dog's age ranged between 2 months and 11 years. Among these animals, 31/117 (26.49 %) dogs and 8/107 (7.47 %) cats were apparently sick. The remaining dogs and cats were apparently healthy. A total of 72/117 (61.53 %) dogs and 65/107 (60.74 %) cats were infested with at least one species of ectoparasites. Mixed infestations with two or more ectoparasites were detected on 61/117 dogs (52.13 %) and 18/107 cats (16.82 %). Among the 117 dogs examined, 68/117 (58.12 %) were found to be infested

with ticks, while fleas and lice were found on 62/117 (52.99 %) and 2/117 (1.71 %) of dogs respectively. About the 107 cats examined, 11/107 (10.28 %) were infested by ticks, 65/107 (60.74 %) by fleas and 4/107 (3.74 %) of cats by lice.

A total of 640 fleas were picked from animals, 305/640 (47.65 %) from dogs and 335/640 (52. 34%) from cats. Three species have been morphologically identified, including 369/640 (57.7 %) *Ctenocephalides felis* which was the most abundant, followed by 149/640 (23.3 %) *Xenopsylla cheopis,* and 122/640 (19%) *Ctenocephalides canis*.

A total of 532 ticks were collected, 520/532 (97.74 %) ticks from dogs and 12/532 (2.25 %) ticks from cats. All ticks belonged to *Rhipicephalus sanguineus* species. In addition, 48 Chewing lice were collected, including 39 *Felicola subrostratus* on cats and 9 *Trichodectes canis* on dogs.

For the present study, a convenient sample of 115 ticks (103 from dogs, 12 from cats) and 225 fleas: 108 *Ctenocephalides felis* (21 from dogs, 87 from cats), 53 *Ctenocephalides canis* (49 from dogs, 4 from cats) and 64 *Xenopsylla cheopis* (62 from dogs, 2 from cats) were selected for molecular screening. Spleen samples collected from all the 117 dogs and the 107 cats were tested for the presence of pathogens.

Detection of Bacteria

Using qPCR targeting the IS30a and the IS1111gene of *C. burnetii* in spleen samples, DNA of *C. burnetii* was identified in 1/117 (0.85 %) of dogs and 1/107 (0.93 %) of cats. Using qPCR targeting the Ecaj 0701 gene of *E. canis,* 17/117(14.52 %) of dogs were positive. 10/17 (58.82 %) of the positive dogs were infested by ticks and fleas and 3/17
(17.64 %) were sick (table 1). *B. henselae* DNA was amplified from the splenic tissue of 1/117 (0.85 %) of dogs and 1/107 (0.93%) of cats for both qPCR systems; genus-specific *Bartonella* spp and *B. henselae*. The dog positive for *B. henselae* harboured ticks and fleas. All animals were tested negative for *Rickettsia* spp. and *Borrelia* spp. DNA.

Using qPCR targeting the RKND03 system for all spotted fever group rickettsiae (SFG), *Rickettsia* spp. DNA was detected in 29 ticks and 2 fleas. *R. massilae* DNA was identified in 28/115 (24.35 %) ticks by q PCR. In our study, *R. massilae* DNA was detected in 27/103 (26.21 %) of ticks from dogs and in 1/12 (8.33 %) of ticks from cats. Only 1/115 ticks (0.87 %) was positive to *R. conorii*-specific qPCR. *R. conorii* DNA was detected in 1/103 (0.97 %) *Rhipicephalus sanguineus* collected from dogs. Using qPCR specific for membrane phosphatase gene, we identified *R. felis* in 2/225 (0.88 %) fleas. DNA of

R. felis was detected in 2/87 (2.30 %) *Ctenocephalides felis* collected from cats.

21/225 (9.33%) fleas tested were positive for *Bartonella* spp. by *Bartonella* qPCR. 11/225 (4.88 %) were positive by *B. henselae*-specific qPCR. *B. henselae* DNA was detected in 11/87 (12.64%) *Ctenocephalides felis* collected from cats. Sequencing of the *gltA* gene fragment from the remaining ten *Bartonella*-positive fleas revealed gene sequences matching those of *B. vinsonii* subsp. *berkhoffii* and *B. clarridgeiae.* A search in GenBank of similar partial sequences from the *gltA* gene indicated that 6/225 (2.66 %) samples were identical to *B. vinsonii* subsp. *Berkhoffii* (GenBank accession no. DQ360833.1, with 99 % similarity) and 4/225 (1.77 %) fleas were in complete homology with the sequences for *B. clarridgeiae* (accession no. FN645454.1, with 100 % similarity). DNA of *B. vinsonii* subsp. *Berkhoffii* was detected in 6/62 (9.67 %) *Xenopsylla cheopis* collected from dogs. *B. clarridgeiae* was identified in 4/87 (4.59 %) *Ctenocephalides felis* collected from cats.

Using qPCR targeting the Ecaj 0701 gene of *E. canis*, 8/115 (6.95%) ticks were positive. DNA of *E. canis* was detected in 8/103 (7.77 %) *Rhipicephalus sanguineus* collected from dogs,

these positive ticks were taken from 4/10 (40 %) infested dogs positive by *E. canis.*

Moreover, four from these positive ticks were coinfected with *R. massilae.* All ticks tested negative for *C. burnetii.*

DISCUSSION

In this study we investigated the occurrence and diversity of canine and feline vector-borne diseases in Algeria using molecular techniques. In total, 18/177 (15.38 %) dogs and 2/107 (1.87%) cats were positive to at least one vector-borne agent, including one dog co-infected with two agents *E. canis* and *C. burnetii*. We also show that ticks and fleas collected from these animals were infected with several bacteria, including *R. massilae, R. conorii and E. canis* in *Rhipicephalus sanguineus; R. felis, B. henselae* and *B. clarridgeiae* in *Ctenocephalides felis* and finally *B. vinsonii* subsp. *Berkhoffii* in *Xenopsylla cheopis.*

Molecular techniques are more useful for detecting chronic and subclinical infections [39], and are ideally suited to epidemiological investigations as reported here. The validity of our data is based on rigorous methods and techniques with advanced equipments that are commonly used in our laboratory,

including the highest quality of positive and negative controls to confirm the test.

To the best of our knowledge, this is the first molecular study demonstrating the presence of *C. burnetii* in dogs and cats from Algeria. *C. burnetii*, the agent of Q fever, is recognized as one of the most important zoonotic pathogen. This organism infects a large range of animal species and endemic infections are widespread throughout in the world. Ticks are involved in the natural transmission cycle of *C. burnetii* [8]. In addition to domestic animals such as cattle, sheep and goat, which have been considered to be the primary reservoirs of *C. burnetii*, pets such as dogs and cats have recently received attention as a potential source of human exposure [2]. Cats have been implicated in some outbreaks [40] and dogs in only one [41].

C. burnetii was detected in spleen sample of one dog and one cat. Based on qPCR results, *C. burnetii* was also identified in 1/123(0.8%) blood samples from apparently healthy dogs in Hungary [42]. In other survey for *C. burnetii*, 12 dogs and 4 cats housed in an animal hospital were examined in Japan, two dogs were positive at the beginning. About 5 months later, three cats became PCR-positive [43]. Recently, *C. burnetii* was amplified in 2 dogs from rural area in Brazil [6].

C. burnetii DNA was also detected in canine placentas in the Netherlands [44], and in uterine biopsies from clinically normal cats in North America [8].

In Algeria, a recent study showed that *C. burnetii* was the cause of fever in one patient, but cases are rarely documented and the prevalence of the disease is probably underestimated [10]. Our results indicate that apparently healthy dogs and cats from Algeria might serve as a source of human *C. burnetii* infection. It is possible that the positive animals lived in a rural habitat in close contact with domestic ruminants which are the most important reservoirs for this agent. A high contamination rate is possible because *C. burnetii* can be excreted in urine, feces and milk and occurs in large number in amniotic fluid and placenta at birth [49]. Additionally, these bacteria are extremely resistant in the environment [49].

In our study, *E. canis* was the most common pathogen found in spleens of dogs. Infections with *E. canis* causes canine monocytic ehrlichiosis which was first recognized as a distinct clinical entity in Algeria in 1935 [11]. Since then, erhlichiosis has been acknowledged as an important emerging tick-borne disease in both humans and animals [45]. *E. canis*, is primarily transmitted by the brown dog tick *Rhipicephalus sanguineus*, with worldwide distribution [2]. Human infection by *E. canis* has

been reported in Venezuela [46]. The importance of this pathogen as a zoonotic agent remains unknown [47]. In this work, the percentage of positivity to *E. canis* DNA in spleen samples of dogs from Algiers was 14.5 %. In Algeria, *E. canis* was newly detected in blood of dogs in 7/110 (6.4 %) cases in the provinces of Tizi Ouzou and Béjaïa [12] and in 10/213 (4.7 %) in Algiers [13].

Previous studies have demonstrated that the spleen is the organ most likely to store *E. canis* during the subclinical phase and the last organ to harbor these organisms before elimination [48]. The authors also indicated longer persistence of *E. canis* in splenic macrophages than in blood monocytes [49]. These findings reveal that extraction and amplification of DNA from splenic aspirates is a reliable method for determining the carrier state of *E. canis* as reported here [48, 49].

Recently, 27/60 (45%) of spleen samples from naturally infected dogs were positive for *E. canis* by PCR assays in Brazil [47].

In previous reports, *E. canis* has been molecularly detected in blood samples of dogs from Brazil (3.7%) [50], Nigeria (12.7%) [5], Iran (22.5%) [45], India (46.9%) [51] and Central America (47%, 34% and 56%) [2, 52, 53]. The difference in the prevalence values found in these studies can be associated to different geographical origin of dogs and diagnostic test used.

Other factors favoring vector-host interactions play a role in the epidemiology of canine ehrlichiosis, such as immunological status of animals studied and arthropod vector density.

In our investigation, 58.8 % of the positive dogs were infested by ticks. The molecular research of *E. canis* in ticks demonstrated that 8/103 (7.77 %) *Rhipicephalus sanguineus* collected from our dogs were infected. These positive ticks were collected from 4/10 (40 %) infested dogs positive by *E. canis.* We report here the first direct evidence of *E. canis* in ticks from Algeria. The prevalence of *E. canis* in *Rhipicephalus sanguineus* ticks from dogs in other African countries has been reported in Cameroon at 6 % [54] and in Ivory Coast at 27% [33] using molecular tools.

In the present work, one cat was qPCR positive to *B. henselae.* This bacterium is an emerging pathogen of veterinary and medical importance [55]. Domestic cats develop frequently subclinical infection with *B. henselae*, the main agent of cat scratch disease (CSD). They are therefore considered as the principal reservoir for human infection [56, 57]. In CSD patients, *B. henselae* can cause ocular symptoms, encephalitis, endocarditis, hemolytic anemia, hepatosplenomegaly, glomerulonephritis, pneumonia and osteomyelitis [28]. Molecular prevalence of *B. henselae* in the present study 1/107

(0.9%) was similar to a study conducted in blood samples of cats in Portugal 2/649 (0.3%) [4] and to the prevalence obtained in cats from Albania by PCR 1/146 (0.7%) [58], but lower than the one previously reported in cats from Argentina 14/101 (11.9%) [59]. Recently, Whole blood samples from stray cats from Algiers, Algeria, were cultured to detect the presence of *Bartonella* species. *B. henselae* was the only species isolated from 36/211 (17%) cats [19].

Furthermore, we report for the first time detection of *B. henselae* and *B. clarridgeiae* in *Ctenocephalides felis* collected from cats in Algeria. The prevalence rate of *B. henselae* and *B. clarridgeiae* DNA was 12.64% and 4.59 % respectively. This finding suggests that cats are important sources of these bacteria and is consistent with reports of *B. henselae* and *B. clarridgeiae* in cat fleas from several countries worldwide [60-63]. In Algeria, *B. clarridgeiae* was detected in blood of dogs [18] and in fleas collected on hedgehogs [17].

In this study, we detected *B. henselae* in spleen of one dog from Algeria. Dogs sub-clinically infected could transmit *Bartonella* to people by spreading infected arthropods in their environment [64]. Domestic dogs may be excellent epidemiological sentinels for *Bartonella* infection in humans [65]. In accordance with our

findings, *B. henselae* was molecularly detected in a spleen aspirate from a dog [66]. DNA of *Bartonella* species was also amplified from the splenic tissue in a sick dog in Spain [67]. Recently, *B. henselae* was identified in 4/96 (4.16%) blood samples of dogs by PCR in Algeria [13].

Dogs can be infected by a wide variety of *Bartonella* species. Earlier investigators support that dogs are likely accidental hosts for *B. henselae*, just like humans, and are efficient reservoirs for both *B. vinsonii berkhoffii* and *B. rochalimae* [65, 68, 69]. In this work, the dog positive to *B. henselae* was infested by ticks and fleas. Molecular analysis of ectoparasites from our dogs revealed that *B. vinsonii* subsp. *Berkhoffii* was detected in 6/62 (9.67 %) *Xenopsylla cheopis*. These positive fleas belong to dogs that were negative to *Bartonella* spp. *Bartonella vinsonii* subsp. *berkhoffii* was earlier detected infecting Algerian dogs [18]. *Xenopsylla cheopis*, the Oriental rat flea, is distributed worldwide and is suspected to transmit several *Bartonella* species [70]. In Algeria, *B. tribocorum* and *B. elizabethae* were detected in *Xenopsylla cheopis* collected from rodents [17].We detected for the first time the presence of *B. vinsonii* subsp. *berkhoffii* in *Xenopsylla cheopis.* These fleas may play a role in *B. vinsonii* subsp. *Berkhoffii* infections.

In our study, all animals tested negative for *Rickettsia* spp. Molecular research of these bacteria in ectoparasites showed positives results. We identified *R. felis* DNA in 2/87 (2.30 %) *Ctenocephalides felis* collected from cats. In Algeria, the molecular presence of *R. felis* was detected in *Ctenocephalides canis* and *Xenopsylla cheopis* from rodents [23]. Lately, *Ctenocephalides felis* was also shown to harbour this emerging pathogen (data submitted to parasites and vectors Leulmi and *al*., 2015). In previous studies, the DNA of *R. felis* was detected in *Ctenocephalides felis* of cats in Thailand [60], in Malaysia [63] and in Ethiopia [61].

We also reported an infection rate of 24.4 % for *R. massiliae* and 11 % for *R. conorii* in *Rh. sanguineus* ticks. These two spotted fever group rickettsiae were amplified from *Rh. sanguineus* by molecular tools in Algeria [71, 72] and in Tunisia [73]. Overall, we confirm that dogs and cats can act as hosts for ectoparasites infected with several rickettsial agents.

An outdoor housing, the contact with other hosts and the non-use of preventive or therapeutic anti-ectoparasites applications were found to be correlated with PCR-positivity to vector borne infections [5], which is associated to a higher exposure of stray dogs and cats of our study to arthropod vectors and the agents they might transmit.

The findings of this study demonstrate that human and animal pathogens are present in dogs and cats and their ectoparasites in Algeria. Because people could be in close contact with carnivores, it is possible that some unknown diseases may be caused by bacterial species reported here.

Doctors and veterinarians should include these pathogens in the differential diagnoses for diseases with compatible clinical signs. In addition, the use of molecular techniques especially real time PCR is important in making appropriate diagnosis and providing the effective treatment for their patients.

Conclusion

This is the first study to investigate exposure to multiple vector-borne pathogens in stray cats from Algeria. This is also the first time that *C. burnetii* has been described in animals from Algeria. The occurrence of these agents in the country, with zoonotic character, emphasizes the need to alert the veterinary community, owners and public health authorities for the risk of infection. Control measures including chemoprophylaxis against the ectoparasite vectors should be implemented to prevent the infection of domestic carnivores, other vertebrate hosts and people.

This paper has provided interesting new information on zoonotic pathogens emerging from companion animals. Additional epidemiological data using larger numbers of domestic animals from more localities is necessary in order to determine the real prevalence and distribution of these diseases in Algeria, as well as providing analysis on differences between risk factors. Further investigations are also warranted in order to isolate these species and to determine their clinical importance.

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Table 1: Detected pathogens in dogs and cats from Algeria, as determined by quantitative PCRs and information relative to positives animals.

Animals n°	Animal species	Age	Sex	Breed	Clinical status	Presence of ectoparasites	qPCR results
1	Canine	< 1 year	F	Mixed-breed	H	Tick and fleas	*E. canis*
2	Canine	30 months	M	Mixed-breed	H	Tick[a] and fleas	*E. canis*
3	Canine	< 1 year	F	Mixed-breed	H	Tick[a] and fleas	*E. canis*
4	Canine	4 months	M	Mixed-breed	S	Tick, fleas and lice	*E. canis*
5	Canine	8 years	M	*American Staffordshire*	S	Tick and fleas	*E. canis*
6	Canine	4 months	M	Mixed-breed	H	Tick[b] and fleas	*E. canis*
7	Canine	7 months	F	German Shepherd	H	Tick[c] and fleas	*E. canis*
8	Canine	1 year	M	shepherd crosses	S	Tick and fleas	*E.canis*
9	Canine	18 months	M	Mixed-breed	H	-	*E. canis*
10	Canine	< 1 year	F	Mixed-breed	H	Tick[b] and fleas	*E. canis*
11	Canine	-	F	Mixed-breed	H	-	*E. canis*
12	Canine	2 years	M	Mixed-breed	H	-	*E. canis*
13	Canine	1 year	F	Mixed-breed	H	Tick and fleas	*E. canis*
14	Canine	-	F	Mixed-breed	H	-	*E. canis*
15	Canine	3 years	M	Mixed-breed	H	-	Co-infection with *E. canis* and *C. burnetii*
16	Canine	-	M	Mixed-breed	H	-	*E. canis*
17	Canine	-	F	Mixed-breed	H	-	*E. canis*
18	Canine	18 months	M	shepherd crosses	H	Tick and fleas	*B. henselae*
19	Feline	2 years	M	Mixed-breed	H	-	*B. henselae*
20	Feline	< 1 year	M	Mixed-breed	H	-	*C. burnetii*

M = male; F = female; H = healthy; S = sick.

[a] *Rh. sanguineus* positive by q PCR to *E. canis* and *R. massilae*

[b] *Rh. sanguineus* positive by q PCR to *E. canis*

[c] *Rh.* positive by q PCR to *R. massilae*

Reference List

(1) Dantas-Torres F, Otranto D. Dogs, cats, parasites, and humans in Brazil: opening the black box. Parasit Vectors 2014; 7:22.

(2) Wei L, Kelly P, Ackerson K, et al. First report of *Babesia gibsoni i*n Central America and survey for vector-borne infections in dogs from Nicaragua. Parasit Vectors 2014; 7:126.

(3) Claerebout E, Losson B, Cochez C, et al. Ticks and associated pathogens collected from dogs and cats in Belgium. Parasit Vectors 2013; 6:183.

(4) Maia C, Ramos C, Coimbra M, et al. Bacterial and protozoal agents of feline vector-borne diseases in domestic and stray cats from southern Portugal. Parasit Vectors 2014; 7:115.

(5) Kamani J, Baneth G, Mumcuoglu KY, et al. Molecular detection and characterization of tick-borne pathogens in dogs and ticks from Nigeria. PLoS Negl Trop Dis 2013; 7(3):e2108.

(6) Mares-Guia MA, Rozental T, Guterres A, et al. Molecular identification of the agent of Q fever - *Coxiella burnetii* - in domestic animals in State of Rio de Janeiro, Brazil. Rev Soc Bras Med Trop 2014 Mar; 47(2):231-4.

(7) de Rooij MM, Schimmer B, Versteeg B, et al. Risk factors of *Coxiella burnetii* (Q fever) seropositivity in

veterinary medicine students. PLoS One 2012; 7(2):e32108.

(8) Cairns K, Brewer M, Lappin MR. Prevalence of *Coxiella burnetii* DNA in vaginal and uterine samples from healthy cats of north-central Colorado. J Feline Med Surg 2007 Jun; 9(3):196-201.

(9) Hornok S, Denes B, Meli ML, et al. Non-pet dogs as sentinels and potential synanthropic reservoirs of tick-borne and zoonotic bacteria. Vet Microbiol 2013 Dec 27; 167(3-4):700-3.

(10) Angelakis E, Mediannikov O, Socolovschi C, et al. *Coxiella burnetii*-positive PCR in febrile patients in rural and urban Africa. Int J Infect Dis 2014 Nov; 28:107-10.

(11) Donatien ALA. Existance en Algerie d'une rickettsia du chien. 28 ed. Bul Soc Pathol Exot, 1935:418-9.

(12) Dahmani M, Loudahi A, Mediannikov O, Fenollar F, Raoult D, Davoust B. Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. Ticks Tick Borne Dis 2015 Mar; 6(2):198-203.

(13) Azzag N, Petit E, Gandoin C, et al. Prevalence of select vector-borne pathogens in stray and client-owned dogs from Algiers. Comp Immunol Microbiol Infect Dis 2015 Feb; 38:1-7.

(14) de Oliveira LS, Mourao LC, Oliveira KA, et al. Molecular detection of *Ehrlichia canis* in cats in Brazil. Clin Microbiol Infect 2009 Dec; 15 Suppl 2:53-4.

(15) Braga IA, dos Santos LG, de Souza Ramos DG, Melo AL, da Cruz Mestre GL, de Aguiar DM. Detection of *Ehrlichia canis* in domestic cats in the central-western region of Brazil. Braz J Microbiol 2014; 45(2):641-5.

(16) Benslimani A, Fenollar F, Lepidi H, Raoult D. Bacterial zoonoses and infective endocarditis, Algeria. Emerg Infect Dis 2005 Feb; 11(2):216-24.

(17) Bitam I, Rolain JM, Nicolas V, et al. A multi-gene analysis of diversity of *Bartonella* detected in fleas from Algeria. Comp Immunol Microbiol Infect Dis 2012 Jan; 35(1):71-6.

(18) Kernif T, Aissi M, Doumandji SE, Chomel BB, Raoult D, Bitam I. Molecular evidence of *Bartonella* infection in domestic dogs from Algeria, North Africa, by polymerase chain reaction (PCR). Am J Trop Med Hyg 2010 Aug; 83(2):298-300.

(19) Azzag N, Haddad N, Durand B, et al. Population structure of *Bartonella henselae* in Algerian urban stray cats. PLoS One 2012; 7(8):e43621.

(20) Bitam I, Rolain JM, Kernif T, Baziz B, Parola P, Raoult D. *Bartonella* species detected in rodents and hedgehogs from Algeria. Clin Microbiol Infect 2009 Dec; 15 Suppl 2:102-3.

(21) Parola P, Paddock CD, Socolovschi C, et al. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev 2013 Oct; 26(4):657-702.

(22) Bitam I. Vectors of rickettsiae in Africa. Ticks Tick Borne Dis 2012 Dec; 3(5-6):382-6.

(23) Bitam I, Baziz B, Kernif T, Harrat Z, Parola P, Raoult D. Molecular detection of *Rickettsia typhi* and *Rickettsia felis* in fleas from Algeria. Clin Microbiol Infect 2009 Dec; 15 Suppl 2:255-6.

(24) Levin ML, Killmaster LF, Zemtsova GE. Domestic dogs (*Canis familiaris*) as reservoir hosts for *Rickettsia conorii*. Vector Borne Zoonotic Dis 2012 Jan; 12(1):28-33.

(25) Nogueras MM, Pons I, Ortuno A, et al. Molecular detection of *Rickettsia typhi* in cats and fleas. PLoS One 2013; 8(8):e71386.

(26) Segura F, Pons I, Miret J, Pla J, Ortuno A, Nogueras MM. The role of cats in the eco-epidemiology of spotted fever group diseases. Parasit Vectors 2014; 7:353.

(27) Skotarczak B. Why are there several species of *Borrelia burgdorferi* sensu lato detected in dogs and humans? Infect Genet Evol 2014 Apr; 23:182-8.

(28) Krupka I, Straubinger RK. Lyme borreliosis in dogs and cats: background, diagnosis, treatment and prevention of infections with *Borrelia burgdorferi* sensu stricto. Vet Clin North Am Small Anim Pract 2010 Nov; 40(6):1103-19.

(29) Whitaker AP. Fleas (Siphonaptera) . 1/16 ed. Royal Entomological Society, 2007:-178.

(30) Walker A.R. BACJLE-PAHIGLAAPRPPM. Ticks of domestic animals in Africa: a guide to identification of species . Edinburgh: Bioscience Reports , 2007.

(31) Socolovschi C, Reynaud P, Kernif T, Raoult D, Parola P. Rickettsiae of spotted fever group, *Borrelia valaisiana*, and *Coxiella burnetii* in ticks on passerine birds and mammals from the Camargue in the south of France. Ticks Tick Borne Dis 2012 Dec; 3(5-6):355-60.

(32) Socolovschi C, Kernif T, Raoult D, Parola P. *Borrelia*, *Rickettsia*, and *Ehrlichia* species in bat ticks, France, 2010. Emerg Infect Dis 2012 Dec; 18(12):1966-75.

(33) Socolovschi C, Gomez J, Marie JL, et al. *Ehrlichia canis* in *Rhipicephalus sanguineus* ticks in the Ivory Coast. Ticks Tick Borne Dis 2012 Dec; 3(5-6):411-3.

(34) Angelakis E, Roux V, Raoult D, Rolain JM. Real-time PCR strategy and detection of bacterial agents of lymphadenitis. Eur J Clin Microbiol Infect Dis 2009 Nov; 28(11):1363-8.

(35) Leulmi H, Socolovschi C, Laudisoit A, et al. Detection of *Rickettsia felis*, *Rickettsia typhi*, *Bartonella* Species and *Yersinia pestis* in Fleas (Siphonaptera) from Africa. PLoS Negl Trop Dis 2014 Oct; 8(10):e3152.

(36) Bechah Y, Socolovschi C, Raoult D. Identification of rickettsial infections by using cutaneous swab specimens and PCR. Emerg Infect Dis 2011 Jan; 17(1):83-6.

(37) Walter G, Botelho-Nevers E, Socolovschi C, Raoult D, Parola P. Murine typhus in returned travelers: a report of thirty-two cases. Am J Trop Med Hyg 2012 Jun; 86(6):1049-53.

(38) Parola P, Diatta G, Socolovschi C, et al. Tick-borne relapsing fever borreliosis, rural senegal. Emerg Infect Dis 2011 May; 17(5):883-5.

(39) Abd Rani PA, Irwin PJ, Coleman GT, Gatne M, Traub RJ. A survey of canine tick-borne diseases in India. Parasit Vectors 2011; 4:141.

(40) Marrie TJ, Durant H, Williams JC, Mintz E, Waag DM. Exposure to parturient cats: a risk factor for acquisition of Q fever in Maritime Canada. J Infect Dis 1988 Jul; 158(1):101-8.

(41) Buhariwalla F, Cann B, Marrie TJ. A dog-related outbreak of Q fever. Clin Infect Dis 1996 Oct; 23(4):753-5.

(42) Hornok S, Denes B, Meli ML, et al. Non-pet dogs as sentinels and potential synanthropic reservoirs of tick-borne and zoonotic bacteria. Vet Microbiol 2013 Dec 27; 167(3-4):700-3.

(43) Komiya T, Sadamasu K, Toriniwa H, et al. Epidemiological survey on the route of *Coxiella burnetii* infection in an animal hospital. J Infect Chemother 2003 Jun; 9(2):151-5.

(44) Roest HI, van Solt CB, Tilburg JJ, et al. Search for possible additional reservoirs for human Q fever, The Netherlands. Emerg Infect Dis 2013 May; 19(5):834-5.

(45) Maazi N, Malmasi A, Shayan P, Nassiri SM, Salehi TZ, Fard MS. Molecular and serological detection of *Ehrlichia canis* in naturally exposed dogs in Iran: an

analysis on associated risk factors. Rev Bras Parasitol Vet 2014 Mar; 23(1):16-22.

(46) Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Ann N Y Acad Sci 2006 Oct; 1078:110-7.

(47) de Sousa KC, Andre MR, Herrera HM, et al. Molecular and serological detection of tick-borne pathogens in dogs from an area endemic for *Leishmania infantum* in Mato Grosso do Sul, Brazil. Rev Bras Parasitol Vet 2013 Oct; 22(4):525-31.

(48) Harrus S, Kenny M, Miara L, Aizenberg I, Waner T, Shaw S. Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental *Ehrlichia canis* infection. Antimicrob Agents Chemother 2004 Nov; 48(11):4488-90.

(49) Baneth G, Harrus S, Ohnona FS, Schlesinger Y. Longitudinal quantification of *Ehrlichia canis* in experimental infection with comparison to natural infection. Vet Microbiol 2009 May 12; 136(3-4):321-5.

(50) Tanikawa A, Labruna MB, Costa A, et al. *Ehrlichia canis* in dogs in a semiarid region of Northeastern Brazil: serology, molecular detection and associated factors. Res Vet Sci 2013 Jun; 94(3):474-7.

(51) Lakshmanan B JLGSDG. Molecular detection of *Ehrlichia canis* from blood of naturally infected dogs in India. 77 ed. Veterinarski Arhiv ,307-12.

(52) Romero LE, Meneses AI, Salazar L, et al. First isolation and molecular characterization of *Ehrlichia canis* in Costa Rica, Central America. Res Vet Sci 2011 Aug; 91(1):95-7.

(53) Rojas A, Rojas D, Montenegro V, Gutierrez R, Yasur-Landau D, Baneth G. Vector-borne pathogens in dogs from Costa Rica: first molecular description of *Babesia vogeli* and *Hepatozoon canis* infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection. Vet Parasitol 2014 Jan 31; 199(3-4):121-8.

(54) Ndip LM, Ndip RN, Esemu SN, Walker DH, McBride JW. Predominance of *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks from kennel-confined dogs in Limbe, Cameroon. Exp Appl Acarol 2010 Feb; 50(2):163-8.

(55) Tsai YL, Lin CC, Chomel BB, et al. *Bartonella* infection in shelter cats and dogs and their ectoparasites. Vector Borne Zoonotic Dis 2011 Aug; 11(8):1023-30.

(56) Chomel BB, Boulouis HJ, Maruyama S, Breitschwerdt EB. *Bartonella* spp. in pets and effect on human health. Emerg Infect Dis 2006 Mar; 12(3):389-94.

(57) Pennisi MG, Marsilio F, Hartmann K, et al. *Bartonella* species infection in cats: ABCD guidelines on prevention and management. J Feline Med Surg 2013 Jul; 15(7):563-9.

(58) Silaghi C, Knaus M, Rapti D, et al. Survey of *Toxoplasma gondii* and *Neospora caninum*, haemotropic

mycoplasmas and other arthropod-borne pathogens in cats from Albania. Parasit Vectors 2014; 7:62.

(59) Cicuttin GL, Brambati DF, De Gennaro MF, et al. *Bartonella* spp. in cats from Buenos Aires, Argentina. Vet Microbiol 2014 Jan 10; 168(1):225-8.

(60) Parola P, Sanogo OY, Lerdthusnee K, et al. Identification of *Rickettsia* spp. and *Bartonella* spp. in ffrom the Thai-Myanmar border. Ann N Y Acad Sci 2003 Jun; 990:173-81.

(61) Kumsa B, Parola P, Raoult D, Socolovschi C. Molecular detection of *Rickettsia felis* and *Bartonella henselae* in dog and cat fleas in Central Oromia, Ethiopia. Am J Trop Med Hyg 2014 Mar; 90(3):457-62.

(62) Tsai YL, Lin CC, Chomel BB, et al. *Bartonella* infection in shelter cats and dogs and their ectoparasites. Vector Borne Zoonotic Dis 2011 Aug; 11(8):1023-30.

(63) Mokhtar AS, Tay ST. Molecular detection of *Rickettsia felis*, *Bartonella henselae*, and *B. clarridgeiae* in fleas from domestic dogs and cats in Malaysia. Am J Trop Med Hyg 2011 Nov; 85(5):931-3.

(64) Solano-Gallego L, Llull J, Osso M, Hegarty B, Breitschwerdt E. A serological study of exposure to arthropod-borne pathogens in dogs from northeastern Spain. Vet Res 2006 Mar; 37(2):231-44.

(65) Diniz PP, Morton BA, Tngrian M, et al. Infection of domestic dogs in peru by zoonotic bartonella species: a cross-sectional prevalence study of 219 asymptomatic dogs. PLoS Negl Trop Dis 2013; 7(9):e2393.

(66) Ohad DG, Morick D, Avidor B, Harrus S. Molecular detection of *Bartonella henselae* and *Bartonella koehlerae* from aortic valves of Boxer dogs with infective endocarditis. Vet Microbiol 2010 Feb 24; 141(1-2):182-5.

(67) Tabar MD, Maggi RG, Altet L, Vilafranca M, Francino O, Roura X. Gammopathy in a Spanish dog infected with *Bartonella henselae*. J Small Anim Pract 2011 Apr; 52(4):209-12.

(68) Yore K, DiGangi B, Brewer M, Balakrishnan N, Breitschwerdt EB, Lappin M. Flea species infesting dogs in Florida and *Bartonella* spp. prevalence rates. Vet Parasitol 2014 Jan 31; 199(3-4):225-9.

(69) Chomel BB, Ermel RW, Kasten RW, Henn JB, Fleischman DA, Chang CC. Experimental infection of dogs with various *Bartonella* species or subspecies isolated from their natural reservoir. Vet Microbiol 2014 Jan 10; 168(1):169-76.

(70) Billeter SA, Colton L, Sangmaneedet S, Suksawat F, Evans BP, Kosoy MY. Molecular detection and identification of *Bartonella* species in rat fleas from northeastern Thailand. Am J Trop Med Hyg 2013 Sep; 89(3):462-5.

(71) Bitam I, Parola P, Matsumoto K, et al. First molecular detection of *R. conorii*, *R. aeschlimannii*, and *R. massiliae* in ticks from Algeria. Ann N Y Acad Sci 2006 Oct; 1078:368-72.

(72) Khaldi M, Socolovschi C, Benyettou M, et al. Rickettsiae in arthropods collected from the North African

Hedgehog (*Atelerix algirus*) and the desert hedgehog (*Paraechinus aethiopicus*) in Algeria. Comp Immunol Microbiol Infect Dis 2012 Mar; 35(2):117-22.

(73) Khrouf F, M'Ghirbi Y, Znazen A, Ben JM, Hammami A, Bouattour A. Detection of *Rickettsia* in *Rhipicephalus sanguineus* ticks and *Ctenocephalides felis* fleas from southeastern Tunisia by reverse line blot assay. J Clin Microbiol 2014 Jan; 52(1):268-74.

III- ÉVALUATION DES COMPÉTENCES VECTORIELLES DES PUCES ET PUNAISES DE LIT DANS LA TRANSMISSION DE L'AGENT DE LA FIÈVRE DES TRANCHÉES *BARTONELLA QUINTANA*.

Bartonella quintana est une bactérie bacille à Gram négatif considérée comme agent pathogène ré-émergent et responsable de diverses maladies humaines : fièvre des tranchées, angiomatose bacillaire, endocardite...etc. Bien que le pou de corps soit considéré comme le principal vecteur de *B. quintana,* l'ADN de cette bactérie a été détecté dans les puces de chat (*Ctenocephalides felis*) collectées dans diverses régions de France en 2003 et dans les puces de l'homme (*Pulex irritans*) collectées sur un singe (*Cercopithecus cephus*) au Gabon en 2001 [26,27]. Cela pourrait expliquer aussi deux rapports cliniques d'adénopathies chroniques attribuées à des infections causées par *B. quintana* et dont le seul facteur de risque épidémiologique identifié était la présence de puces [29,30]. Cette bactérie est également présente dans d'autres arthropodes tels que les poux de tête [43,44] et les tiques [45].

La détection récente de l'ADN de *B. quintana* dans des *Cimex hemipterus* (punaises de lit tropicale) recueillies dans deux prisons au Rwanda indique que les punaises de lit pourraient être impliquées dans la transmission de *B. quintana* [46]. Et cela soulève la question de savoir si les puces de chat (*Ct. felis*) et les punaise de lit (*C. lectularius*) pourraient acquérir, excréter et transmettre *B. quintana* et constituer ainsi un potentiel vecteur compétent. À cette fin, nous avons utilisé séparément deux

modèles expérimentaux d'infection des puces et punaises de lit en utilisant trois approches différentes: la qPCR, les cultures et l'immunohistochimie. Suivant deux essais séparés, un total de 960 *C. felis* ont été divisées en 12 groupes (2 groupes témoins et 10 groupes infectés) contenant chacun 80 puces. Les puces ont été nourris avec un repas sanguin contenant *B. quintana* à différentes dilutions (≈$3.6x10^4$ - $8.4x10^9$ bactéries). Au 3ème jour post infection, *B. quintana* a été détectée avec deux gènes spécifiques par qPCR dans 60 à 100% des puces choisies au hasard par dilution: 52% (26/50) des puces infectées de l'essai 1, et de 90% (45/50) des puces de l'essai 2. *B. quintana* a également été identifiée par des analyses moléculaires et culture des excréments des puces. Le nombre moyen de bactérie, tel que déterminé par qPCR diminue jusqu'au 11ème jour post infections et se négativent dans les deux essais au 13ème jour post infection. Par immunohistochimie, les bactéries ont été localisées dans le tube gastro-intestinal. Nos résultats indiquent que les puces de chat peuvent acquérir *B. quintana* par voie artificielle et excréter des organismes viables dans leurs excréments. Par conséquent, les puces peuvent jouer un rôle important en tant que vecteurs de la fièvre des tranchées ou d'autres manifestations cliniques qui sont causées par *B. quintana*. Cependant, le rôle biologique de *C. felis* dans la transmission de *B. quintana* dans des conditions

naturelles est encore à définir (***Article N° 7***). Concernant l'infection des punaises de lit par *B. quintana*, nous avons mis au point un élevage artificiel en utilisant la machine Hemotek et la membrane artificiel parafilm M. Une fois l'élevage maitrisé, nous avons procédé à 2 essais séparés d'infection : à l'aide de deux cellules de 30 punaises de lit (adulte et immature larve 1) chacune. A J3 post infection nous avons obtenu 100% d'acquisition de la bactérie chez les adultes et les immatures, et la bactérie était éliminée vivante dans les fèces. Les punaises de lit ont pu maintenir la bactérie plus longtemps que les puces soit jusqu'à J17, J19 post infection. Elles peuvent donc être considérées comme vecteur potentiel de *B. quintana.* Les deux modèles expérimentaux d'infections utilisés ont pu incriminer les puces et les punaises dans la transmission de l'agent de la fièvre des tranchées *B. quintana*, néanmoins sous conditions expérimentales.

ARTICLE 6

Article N.7. Kernif T*, **Leulmi H***, Socolovschi C, Bérenger J-M, Lepidi H, Bitam I, Rolain J-M, Raoult D and Parola P. **Acquisition and excretion of *Bartonella quintana* by the cat flea, *Ctenocephalides felis felis***. Mol Ecol. 2014 Mar;23(5):1204-12. doi: 10.1111/mec.12663. Epub 2014 Feb 20.

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Acquisition and excretion of *Bartonella quintana* by the cat flea, *Ctenocephalides felis felis*

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Abstract

Bartonella quintana* is transmitted by the infected faeces of body lice. Recently, this bacterium was detected in cat fleas (*Ctenocephalides felis*) and in two humans with chronic adenopathy whose only risk factor was contact with cat fleas. In this study, a total of 960 *C. felis* were divided into 12 groups (2 control groups and 10 infected groups) each containing 80 fleas. The fleas were fed *B. quintana*-inoculated human blood at different dilutions ($\approx 3.6 \times 10^4 - 8.4 \times 10^9$ bacteria) for 4 days via an artificial membrane. Subsequently, all flea groups were fed uninfected blood until day 13 postinfection (dpi). On day 3 pi, *B. quintana* was detected with two specific genes by quantitative PCR in 60–100% of randomly chosen fleas per dilution: 52% (26/50) in the infected fleas in Trial 1 and 90% (45/50) of the fleas in Trial 2. *B. quintana* was also identified by molecular and culture assays in flea faeces. The average number of *B. quintana* as determined by qPCR decreased until the 11th dpi and was absent in both trials at the 13th dpi. Bacteria were localized only in the flea gastrointestinal gut by specific immunohistochemistry. Our results indicate that cat fleas can acquire *B. quintana* by feeding and release viable organisms into their faeces. Therefore, fleas may play a role as vectors of trench fever or other clinical manifestations that are caused by *B. quintana*. However, the biological role of *C. felis* in the transmission of *B. quintana* under natural conditions is yet to be defined.

Keywords: cat fleas, artificial flea feeding, acquisition, faeces excretion, transmission, *Bartonella quintana*

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Introduction

Bartonella quintana, the causative agent of trench fever, is a fastidious Gram-negative bacterium and is a reemerging human pathogen (Anderson & Neuman 1997). Trench fever was described during World Wars I and II when thousands of soldiers suffered from the disease (McNee *et al*. 1916; Kostrzewski 1949). In 1990, *B. quintana* was identified as an agent of bacillary angiomatosis in patients with AIDS (Relman *et al*. 1990), endocarditis (Drancourt *et al*. 1995; Spach *et al*. 1995a), chronic bacteraemia (Spach *et al*. 1995b; Brouqui *et al*. 1999) and chronic lymphadenopathy (Raoult *et al*. 1994). As early as 1920, *B. quintana* has been observed in body lice, which are considered the main vector of this bacterium (Byam & Lloyd 1920). *B. quintana* has recently been found in head lice (Bonilla *et al*. 2009; Angelakis *et al*. 2011), fleas (Rolain *et al*. 2003b, 2005), ticks (Chang *et al*. 2001) and mites (Melter *et al*. 2012). However, most of these studies detected *B. quintana* by the use of molecular tools but lacked proof of arthropod vector competence.

Several flea species, including *Pulex irritans*, *Ctenocephalides felis felis*, *C. canis*, *Ceratophyllus gallinae*, *Ceratophyllus columbae* and *Archaeopsylla erinacei*, may infest

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humans (Rolain *et al.* 2005). The presence of *B. quintana* DNA has been reported in cat fleas (*C. felis*) that were collected from various regions in France and in human fleas (*P. irritans*) that were collected from a pet monkey (*Cercopithecus cephus*) in Gabon, Africa (Rolain *et al.* 2003b, 2005). These results confirm that *B. quintana* may be found in fleas and may explain two clinical reports of chronic adenopathy that were attributed to *B. quintana* infection for which the only epidemiologic risk factor that was identified was the presence of fleas (Raoult *et al.* 1994; Drancourt *et al.* 1996). Some studies suggest that few animals (such as dogs and cynomolgus and rhesus macaques) can serve as reservoir hosts for *B. quintana* (Chomel *et al.* 2006; Huang *et al.* 2011; Li *et al.* 2013). Our objective was to evaluate whether cat fleas could acquire *B. quintana* and excrete viable bacteria in their faeces, thus constituting a potential vector for *B. quintana* transmission. For this purpose, we used an experimental model of cat flea infection.

Materials and methods

Rearing fleas

Since 2010, cat fleas (*C. felis felis*, strain Bristol) have been maintained by our team at the Rickettsioses laboratory of the Medicine Faculty in Marseille, France. Adult fleas were fed *in vitro* using human citrated blood via an artificial membrane of Parafilm®M (Sigma-Aldrich, Saint-Louis, Missouri, USA). The flea larvae were maintained at 80% humidity in containers with 40 g of sand, 3 g of spray-dried human blood, 20 g of rat food and 2 g of brewer's yeast (T. Kernif, K. Stafford, G. C. Coles, I. Bitam, P. Kassim, J. Chironi, D. Raoult & P. Parola, submitted). The fleas and their faeces were proven to be free from *B. quintana* using a quantitative real-time PCR (qPCR) assay with primers that are described below (section: Real-time PCR amplification). To prevent contamination, the feeder machine as described by Wade and Georgi (1988) was introduced in a clear acrylic glove box, reference number '830 ABD/EXP/SP' (Fisher® Scientific, USA), to raise fleas in adapted containment.

Bartonella quintana *strain*

Culturing of *B. quintana* and all procedures involving experimental infections of fleas were conducted in a Biosafety Level 2 (BSL2) room. The *B. quintana* strain Oklahoma (ATCC 49793) was used. The strain was cultured on Columbia sheep blood agar plates (5%, BioMerieux®, Marcy l'Etoile, France) and incubated at 37°°C under a 5% CO_2 atmosphere as described previously (Rolain *et al.* 2003a). Between 8 and 12 days after inoculation of blood culture, the bacteria were harvested by adding 400 μL of phosphate-buffered saline (PBS), pH 7.2 (BioMerieux®, Craponne, France), followed by a serial dilution of the isolates from 10 to 10^{-4}. Subsequently, 200 μL of the pure bacterial suspension and each dilution was mixed with 2 mL of whole blood and used as bloodmeals for fleas. We tested the inocula by qPCR to ensure the presence of bacteria. In addition, we cultured 150 μL of the initial dilutions and other suspensions (up to 10^{-10}) on sheep blood agar plates to estimate the number of colony forming bacteria per microlitre and the viability of the bacteria.

Infection of fleas and sampling strategy

Two separate trials were conducted using fleas drawn from the same colony and with the same age. We formed 6 groups for each trial (including 1 control group and 5 infected batches) that consisted of 80 fleas in each group with approximately 25 males and 55 females. The concentration of *B. quintana* in pure suspension was 1.8×10^6 bacteria in group 1 (G1) and 4.2×10^7 bacteria in group 1′ (G1′) per μL of PBS in Trials 1 and 2, respectively, and this bacterial suspension was diluted to a concentration of 10^{-4}. Each group of fleas (G1 to G5 of Trial 1 and G1′ to G5′ of Trial 2) was fed for 4 days with infected blood containing 2 mL of blood and 200 μL of the bacterial suspension at the different dilutions ($\approx 3.6 \times 10^4 - 8.4 \times 10^9$ bacteria). The control groups were fed 2 mL of uninfected blood containing 200 μL of PBS. Subsequently, all flea groups were fed uninfected blood beginning on the 3rd day postinfection (dpi) until the 13th dpi. At the 3rd dpi, 10 viable fleas and approximately 50 mg of faeces from each group were recovered for analysis by qPCR and culturing to determine the acquisition and viability of the *B. quintana* in cat fleas. Five fleas from each dilution were immunohistochemically analysed to determine bacterial localization in the fleas (Table 1 and Fig. 1). We recovered the faeces from fleas that had been infected by inocula at $\approx 8.4 \times 10^9$ bacteria every 48 h to monitor the excretion of *B. quintana* (Table 2). At the end of the experiment (13th dpi), 10 fleas of each dilution group were analysed by qPCR.

Extraction of DNA from samples

The fleas from each dilution group were individually surface-decontaminated by 5-min immersion in ethanol alcohol (COOPER®, Paris, France), followed by three 5-min immersions in sterile PBS as described previously (La Scola *et al.* 2001). Each flea was incised using a scalpel and then incubated overnight at 56°°C in 180 μL of buffer G2 (30 mM Tris-Cl; 30 mM EDTA; 5% Tween 20; 0.5% Triton X-100; 800 mM GuHCl) containing 20 μL of proteinase K (activity = 600 mAU/mL solution or 40 mAU/mg

Table 1 Molecular, culture and immunohistological methods of detection and isolation of *B. quintana* in bloodmeals, fleas and their faeces in Trial 1 and Trial 2

			Day 3 Postinfection (P.I.)				Day 13 P.I.
			qPCR (*fabF3*)				
Trials	Group of Fleas (No)	Sampling (Quantity)	Bacteria(≈)	No. positive (%)	Culture	Immunohistochemistry	qPCR (*fabF3*)
Trial 1	Group 1 (80)	Bloodmeal* (2 mL)	3.6×10^8		+		
		Fleas (10)	6×10^2 to 9.6×10^3	10 (100%)	+‡	+†	–
		Faeces (≈ 50 mg)	1.9×10^6		–		–
	G 2 (80)	Bloodmeal*(2 mL)	3.6×10^7		+		
		Fleas (10)	5.4×10 to 3.4×10^3	10 (100%)	ND	+†	–
		Faeces (≈ 50 mg)	1.1×10^3		–		–
	G 3 (80)	Bloodmeal*(2 mL)	3.6×10^6		+		
		Fleas (10)	3.8×10 to 7.6×10	6 (60%)	ND	+†	–
		Faeces (≈ 50 mg)	2.1×10^3		–		–
	G4 (80)	Bloodmeal*(2 mL)	3.6×10^5		+		
		Fleas (10)	–	0 (0%)	ND	+†	–
		Faeces (≈ 50 mg)	1.4×10^2		–		–
	G5 (80)	Bloodmeal*(2 mL)	3.6×10^4		+		
		Fleas (10)	–	0 (0%)	ND	+†	–
		Faeces (≈ 50 mg)	–		–		–
	Group Control (80)	Bloodmeal*(2 mL)	–	–	–		
		Fleas (10)	–	0 (0%)	ND	–	–
		Faeces (≈ 50 mg)	–	–	–		–
Trial 2	Group 1′ (80)	Bloodmeal*(2 mL)	8.4×10^9		+		
		Fleas (10)	1.5×10^2 to 7×10^4	10 (100%)	ND	ND	–
		Faeces (≈ 50 mg)	9.7×10^6		+‡		–
	G 2′ (80)	Bloodmeal*(2 mL)	8.4×10^8		+		
		Fleas (10)	3.8×10 to 9.6×10^3	10 (100%)	ND	ND	–
		Faeces (≈ 50 mg)	3×10^5		–		–
	G3′ (80)	Bloodmeal*(2 mL)	8.4×10^7		+		
		Fleas (10)	7.6×10 to 4.5×10^3	9 (90%)	ND	ND	–
		Faeces (≈ 50 mg)	1.9×10^4		–		–
	G4′ (80)	Bloodmeal*(2 mL)	8.4×10^6		+		
		Fleas (10)	7.6×10 to 4.5×10^3	8 (80%)	ND	ND	–
		Faeces (≈ 50 mg)	6×10^2		–		–
	G 5′ (80)	Bloodmeal*(2 mL)	8.4×10^5		+		
		Fleas (10)	3.8×10 to 3×10^2	8 (80%)	ND	ND	–
		Faeces (≈ 50 mg)	2.4×10^3		–		–
	Group Control′ (80)	Bloodmeal*(2 mL)	–		–		
		Fleas (10)	0 (0%)	0 (0%)	ND	ND	–
		Faeces (≈ 50 mg)	–		–		–

No, number of fleas; (+) positive; (–) negative; ND, not done; (≈), approximately; qPCR, quantitative real-time polymerase chain reaction; *fabF3*, 3-oxoacyl-[acyl-carrier-protein] synthase.
*Infected or uninfected blood.
†Observed in gut.
‡Confirmation by qPCR.

of protein) until lysis and then homogenized. In addition, 50 mg of faeces was homogenized in 200 μL of PBS. After the prelysing steps, DNA extraction for 200 μL of both homogenization types was performed using an automatic EZ1 robot (QIAGEN-BioRobot® EZ1, Tokyo, Japan), according to the manufacturer's instructions (EZ1 DNA Tissue Kit, QIAGEN®, Hilden, Germany).

Real-time PCR amplification

DNA was the template in qPCR assays targeting two specific *B. quintana* genes that encoded 3-oxoacyl-[acyl-carrier-protein] synthase (*fabF3*) and a hypothetical intracellular effector (*yopP*). The primer sequences were as follows: Bqui05300F/FabF3/5′-GCT-GGC-CTT-GCT-

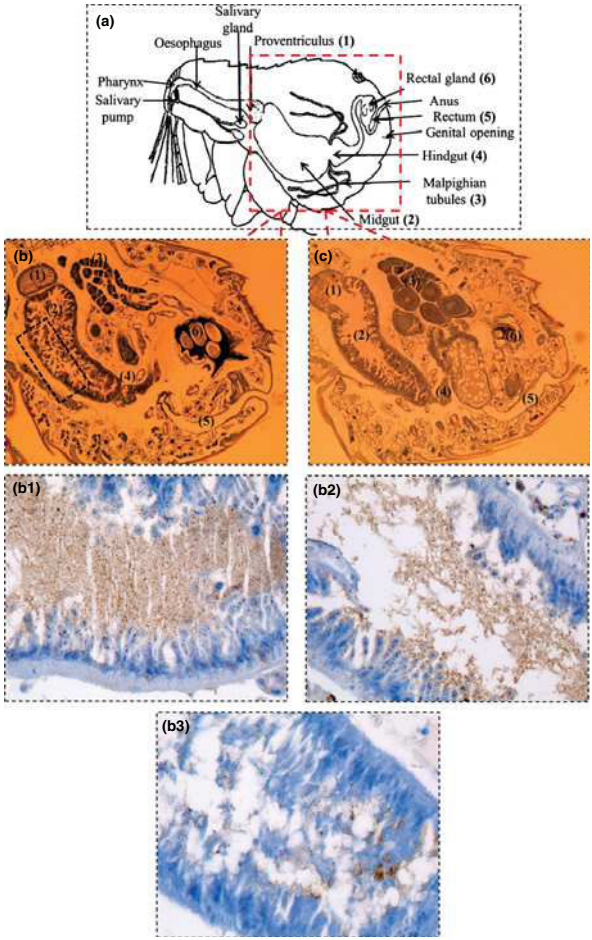

Fig. 1 Localization of *B. quintana* in the body of a flea by immunohistological analysis. (a) Generalized, internal anatomy of an adult flea with mouthparts and alimentary tract denoted [From Marshall, 1981. Presented by Durden L.A. & Traub R. (2002) in the Medical and Veterinary Entomology Book in Part 7 – Fleas (*Siphonaptera*), pages 103–125. Elsevier Inc.]; (b) immunohistochemical detection of *B. quintana* in the gut lumen of infected flea on the 3rd dpi using polyclonal rabbit anti-*B. quintana* antibody that was diluted 1:5000 and counterstained with haematoxylin. The original magnification was ×40; (b1, b2, b3) observation of *B. quintana* in the gut lumen of infected flea of Trial 1 (group 1, group 3 and group 5, respectively), magnification of ×400; (c) no detection of *B. quintana* in the gut lumen of uninfected flea on the 3rd dpi. The original magnification was ×40.

CTT-GAT-GA-3′, Bqui05300R/FabF3/5′-GCT-ACT-CTG-CGT-GCC-TTG-GA-3′, probed with Bqui05300P/FabF3/6-FAM-TGC-AGC-AGG-TGG-AGG-AGA-ACG-TG-TAMRA, and Bqui11580F/yopP/50-TAA-ACC-TCG-GGG-GAA-GCA-GA-30, Bqui11580R/yopP/5′-TTT-CGT-CCT-CAA-CCC-CAT-CA-3′, probed with Bqui11580P/yopP/6-FAM-CGT-TGC-CGA-CAA-GAC-GTC-CTT-GC-TAMRA (Angelakis *et al.* 2011). Briefly, a 20-μL qPCR reaction mixture was set up containing 2 × Reaction MasterMix for Fast qPCR with Taq DNA polymerase (Eurogentec®), 20 μM of each primer, 2.5 μM of each probe, DNase/RNase-free dH_2O and 5 μL of DNA from each sample. The reaction components were mixed in 96-well plates, and the assay was performed using the CFX96® qPCR Detection System (Bio-Rad, France). The qPCR conditions were as follows: the reaction mixtures were kept at 50°°C for 2 min, then 95°°C for 5 min and subsequently put through 40 cycles of 95°°C for 1 s and 60°°C for 35 s. The qPCR was considered positive when the cycle

threshold (Ct) was lower than 36 (Fig. 2). The number of *B. quintana* in each sample was calculated based on the DNA copy numbers of *B. quintana*. A qPCR standard curve was obtained by analysing the *fabF3* primers in serial dilutions of *B. quintana* culture, and the standard value was determined for duplicate trials. The *B. quintana* infection density was quantified as the ratio of the log of the transformed *fabF3* copy numbers per individual flea, faeces and bloodmeal (Table 1 and Fig. 2).

Table 2 Detection and isolation of *B. quintana* in faeces of fleas from groups G1 (Trial 1) and G1′ (Trial 2)

Day Postinfection	qPCR (*fabF3* 'bacteria')		Culture	
	G1 (Trial 1)	G1′ (Trial 2)	G1 (T1)	G1′(T2)
D3	1.9×10^6	9.7×10^6	–	+
D5	ND	2.5×10^6	ND	ND
D7	ND	3.3×10^2	ND	ND
D9	ND	1.5×10^2	ND	ND
D11	3.8×10	7.6×10	–	–
D13	–	–	ND	ND

Control groups	Trial 1	Trial 2	T1	T2
D3	–	–	–	–
D11	–	–	–	–

D, day; (+) positive; (–) negative; ND, not done.

Culture sampling

Bartonella quintana colonies are typically small, creamy white colonies that allow them to be readily distinguished from those formed by other flea gut commensals, but some colonies are very large and invasive. Approximately 300 µL of homogenized faeces (50 mg in 500 µL of PBS) from infected and uninfected fleas in 5% sheep's blood was filtered using a 0.8-µm filter (Millex Ø 33 mm, Dominique Dutscher®) before culturing. These filtrates were grown on agar plates (sheep blood 5%, BioMerieux®) and incubated at 37°°C under an atmosphere of 5% CO_2. After formation of the first colonies on the agar plate, the culturing procedure was repeated to isolate the bacterial species and for qPCR confirmation. Ten fleas fed the ≈3.6×10^8 bacteria dilution were decontaminated for 5 min in ethanol alcohol (COOPER®, Paris, France) and rinsed three times for 5 min in sterile PBS as described elsewhere (La Scola *et al.* 2001). These fleas were subsequently crushed in 500 µL of PBS and 300 µL of the resulting suspension was cultured on blood agar plates; the remaining homogenate was analysed by qPCR.

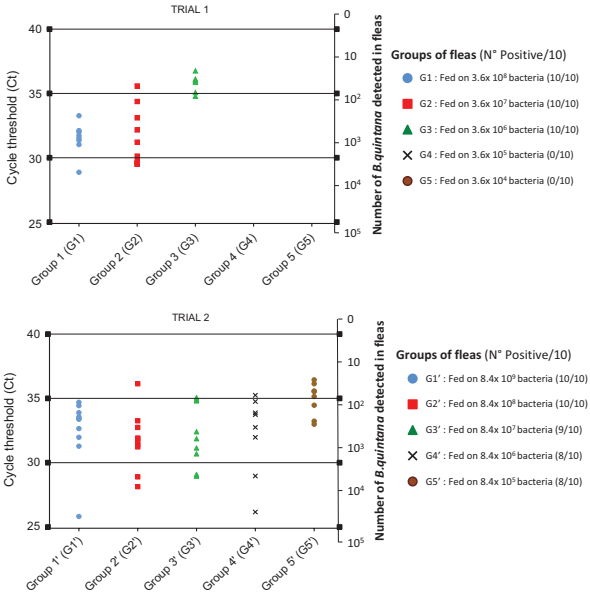

Fig. 2 The *B. quintana* infection load per group and individual flea level in Trials 1 and 2 as analysed by qPCR of the *fabF3* gene are represented with a cycle threshold (Ct) and bacteria number. The conversion of Ct at bacteria number was realized by the regression formula following: $y = -0.3005x + 12.399$ ($R^2 = 0.9924$) [the data of standard curve of the range not shown].

Immunohistological analysis

Immunohistochemistry was performed on 3-μm-thick, paraffin-embedded sections of formalin-fixed fleas using the Ventana Benchmark autostainer (Ventana Medical Systems, Inc.), and uninfected fleas were used as a negative control. After deparaffinization, each tissue section was incubated with polyclonal rabbit anti-*B. quintana* antibody, diluted 1:5000 as previously described (Lepidi *et al.* 2000).

Statistical analysis

To determine whether *B. quintana* influenced flea mortality, statistical analysis was performed with SPSS, version 17.2, for Windows. The number of dead fleas in uninfected and infected groups was compared using the chi-squared (χ^2) and Fisher's exact tests. One-way ANOVA was performed to analyse the differences in the mean scores of bacterial DNA copy number per individual flea in ten groups for each gene (*fabF3* and *yopP*) using Open Epi version 3.01. The differences were considered statistically significant at *P*-value <0.05 for all analyses.

Results

Bartonella quintana *in bloodmeal and surviving fleas fed on infected blood*

Using culture-based techniques, we confirmed that *B. quintana* grew 8 to 10 days postplating on blood agar, and DNA extraction from these colonies resulted in positive qPCR products. The average number of *B. quintana* in each sample of bloodmeal was calculated (Table 1).

To determine whether *B. quintana* influenced flea mortality, 80 fleas infected with inocula at 3.6×10^8 bacteria were compared with 80 uninfected fleas at 3, 11 and 13 dpi. The mortality rates of infected fleas were 5/80 (10 viable fleas recovered for analysis), 12/65 and 21/53 fleas at 3 dpi, 11 dpi and 13 dpi, respectively. The mortality rates for uninfected fleas were 3/80 (10 viable fleas recovered for analysis), 13/67 and 18/54 fleas at 3 dpi, 11 dpi and 13 dpi, respectively. We did not observe a difference in the mortality rates of infected and control fleas using chi-squared (χ^2) test ($P > 0.13$).

Acquisition of B. quintana *by cat fleas*

Fleas were exposed to *B. quintana*-infected bloodmeal for 4 days. On the 3rd dpi, the *B. quintana* infection load was examined at the whole individual flea level in both trials. We detected *B. quintana* in 52% (26/50) in the infected fleas in Trial 1 and in 90% (45/50) of the fleas in Trial 2. The control fleas were negative for the bacterium in both trials. We detected *B. quintana*-positive fleas in all groups (G1′ to G5′) of Trial 2 and flea groups of G1, G2 and G3 for Trial 1 (Table 1). The greatest *B. quintana* infection loads for each group of fleas in both trials are reported in Table 1. Fleas that were infected with pure inoculum (G1; G1′) exhibited the greatest quantities of *B. quintana* per individual flea sample (6×10^2 to 9.6×10^3 bacteria in Trial 1 and 1.5×10^2 to 7×10^4 bacteria in Trial 2). Fleas from both trials had significantly different *B. quintana* infection loads, and those values decreased in the groups of fleas that were fed pure to 10^{-4} dilutions of *B. quintana*. The fleas in the two last groups (G4 and G5) of Trial 1 were negative for *B. quintana* after they were fed bloodmeal with $< 8 \times 10^5$ bacteria. The quantities of *B. quintana* in each group per individual flea sample per trial analysed by qPCR of the *fabF3* gene are represented with bacteria (3.8×10 to 7×10^4) and a Ct value ranging from 25.92 to 36.5 in Fig. 2 (*yopP* gene results are shown in Table S1a,b, Supporting Information). Finally, there was no significant difference in the mean DNA copy number per individual flea in the ten groups using the *fabF3* gene ($P = 0.76244$) or the *yopP* gene ($P = 0.51222$).

Localization of B. quintana *in the bodies of* Ctenocephalides felis felis

Immunohistochemical results of the 5 tested fleas (from the 3rd dpi) from Trial 1 demonstrated the presence of *B. quintana* as dense clusters of immunopositive microorganisms in the infected flea gut tract (Fig. 1). Immunopositive microorganisms were found in all flea dilution groups except for the control fleas; however, the density of the clusters was low in fleas of the G4 and G5 from Trial 1.

Evaluation of B. quintana *in fleas and their faeces*

Detection of B. quintana *in faeces.* On the 3rd dpi, we separately tested the faeces of the infected fleas and controls from both trials by qPCR. The results indicated the presence of *B. quintana* in *C. felis felis* faeces in all groups of Trial 2, and the levels decreased from fleas of group G1′ (9.7×10^6 bacteria) to G5′ (2.4×10^3 bacteria). In Trial 1, the presence of *B. quintana* in *C. felis felis* faeces was found in all groups except G5, and the levels ranged from 1.9×10^6 bacteria (G1) to 1.4×10^2 bacteria (G4) (Table 1).

Viability of B. quintana *in fleas and their faeces.* We used MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis to detect faecal bacteria after culturing as described previously

(Fournier *et al.* 2009). To avoid the detection of flea gut commensal bacteria such as *Serratia marcescens* (family: Enterobacteriaceae, Gram negative), we filtered the different dilutions of faeces using a 0.8-μm filter before culturing. On the 3rd dpi, we grew *B. quintana* from the faeces of fleas that had been fed inocula with 8.4×10^9 bacteria in Trial 2 on agar plates. Colonies formed after 10 days postplating; however, none colonies have grown in the other dilutions, including the group G1 ($\approx 3.6 \times 10^8$ bacteria) of Trial 1 (Table 1). Furthermore, we only observed a positive culture from one of 10 group G1 fleas (infected with $\approx 3.6 \times 10^8$ bacteria) from Trial 1 at 30 days postplating. In Trial 2, the existence of *B. quintana* colonies was detected by culturing group G1′ flea faeces (infected with $\approx 8.4 \times 10^9$ bacteria) and was confirmed using a second culture step (direct and indirect cultures); these results were verified by qPCR for *fabF3 and yopP* genes.

Persistence of B. quintana *in faeces.* Using qPCR, we followed the presence and elimination of faecal bacteria in fleas that were fed pure suspensions (Trials 1 and 2, 3.6×10^8 bacteria and 8.4×10^9 bacteria, respectively). The results reported in Table 2 demonstrate that the average number of *B. quintana* in the flea faeces increased in Trials 1 and 2 until the 3rd dpi to a maximal number of 9.7×10^6 bacteria, and this value then decreased logarithmically until the 11th dpi to a minimum of 7.6×10 bacteria in both trials. At the 13th dpi, the faeces of all inocula-infected fleas were negative for *B. quintana* in both trials (Table 2). On the 13th dpi, we tested 10 viable fleas from each group by qPCR, and those fleas were negative for *B. quintana*, which indicates that the bacteria were completely eliminated from infected fleas (Table 1).

Discussion

The cat flea, *C. felis felis*, is found worldwide and has been reported to parasitize many species of wild and domestic animals (Rust & Dryden 1997). The cat flea is the only arthropod that has been demonstrated to date to biologically transmit *Rickettsia felis* (Reif & Macaluso 2009). In addition, the cat flea has been confirmed as a vector for *Bartonella henselae*, the causative agent of cat scratch disease (Zangwill *et al.* 1993; Chomel *et al.* 1996). Foil *et al.* (1998) demonstrated the experimental infection of cats with *B. henselae* after inoculation with cat flea faeces (Foil *et al.* 1998). Recently, this flea was suspected to be a potential vector for transmitting *B. quintana*, *B. clarridgeiae*, *B. koehlerae*, *B. birtlesii* and *B. tribocorum* to mammals (Bouhsira *et al.* 2013a).

A symbiont–host relationship has been observed in the human body louse *Pediculus humanus corporis* vector, which excretes the agent of trench fever, *B. quintana*, in its faeces (Higgins *et al.* 1996). The first clinical manifestation of trench fever was attributed to *B. quintana* and was characterized by a sudden onset of headache, pain in the shins, dizziness and fever. Thereafter, the primary infection resolved but had frequent relapses (Foucault *et al.* 2006). Chronic bacteraemia developed in some patients (Brouqui *et al.* 1999), and chronic asymptomatic bacteraemia in humans indicated that humans may be the natural reservoir of *B. quintana* (Foucault *et al.* 2002). Although humans are a primary reservoir host for *B. quintana*, some recent reports have found *B. quintana* in cat teeth (La *et al.* 2005), in dogs and in cynomolgus and rhesus macaques (Chomel *et al.* 2006; Huang *et al.* 2011; Li *et al.* 2012, 2013). Our results indicate that other arthropods, such as fleas, may acquire *B. quintana* and then excrete it in its faeces.

Several studies suggested that biting arthropods such as flies, lice, fleas or ticks can transmit *Bartonella* spp. during their bloodmeal (Tsai *et al.* 2011), but few studies have described the details of the growth kinetics and transmission of these bacteria. For example, human infection by *B. quintana* is presumed to ensue from the inoculation of faeces into louse bites by scratching (Maurin & Raoult 1996). Another organism, *B. henselae*, is also thought to be transmitted by contamination of a bite wound with infected faeces, and the *C. felis* flea is capable of ingesting *B. henselae*, supporting replication of these bacteria in their digestive tract and excreting viable organisms in their faeces (Higgins *et al.* 1996). As suggested by Morick *et al.* (2013), *Bartonella* spp. are likely not pathogenic to fleas and are well adapted to their vectors. Our study indicates that cat fleas could maintain *B. quintana* in their gastrointestinal tract and viable colonies could be produced from faeces upon inoculation on sheep blood agar plates. We suggest a similar scenario for the transmission of *B. quintana* by cat fleas to cats and humans.

Our experimental results corroborate data reported by Bouhsira *et al.* (2013a), which used artificial conditions for feeding *Bartonella* species including *B. quintana*, and found no difference in the persistence of the cat flea or of its faeces excretion up to the 3rd dpi. In a study by Seki *et al.* (2007), a quantitative analysis of bacterial multiplication rate was performed – the proliferation of *B. quintana* in body lice was observed to begin 4 days after ingestion. Furthermore, the bacteria were constantly excreted into the faeces for at least 3 weeks (Seki *et al.* 2007). However, in our study, viable *B. quintana* in faeces were observed in fleas that were fed a suspension of bacteria $\geq 4.2 \times 10^9$ per mL of blood and bacterial load in faeces decreased gradually after 3rd dpi. Our findings suggest that infectivity did not remain in a faecal environment for a prolonged time. In

addition, we suggest that *B. quintana* was completely shed in flea faeces until the 13th dpi or *B. quintana* was disseminated in the different body cavity of the cat fleas. Nonetheless, we observed the absence of *B. quintana* in all fleas and in their faeces on day 13. The absence of the *Bartonella* was observed in previous studies: no *B. quintana* DNA was detected in louse faeces on either day 5, 7, 9 or 11 after infection (Seki *et al*. 2007) and no *B. henselae* DNA was detected in cat fleas and their faeces on day 9 (Bouhsira *et al*. 2013b). Further studies are required to determine whether the *B. quintana* was completely eliminated after 13 dpi.

Our immunohistological approach assessed *B. quintana* localization only in the flea gut at the 3rd dpi. Unfortunately, the immunohistological method was not used on cat fleas at 13 dpi to evaluate *B. quintana* dissemination from the gut to other cat flea organs. Recently, *R. felis* has been shown to replicate in the cat fleas' digestive tract, migrate to the hemolymph and then disseminate through the excretory system (such as the Malpighian tubules, hindgut and rectal ampulla) and reproductive tissues (Thepparit *et al*. 2013). However, the method of *Bartonella* migration (e.g. *B. quintana* or *B. henselae*) from the digestive tract of arthropods (such as the louse or flea) to other tissues has not yet been elucidated (Seki *et al*. 2007; Bouhsira *et al*. 2013b).

Although no clear replication pattern or dissemination of *B. quintana* in cat fleas was reported throughout the study, we showed that cat fleas can acquire *B. quintana*, a known human pathogen, by feeding and releasing viable organisms into their faeces. Therefore, fleas may play a role as vectors of trench fever or other infections caused by *B. quintana*. More studies are required to better understand *B. quintana* persistence in both fleas and their faeces and also to understand the role of fleas in *B. quintana* infections.

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Conflict of interests

The authors declare that they have no competing interests.

References

Anderson BE, Neuman MA (1997) *Bartonella* spp. As emerging human pathogens. *Clinical Microbiology Reviews*, **10**, 203–219.

Angelakis E, Rolain JM, Raoult D, Brouqui P (2011) *Bartonella quintana* in head louse nits. *FEMS Immunology and Medical Microbiology*, **62**, 244–246.

Bonilla DL, Kabeya H, Henn J, Kramer VL, Kosoy MY (2009) *Bartonella quintana* In body lice and head lice from homeless persons, San Francisco, California, USA. *Emerging Infectious Diseases*, **15**, 912–915.

Bouhsira E, Ferrandez Y, Liu M *et al*. (2013a) *Ctenocephalides felis* an in vitro potential vector for five *Bartonella* species. *Comparative Immunology, Microbiology & Infectious Diseases*, **36**, 105–111.

Bouhsira E, Franc M, Boulouis HJ, Jacquiet P, Raymond-Letron I, Liénard E (2013b) Assessment of Persistence of *Bartonella henselae* in *Ctenocephalides felis*. *Applied and Environmental Microbiology*, **79**, 7439–7444.

Brouqui P, Lascola B, Roux V, Raoult D (1999) Chronic *Bartonella quintana* bacteremia in homeless patients. *New England Journal of Medicine*, **340**, 184–189.

Byam W, Lloyd LL (1920) Trench Fever: its epidemiology and endemiology. *Proceedings of the Royal Society of Medicine*, **13**, 1–27.

Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N (2001) Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. *Journal of Clinical Microbiology*, **39**, 1221–1226.

Chomel BB, Kasten RW, Floyd-Hawkins K *et al*. (1996) Experimental transmission of *Bartonella henselae* by the cat flea. *Journal of Clinical Microbiology*, **34**, 1952–1956.

Chomel BB, Boulouis HJ, Maruyama S, Breitschwerdt EB (2006) *Bartonella* spp. in pets and effect on human health. *Emerging Infectious Diseases*, **12**, 389–394.

Drancourt M, Mainardi JL, Brouqui P *et al*. (1995) *Bartonella (Rochalimaea) quintana* endocarditis in three homeless men. *New England Journal of Medicine*, **332**, 419–423.

Drancourt M, Moal V, Brunet P *et al*. (1996) *Bartonella (Rochalimaea) quintana* infection in a seronegative hemodialyzed patient. *Journal of Clinical Microbiology*, **34**, 1158–1160.

Foil L, Andress E, Freeland RL *et al*. (1998) Experimental infection of domestic cats with *bartonella henselae* by inoculation of *Ctenocephalides felis* (Siphonaptera: Pulicidae) feces. *Journal of Medical Entomology*, **35**, 625–628.

Foucault C, Barrau K, Brouqui P, Raoult D (2002) *Bartonella quintana* bacteremia among homeless people. *Clinical Infectious Diseases*, **35**, 684–689.

Foucault C, Brouqui P, Raoult D (2006) *Bartonella quintana* characteristics and clinical management. *Emerging Infectious Diseases*, **12**, 217–223.

Fournier PE, Couderc C, Buffet S, Flaudrops C, Raoult D (2009) Rapid and cost-effective identification of *Bartonella* species using mass spectrometry. *Journal of Medical Microbiology*, **58**, 1154–1159.

Higgins JA, Radulovic S, Jaworski DC, Azad AF (1996) Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera:Pulicidae). *Journal of Medical Entomology*, **33**, 490–495.

Huang R, Liu Q, Li G, Li D, Song X, Birtles RJ (2011) *Bartonella quintana* infections in captive monkeys, China. *Emerging Infectious Diseases*, **17**, 1707–1709.

Kostrzewski J (1949) The epidemiology of trench fever. *Bulletin of the Polish Academy of Sciences*, **7**, 233–263.

La Scola B, Fournier PE, Brouqui P, Raoult D (2001) Detection and culture of *Bartonella quintana, Serratia marcescens* and *Acinetobacter* spp. from decontaminated human body lice. *Journal of Clinical Microbiology*, **39**, 1707–1709.

La VD, Tran-Hung L, Aboudharam G, Raoult D, Drancourt M (2005) *Bartonella quintana* In Domestic Cat. *Emerging Infectious Diseases*, **11**, 1287–1289.

Lepidi H, Fournier PE, Raoult D (2000) Quantitative analysis of valvular lesions during *Bartonella* endocarditis. *American Journal of Clinical Pathology*, **114**, 880–889.

Li H, Tong Y, Huang Y *et al.* (2012) Complete genome sequence of *Bartonella quintana*, a bacterium isolated from rhesus macaques. *Journal of Bacteriology*, **194**, 6347.

Li H, Liu W, Zhang GZ *et al.* (2013) Transmission and maintenance cycle of *Bartonella quintana* among rhesus macaques, China. *Emerging Infectious Diseases*, **19**, 297–300.

Maurin M, Raoult D (1996) *Bartonella (Rochalimaea) quintana* infections. *Clinical Microbiology Reviews*, **9**, 273–292.

McNee JW, Renshaw A, Brunt EH (1916) "Trench Fever"**:** a relapsing fever occurring with the British forces in France. *British Medical Journal*, **12**, 225–234.

Melter O, Arvand M, Votypka J, Hulinska D (2012) *Bartonella quintana* transmission from mite to family with high socioeconomic status. *Emerging Infectious Diseases*, **18**, 163–165.

Morick D, Krasnov BR, Khokhlova IS *et al.* (2013) Effects Of *Bartonella* spp. on flea feeding and reproductive performance. *Applied and Environmental Microbiology*, **79**, 3438–3443.

Raoult D, Drancourt M, Carta A, Gastaut JA (1994) *Bartonella (Rochalimaea) quintana* isolation in patient with chronic adenopathy, lymphopenia. *And A Cat*. *Lancet*, **343**, 977.

Reif KE, Macaluso KR (2009) Ecology of *Rickettsia felis*: a Review. *Journal of Medical Entomology*, **46**, 723–736.

Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS (1990) The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. *New England Journal of Medicine*, **323**, 1573–1580.

Rolain JM, Arnoux D, Parzy D, Sampol J, Raoult D (2003a) Experimental infection of human erythrocytes from alcoholic patients with *Bartonella quintana*. *Annals of the New York Academy of Sciences*, **990**, 605–611.

Rolain JM, Franc M, Davoust B, Raoult D (2003b) Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* In cat fleas, France. *Emerging Infectious Diseases*, **9**, 338–342.

Rolain JM, Bourry O, Davoust B, Raoult D (2005) *Bartonella quintana* and *Rickettsia felis* in Gabon. *Emerging Infectious Diseases*, **11**, 1742–1744.

Rust MK, Dryden MW (1997) The biology, ecology, and management of the cat flea. *Annual Review of Entomology*, **42**, 451–473.

Seki N, Kasai S, Saito N *et al.* (2007) Quantitative analysis of proliferation and excretion of *Bartonella quintana* In body Lice, *Pediculus humanus L. American Journal of Tropical Medicine and Hygiene*, **77**, 562–566.

Spach DH, Kanter AS, Daniels NA *et al.* (1995a) *Bartonella (Rochalimaea)* species as a cause of apparent "culture-negative" endocarditis. *Clinical Infectious Diseases*, **20**, 1044–1047.

Spach DH, Kanter AS, Dougherty MJ *et al.* (1995b) *Bartonella (Rochalimaea) quintana* bacteremia in inner-city patients with chronic alcoholism. *New England Journal of Medicine*, **332**, 424–428.

Thepparit C, Hirunkanokpun S, Popov VL, Foil LD, Macaluso KR (2013) Dissemination of bloodmeal acquired *Rickettsia felis* in cat fleas, *Ctenocephalides felis*. *Parasites & Vectors*, **6**, 149.

Tsai YL, Chang CC, Chuang ST, Chomel BB (2011) *Bartonella* species and their ectoparasites: selective host adaptation or strain selection between the vector and the mammalian host? *Comparative Immunology, Microbiology & Infectious Diseases*, **34**, 299–314.

Wade SE, Georgi JR (1988) Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouche (Siphonaptera: Pulicidae). *Journal of Medical Entomology*, **25**, 186–190.

Zangwill KM, Hamilton DH, Perkins BA *et al.* (1993) Cat Scratch Disease in connecticut - epidemiology, risk factors, and evaluation of a new diagnostic test. *New England Journal of Medicine*, **329**, 8–13.

K.T., C.S., B.I., R.D. and P.P. conceived and designed the experiments. K.T. and Leu. H. performed the experiments. K.T., Leu. H., C.S., R.J.-M., R.D. and P.P. analysed the data. Lep. H. contributed immunohistochemistry tools. C.S., B.J.-M. and R.D. contributed reagents/materials/analysis tools. K.T., C.S., R.J.-M. and P.P. wrote the paper. K.T., C.S., R.J.-M., R.D. and P.P. critically discussed the manuscript.

Data accessibility

The data for a Ct and copy number of the *fabF3* and *yopP* genes of *Bartonella quintana* in individual fleas of each group at 3 days postinfection in Trial 1 and Trial 2 may be found as supplemental material in the online version of this article.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. (a) Cycle threshold (Ct) and copy number of *Bartonella quintana* detected by *fabF3* and *yopP* genes in individual fleas per group at 3 days postinfection in Trial 1. (b) Cycle threshold (Ct) and copy number of *Bartonella quintana* detected by *fabF3* and *yopP* genes in individual fleas per group at 3 days postinfection in Trial 2.

ARTICLE 7

Article N.8. **Leulmi H**, Bitam I, Berenger J-M, Lepidi H, Rolain J-M, Almeras L, Raoult D, Parola P. **Competence of *Cimex lectularius* Bed Bugs for the Transmission of *Bartonella quintana*, the Agent of Trench Fever**. PLoS Negl Trop Dis. 2015 May 22;9(5):e0003789. doi: 10.1371/journal.pntd.0003789. eCollection 2015.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Competence of *Cimex lectularius* Bed Bugs for the Transmission of *Bartonella quintana*, the Agent of Trench Fever

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Abstract

Background

Bartonella quintana, the etiologic agent of trench fever and other human diseases, is transmitted by the feces of body lice. Recently, this bacterium has been detected in other arthropod families such as bed bugs, which begs the question of their involvement in *B. quintana* transmission. Although several infectious pathogens have been reported and are suggested to be transmitted by bed bugs, the evidence regarding their competence as vectors is unclear.

Methodology/Principal Findings

Bed bugs at the adult and instar developmental stages were fed three successive human blood meals inoculated with *B. quintana* bacterium from day one (D1) to D5; subsequently they were fed with pathogen-free human blood until the end of the experiment. Bed bugs and feces were collected in time series, to evaluate their capacities to acquire, multiply and expel viable *B. quintana* using molecular biology, immunohistochemistry and cultures assays. *B. quintana* was detected molecularly in 100% of randomly selected experimentally infected bed bug specimens (D3). The monitoring of *B. quintana* in bed bug feces showed that the bacterium was detectable starting on the 3rd day post-infection (pi) and persisted until day 18±1 pi. Although immunohistochemistry assays localized the bacteria to the gastrointestinal bed bug gut, the detection of *B. quintana* in the first and second instar larva stages suggested a vertical non-transovarial transmission of the bacterium.

Conclusion

The present work demonstrated for the first time that bed bugs can acquire, maintain for more than 2 weeks and release viable *B. quintana* organisms following a stercorarial shedding. We also observed the vertical transmission of the bacterium to their progeny. Although

the biological role of bed bugs in the transmission of *B. quintana* under natural conditions has yet to be confirmed, the present work highlights the need to reconsider monitoring of these arthropods for the transmission of human pathogens.

Author Summary

Bartonella quintana, the etiologic agent of trench fever and other human diseases, is known to be transmitted by the feces of body lice. Recently, the DNA of this bacterium has been detected in bed bugs. Several pathogens have been associated and suggested to be transmitted by bed bugs, despite the insufficient evidence to support this vector role. The aim of the present study was to assess the competence of bed bugs in the transmission of *B. quintana* using an experimental artificial model of infection. To this end, bed bugs were fed with human infected blood meals. On the 3rd day post-infection (dpi) *B. quintana* was detected molecularly in 100% of experimentally infected bed bug. The bacterium was also detectable in bed bug feces starting on the 3rd dpi and persisted until 18±1 dpi. Although immunohistochemistry assays localized the bacteria to the gastrointestinal bed bug gut, *B. quintana* was also detected in the first and second instars larva. The present work highlights the need to reconsider monitoring of bed bugs for the transmission of pathogens.

Introduction

Bartonella quintana is a fastidious gram-negative bacterium that is regarded as a re-emerging human pathogen [1]. *B. quintana* DNA has been detected in the dental pulp of a 4000-year-old man [2] and in lice found in a mass grave of Napoleon's soldiers in Lithuania, which suggests that many of the soldiers were affected by trench fever [3]. Trench fever was the first described clinical manifestation of *B. quintana* infection, and it affected thousands of soldiers during World Wars I and II [3]. Subsequently, *B. quintana* has been identified as an agent of bacillary angiomatosis in AIDS patients [4], endocarditis [5,6], chronic bacteremia [7,8], and chronic lymphadenopathy [9]. The severity of *Bartonella* infection correlates with the immune status of the patient; the clinical manifestations can range from benign and self-limited to severe and life-threatening disease [10]. Although body lice are considered as the main vector of *B. quintana* [11], this bacterium has also been found in other arthropods such as head lice [12,13], ticks [14] and mites [15]. Recently, after the detection of *B. quintana* DNA in fleas [16], it was experimentally demonstrated that the cat flea, *Ctenocephalides felis*, could acquire and excrete viable *B. quintana* in their feces [17]. These results supported the likely vector role of fleas in trench fever or other clinical manifestations caused by *B. quintana* [17].

The recent detection of *B. quintana* DNA in *Cimex hemipterus* (tropical bed bugs) collected from two prisons in Rwanda indicated that bed bugs could be involved in the transmission of *B. quintana* [18]. This raises the question of whether *C. lectularius* (common bed bug) could acquire and excrete viable *B. quintana* and thus constitute a potential competent vector. For this purpose, we used an experimental model infection of *C. lectularius* bed bugs using three different approaches: qPCR, culture and immunohistochemistry.

Materials and Methods

Bacterial strain

B. quintana strain Oklahoma (ATCC 49793) [17] was used to infect the blood used to feed the bed bugs. The use, culturing and all procedures involving experimental infections of *B. quintana* were conducted in a Biosafety Level 2 room.

Medium and growth condition

B. quintana strain was grown as described previously [19] on 5% Columbia sheep blood agar plates (BioMerieux, Marcy l'Etoile, France) in a humidified atmosphere at 37°C supplemented with 5% carbon dioxide (CO_2) using the pouch of atmosphere generation system CO_2 Gen (Oxoid Ltd by Mitsubishi Gas chemical Company Inc, Japan). After 8 to 10 days of culture, the bacteria were harvested by adding four-hundred μL of phosphate buffered saline (PBS), pH 7.2 (BioMerieux, Craponne, France). Two-hundred microliters of the pure bacterial suspension were mixed with 2 mL of whole blood, and this was used as the blood meal to infect the bed bugs. The remaining 200 μL of the bacterial suspension were diluted up to 10^{-10} and cultured to estimate the number of colony-forming units (CFU) per microliter.

Bed bugs maintenance and supply

Since 2012, bed bugs (*Cimex lectularius*) have been maintained in a laboratory insectarium by our team at the WHO collaborative center for rickettsioses and other arthropod borne bacterial diseases in Marseille, France. This colony originated from bed bugs collected at the adult and the five instar stages from an infested apartment (Aix-en-Provence, France) using a modified Dyson DC34 hand vacuum system. They were maintained in containers kept in incubator at 60% humidity and 22°C. The bed bugs were fed once a week using citrated human blood obtained from the French Blood Establishment. Ethical approval for the use of in vitro human blood was obtained from the laboratory research ethics board of Molecular Hematology, French Blood Establishment. Two mL of blood was placed in a Hemotek artificial feeder machine (Hemotek 5W1; Discovery Workshops, Accrington, UK) covered by an artificial membrane of Parafilm M (Sigma-Aldrich, Saint-Louis, Missouri, USA) that was stretched to the twice of its length and width [20]. To prevent contamination during the experimental infection model, the Hemotek feeder and the recipient's containers of bed bugs were introduced in a clear acrylic box.

Bed bug infections

Two separate trials were conducted using *C. lectularius* drawn from the same colony at the same age. Prior to initiation of the infection, the bed bugs and their feces were shown to be free from *B. quintana* using qPCR.

We formed 4 groups for each trial including 2 infected (1 adults and 1 larva group) and 2 control groups (1 adults and 1 larva group); each group consisted of 30 bed bugs. In the adult vials we used 10 males and 20 females, and also larval group was composed of 30 Larva 1 (L1) bed bugs.

The concentration of *B. quintana* in the infected suspension composed by the bacterial suspension and the blood meal was 6×10^8 CFU/mL bacteria in trial 1 and 8×10^5 CFU/ mL in trial 2. Each group of bed bugs was fed 3 times in 5 days (every other day) with 200 μL of the bacterial suspension mixed with 2 mL of blood meal. The control groups were fed with 2 mL of uninfected blood mixed with 200 μL of PBS. Subsequently, all bed bug groups were fed with

uninfected blood every other day starting on the 3rd day post-infection (dpi) until the end of the experiment.

We tested 200 μL of the infected inoculum (the infected blood suspension that the bed bugs fed on) to ensure the presence of *B. quintana* in the infected blood meal using qPCR. We cultured 150 μL of the inoculum and plated dilutions up to 10^{-10} to ensure the viability and to determine the concentration of *B. quintana* in the infected inoculum.

Sampling strategy

At the 3rd dpi, five viable bed bugs and approximately 20 mg of feces from each group (from *B. quintana* exposed group of adults and instars and also from the control groups) were recovered for analysis by qPCR. Feces were collected from a sheet of paper placed on the bottom of the bed bugs containers. Culture analysis of feces and two bed bugs were also performed; both tests were used to determine the acquisition and viability of *B. quintana* in bed bugs and in their feces. Four adult *C. lectularius* from the *B. quintana*-exposed group were immunohistochemically analyzed to determine the bacterial localization. Four bed bugs from the control group were also analyzed and served as controls. Starting on the 5th dpi, we recovered two adults and feces every 48 h to monitor the excretion of *B. quintana* through the end of the experiment (21st dpi). We screened five eggs from the container housing the infected adults by qPCR at the 3rd dpi to determine if the eggs were infected. Simultaneously, we recovered ten eggs to be reared in separate vials to obtain L1 and L2 larvae. The larvae were analyzed by qPCR to determine if any *B. quintana* acquisition occurred.

DNA extraction

The DNA of individual bed bugs and their feces were extracted using an automatic EZ1 robot (QIAGEN-BioRobot_ EZ1, Tokyo, Japan) according to the manufacturer's instructions (EZ1 DNA Tissue Kit, QIAGEN, Hilden, Germany). First, we decontaminated the surface of the bed bugs by 5 min immersion in ethanol alcohol (COOPER, Paris, France), followed by three 5 minutes immersions in sterile PBS as described previously [21]. Each bed bug was incubated overnight at 56°C in 180 μL of buffer G2 and 20 μL of proteinase K for pre-lysis followed by extraction using EZ1 robot. For all samples, the final elution volume was 100 μL.

Real time PCR amplification

Template DNA was used in the qPCR assays targeting two specific *B. quintana* genes that encoded 3oxoacyl-[acyl-carrier-protein] synthase (*fabF3*) and a hypothetical intracellular effector (*yopP*) [13], which are both *B. quintana*-specific genes. The CFX96 (Bio-Rad, France) was used to perform each real time PCR. The qPCR was considered positive when the cycle threshold (Ct) was lower than 36 [17]. The number of *B. quintana* in each sample was calculated based on the DNA copy numbers. A qPCR standard curve was obtained by analyzing the *fabF3* and *yopP* systems in serial dilutions of *B. quintana* culture, and the standard value was determined for duplicate trials [17]. The *B. quintana* infection density was quantified as the ratio of the log of the transformed *fabF3* and *yopP* copy numbers per individual bed bug, feces, and blood meal. The cycle thresholds (Ct) values of [12.9; 14.5; 17.8; 22.0; 25.7; 28.9; 30.9; 34.3 and 36.0] correspond, respectively, to [4 x 10^9; 4 x 10^8; 4 x 10^7 4 x 10^5 4 x 10^4; 4 x 10^2; 4 x 10^1 and 4] CFU/mL. Regressions formula was realized as following: $Y = -0.377X + 14.236$ ($R^2 = 0.996$) for *fabF3* gene and $Y = -0.372X + 14.158$ ($R^2 = 0.996$) for *yopP* gene.

Culture sampling

Approximately 500 μL of homogenized feces (20 mg in 500 μL of PBS) from groups of infected and uninfected bed bugs with 5% sheep's blood were filtered using a 0.8 μm filter (Millex Ø 33 mm, Dominique Dutscher) and were cultured on agar plates [17]. The bodies of the bed bugs were also cultured using the same method described for the culturing of feces.

Immunohistological analysis

Immunohistochemistry was performed on 3 μm-thick, paraffin-embedded sections of formalin-fixed bed bugs using the Ventana Benchmark autostainer (Ventana Medical Systems, Inc.) [17]. Four infected bed bugs (2 from each trial) and 4 uninfected bed bugs were analyzed (2 from each trial). After deparaffinization, each tissue section was incubated with polyclonal rabbit anti-*B. quintana* antibody, which was diluted 1:5000 as previously described [22].

Results

Acquisition of *B. quintana* by bed bugs

In the two trials, adults and L1 bed bugs were exposed to *B. quintana* three times in 5 days using *B. quintana*-infected blood meal. On the 3rd dpi, we individually analyzed five adults and five L1 *C. lectularius* by qPCR. The control groups (fed on blood meal with 200 μL of PBS) were negative by qPCR for the presence of the bacterium in both trials. In the *B. quintana*-exposed groups, we detected *B. quintana* in 100% (5/5) of the adult bed bugs and in 100% (5/5) of the L1 bed bugs in both trials. The quantities of *B. quintana* in each individual bed bug sample per trial as determined by qPCR of the *fabF3* and *yopP* genes are given in Tables 1 and 2. Bacterial quantities ranged between 5.8 x 10^7 CFU/ mL and 4.8 x 10^2 CFU/ mL in trial 1 and from 2.8 x 10^6 CFU/ mL to 6 x 10 CFU/ mL in trial 2. Feces of adults and larva bed bugs were also tested by qPCR to evaluate the presence of *B. quintan*a and to confirm the route of way of elimination. The results indicated the presence of the bacterium in the feces in both trials

Table 1. Cycle threshold (Ct) and copy number of *Bartonella quintana* detected by targeting *fabF3* and *yopP* genes in individual bed bugs per group at the 3rd day post-infection in trial 1.

Bed bugs	*fabF3* gene	G1		G2		G3	G4
		Ct	Copy	Ct	Copy	Ct	Ct
	Bed bug 1	18.7	1.5 x 10^7	22.0	8.5 x 10^5	>36	>36
	Bed bug 2	20.2	4.1 x 10^6	21.3	1.6 x 10^6	>36	>36
	Bed bug 3	17.2	5.8 x 10^7	21.5	1.3 x 10^6	>36	>36
	Bed bug 4	17.8	3.5 x 10^7	27.8	5.7 x 10^3	>36	>36
	Bed bug 5	21.0	2.2 x 10^6	30.6	4.8 x 10^2	>36	>36
	Feces	15.4	2.8 x 10^8	18.2	5.5 x 10^7	>36	>36
Bed bugs	***yopP* gene**	**Ct**	**Copy**	**Ct**	**Copy**	**Ct**	**Ct**
	Bed bug 1	18.3	2.2 x 10^7	22.4	6.9 x 10^5	>36	>36
	Bed bug 2	21.1	2.0 x 10^6	22.8	4.5 x 10^5	>36	>36
	Bed bug 3	17.5	4.3 x 10^7	20.2	4.0 x 10^6	>36	>36
	Bed bug 4	16.4	1.2 x 10^8	27.7	6.2 x 10^3	>36	>36
	Bed bug 5	20.5	3.2 x 10^6	30.5	5.2 x 10^2	>36	>36
	Feces	15.6	2.3 x 10^8	18.3	2.2 x 10^7	>36	>36

G1: group of infected bed bugs adult, **G2:** group of infected bed bugs instar, **G3:** group of bed bugs adults control, **G4:** group of bed bugs instar control. The conversion of Ct to bacteria number in positive samples was realized by the regression formula following: **y = -0.377x + 14.236** (R^2 = 0.996) **for *fabF3* gene** and **y = -0.372x + 14.158** (R^2 = 0.996) for ***yopP* gene,** Ct > 36: sample considered to be negative.

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Table 2. Cycle threshold (Ct) and copy number of *Bartonella quintana* detected by targeting *fabF3* and *yopP* genes in individual bed bugs per group at the 3rd day post-infection in trial 2.

Bed bugs	*fabF3* gene	G1		G2		G3	G4
		Ct	Copy	Ct	Copy	Ct	Ct
	Bed bug 1	21.4	1.4×10^6	21.5	1.3×10^6	>36	>36
	Bed bug 2	20.7	2.8×10^6	25.1	5.8×10^4	>36	>36
	Bed bug 3	27.5	7.3×10^3	22.5	5.6×10^5	>36	>36
	Bed bug 4	21.6	1.2×10^6	23.8	1.9×10^5	>36	>36
	Bed bug 5	27.0	1.2×10^4	33.0	6.0 x10	>36	>36
	Feces	27.3	9.1×10^3	20.6	2.8×10^6	>36	>36
Bed bugs	*yopP* gene	Ct	Copy	Ct	Copy	Ct	Ct
	Bed bug 1	22.5	5.8×10^5	22.0	8.7×10^5	>36	>36
	Bed bug 2	20.5	3.3×10^6	24.6	9.3×10^4	>36	>36
	Bed bug 3	27.5	7.3×10^3	21.0	2.1×10^6	>36	>36
	Bed bug 4	21,7	1.1×10^6	23.7	2×10^5	>36	>36
	Bed bug 5	26.9	1.2×10^4	34.2	2.3 x 10	>36	>36
	Feces	27.2	5.5×10^4	22.2	7.4×10^5	>36	>36

G1: group of infected bed bugs adult, **G2:** group of infected bed bugs instar, **G3:** group of bed bugs adult control, **G4:** group of bed bugs instar control. The conversion of Ct to bacteria number in positive samples was realized by the regression formula following: **$y = -0.377x + 14.236$** ($R^2 = 0.996$) for ***fabF3* gene** and **$y = -0.372x + 14.158$** ($R^2 = 0.996$) for ***yopP* gene,** Ct > 36: sample considered to be negative.

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with 2.8×10^8 CFU/ mL in the adult feces and 5.5×10^7 CFU/ mL in the L1 feces in trial 1 and 9.1×10^3 CFU/ mL in the adult feces and 2.8×10^6 CFU/ mL in the L1 feces in trial 2 (Table 3).

Localization of *B. quintana* in the bodies of bed bugs

Immunohistochemical analysis of the 4 tested *C. lectularius* (from the 3rd dpi) from trial 1 and trial 2 demonstrated the presence of *B. quintana* as dense clusters of immunopositive microorganisms in the midgut and hindgut of the gut tract (Fig 1, Table 3).

Evolution of *B. quintana* in bed bugs and their feces

Viability of *B. quintana* in bed bugs and in their feces. Cultures of homogenized and filtered feces and whole organisms from the infected adult and L1 bed bug groups were positive on the 3rd dpi in both two trials. The presence of viable *B. quintana* was confirmed using a second culture (direct and indirect culture) and was corroborated by qPCR (Table 3).

Persistence of *B. quintana* in the body of bed bugs. Using qPCR, we followed the presence of the bacterium in adult bed bugs from the 3rd dpi until the end of the experiment. The results reported in Fig 2 demonstrate that the average number of *B. quintana* in the bed bugs decreased during both trials. In trial 1 (bed bugs fed with 6×10^8 CFU/mL), *B. quintana* persist up to the 19th dpi; however, in trial 2 (bed bugs fed with 8×10^5 CFU/mL), *B. quintana* was detected until the 17th dpi. We analyzed 5 eggs (recovered from the *B. quintana*-exposed group of adult bed bugs) at the 3rd dpi and found that 2 of them were positive by qPCR (Ct [24.4, +/-2.2]). Culture analysis of the egg suspension was also positive. Ten L1 stage larvae were obtained after incubation of the eggs for 6 days; five were analyzed by qPCR and all were positive for *B. quintana* in both trials (the mean Ct in trial 1 was [24.4, +/-3] and [29.4, +/-1.1] in trial 2). We maintained the five remaining L1 larvae (they molt to L2 after 10 days of incubation), and one was positive in each of the trials (Ct = 21.6 in trial 1 and Ct = 31.6 in trial 2).

Table 3. Molecular, culture, and immunohistologic methods for detection and isolation of *B. quintana* in blood meals, bed bugs, and their feces.

Trials	Group of bed bugs (n)	Sampling (Quantity)	Day 3 Post-Infection (P.I.)				Day 21 P.I.
			qPCR		Culture	Immunohisto -chemistry	qPCR (*fabF3*)
			Ct/Bacteria(≈)	No. positive (%)			
Trial 1	**G 1(30)**	Blood meal‡ (2ml)	11.4≈6 x 10^8CFU		+		
		Bed bugs (5)	19.0	5 (100%)	+(¥)	+(*)	-
		Feces (≈30mg)	15.4		+(¥)		-
	G 2 (30)	Blood meal‡ (2ml)	11.4≈6 x 10^8CFU		+		
		Bed bugs (5)	24.6	5 (100%)	ND	+(*)	-
		Feces (≈30mg)	18.2		ND		-
	G 3	Blood meal‡ (2ml)	-	-	-		
	Control	Bed bugs (5)	-	0 (0%)	-	-	-
	(30)	Feces (≈30mg)	-	-	-		-
	G 4	Blood meal‡ (2ml)	-	-	-		
	Control	Bed bugs (5)	-	0 (0%)	ND	-	-
	(80)	Feces (≈30mg)	-	-	ND		-
Trial 2	**G 1'**	Blood meal‡ (2ml)	22.9≈8 x 10^5 CFU		+		
	(30)	Bed bugs (5)	23.6	5 (100%)	+(¥)	+(*)	-
		Feces (≈50mg)	27.3		+(¥)		-
	G 2' (30)	Blood meal‡ (2ml)	22.9≈8 x 10^5 CFU		+		
		Bed bugs (5)	25.0	5 (100%)	+(¥)	+(*)	-
		Feces (≈50mg)	20.6		+(¥)		-
	G3'	Blood meal‡ (2ml)	-		-		
	Control	Bed bugs (5)	0 (0%)	0 (0%)	-	-	-
	(30)	Feces (≈50mg)	-		-		-
	G 4'	Blood meal‡ (2ml)	-		-		
	Control	Bed bugs (5)	0 (0%)	0 (0%)	-	-	-
	(30)	Feces (≈50mg)	-		-		-

Group 1: 30 infected bed bugs adults; **Group 2:** 30 infected bed bugs instars L1; **Group Control 1:** 30 uninfected bed bugs adults; **Control 2:** 30 uninfected bed bugs instars L1

No: number of bed bugs; **(+)** positive **; (-)** negative

ND: Not Done (because we haven't enough feces to be cultured)**; (**≈**)**: approximately

qPCR: Quantitative real-time polymerase chain reaction

(‡) Infected or uninfected blood

(*) observed in gut

(¥) Confirmation by qPCR

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Persistence of *B. quintana* elimination in the feces of bed bugs. Using qPCR, we determined the presence of the bacterium in feces of adult bed bugs. The results reported in Fig 3 demonstrate that the average number of *B. quintana* bacteria in the bed bugs decreased in trial 1 up to the 19th dpi and up to the 17th dpi in the second trial. In addition, we noted a decreasing Ct value on the 13th dpi and 15th dpi compared to the 11th dpi, which indicates bacterial multiplication inside the body of the bed bugs resulting in elimination at a high concentration.

Discussion

Here, we report two experimental trials to investigate potential acquisition and transmission of *B. quintana* (the agent of trench fever and other diseases) by bed bugs (*C. lectularius*). The

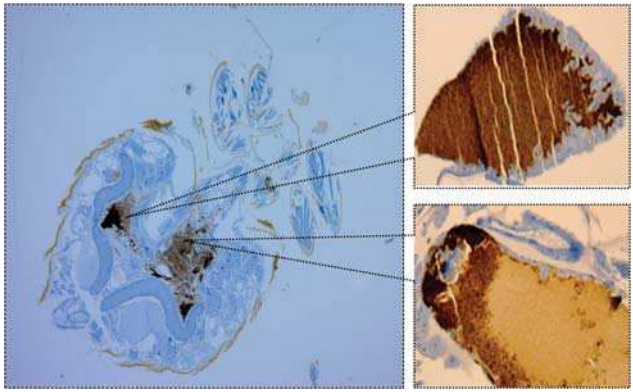

Fig 1. Immunohistochemistry localization of *B. quintana* inside the digestive tract of infected bed bugs.

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results show that bed bugs (adults and larva) exposed to *B. quintana* can acquire the bacterium and eliminate it in feces. The bed bugs maintain and shed stercorarially *B. quintana* for up to 17^{th} or 19^{th} dpi depending on the inoculum concentration. However, *B. quintana* was detected viable in feces and was shown to be alive inside the body of the bed bugs at the 3^{rd} dpi. Using immunohistochemistry, the bacterium was localized in the midgut and hindgut of the bed bugs digestive tract. Surprisingly, *B. quintana* was detected in eggs, L1 and L2 larvae.

In this study, we used three validated approaches. First, qPCRs was perfomed to study the acquisition and elimination of the bacterium by *C. lectularius*. This technique is reliable because we used a set of two qPCR systems targeting *yopP* and *fabF3*, which are known to be specific for *B. quintana* DNA detection, and we used negative and positive controls. Second, we cultivated the bacteria from the samples to determine if the eliminated bacteria were viable. This approach was also a validated technique [17] containing a negative and positive control. The third method was immunohistochemistry, which was used to localize the bacterium inside

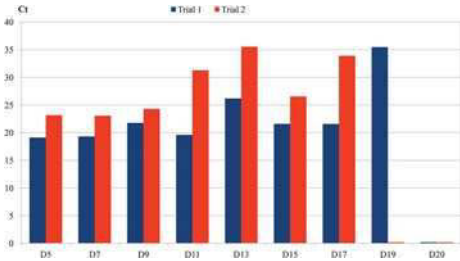

Fig 2. Persistence of *Bartonella quintana* in the bodies of bed bugs.

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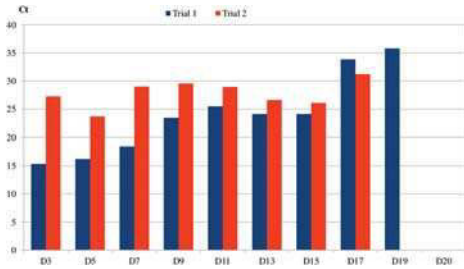

Fig 3. Persistence of *Bartonella quintana* in the feces of bed bug.

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the body of the bed bugs. The immunohistochemistry experiments were conducted in a blinded fashion by one of us (HLi), and the results were concordant with the qPCRs results.

Cimex lectularius and *C. hemipterus* (Cimicidae: Hemipetra), commonly called bed bugs, continue to increase in scope [23,24]. In recent years, these hematophagous arthropods have undergone a major resurgence in frequency and in geographic distribution leading to clinical problems. An increasing number of infestations have been reported in Europe [25,26] [23,27] America [28], Australia [23], Asia [29,30] [31,32] and Africa [18,33].

A bite causing cutaneous lesions is the most common clinical consequence of bed bugs on public health. In addition, mental health can be affected by knowledge of a bed bug infestation in one's living environment [23]. Bed bugs are suspected of transmitting infectious agents, however there is little evidence that such transmission has ever occurred. More than 45 pathogens associated with human infection and disease have been suspected to be transmitted by bed bugs [34]. Older scientific literature cited by Goddard and de Shaso [35] suggested that bed bugs may be vectors of yellow fever, tuberculosis, relapsing fever, leprosy, filariasis [36], *kala azar* (leishmaniasis), smallpox and HIV [37,38]. *Yersinia pestis* has also noted to develop inside the body of bed bugs, *C. lectularius* [39,40]. Verjbitzki [40] found with animal model infection of bed bugs with high virulence strain of *Y. pestis* can induce death of the guinea-pigs. They found also that three bed bugs are able to convey infection [39,40]. Jordansky and Klodnitsky [41] found that the number of *Y. pestis* bacilli in the bed bug's stomach increased from the third to the sixth day after the infected meal [39,41]. Throughout these animal models, it may be appear that bed bugs can play an important role to convey infection of plague and perhaps other pathogens. Hepatitis B virus has also been postulated as likely candidate for possible transmission by bed bugs [42–45]. Blow et al. 2001 [45], offered evidence for stercorarial transmission of Hepatitis B viral agents from bed bugs in a time series and with transtadial transmission. Recently Salazar et al [46] assessed the vector competence of *C. lectularius* against *Trypanosoma cruzi* and it has been confirmed that *T. cruzi* was viable in bed bug feces. Goddard et al [47] have experimentally infected bed bugs with *Rickettsia parkeri* and found using immunofluorescence that the bacterium was present in the salivary gland at 15 days post infection [47]. Moreover, our laboratory recently detected *B. quintana* DNA in *C. hemipterus* collected from two prisons in Rwanda [18]. The only confirmed and known vector of *B. quintana* is body lice (spread through feces). However, several studies suggested that hematophagous arthropods, such as flies, lice, fleas, or ticks can acquire or transmit *Bartonella* spp. [14]. Few studies have described the kinetics of elimination and the details of transmission of these bacteria.

The results of our experiments are in agreement with many experimental infection models, such as the experimental infection of fleas with *B. quintana* [48], where they found that *B. quintana* was detected in the beginning of the 3rd dpi, in fleas, as in our bed bug experimental model,. We also found that *B. quintana* was viable in feces and decreased gradually after the 3rd dpi, which was similarly observed using the experimental cat flea *B. quintana* infection model [17].

Concerning the detection of *B. quintana* in eggs, L1 and L2 larvae, the vertical transmission of *Bartonella* species was suggested to occur, but the transmission routes were unknown [49]. Using IHC, in the four specimens we localized the bacterium to the digestive tract but not in the ovary. The presence of *B. quintana* in eggs, L1 and L2 larvae may be, due to vertical non-transovarial or horizontal transmission. In our context, the transmission may have occurred by external contact of the eggs, L1 and L2 larvae with the viable *B. quintana* released in adult's feces which could be strongly considered as horizontal transmission. However Morick et al, demonstrate that Bartonella-positive flea feces and gut voids are proper infection sources for flea larvae and indicate that is considered as vertical non transovarial transmission [49].

In conclusion, we showed that the bed bug *C. lectularius* can acquire *B. quintana* by feeding and release viable organisms into their feces. Therefore, bed bugs may play a role as vectors of trench fever or other diseases caused by *B. quintana*. Knowing that stringent criteria exist in biomedical research for indicting the roles of living agents as biologically significant reservoirs and/or vectors of pathogens [50], more studies are required to better understand *B. quintana* persistence in both bed bugs and their feces and to understand the potential vector role of bed bugs in *B. quintana* other bacterial infections.

Author Contributions

Conceived and designed the experiments: DR PP. Performed the experiments: HLeu IB JMB HLep PP. Analyzed the data: DR PP IB LA JMR HLeu. Contributed reagents/materials/analysis tools: DR HLep. Wrote the paper: HLeu IB JMB LA PP.

References

1. Rolain JM, Foucault C, Guieu R, La SB, Brouqui P, Raoult D (2002) *Bartonella quintana* in human erythrocytes. Lancet 360: 226–228. S014067360209462X [pii]. PMID: 12133660
2. Drancourt M, Tran-Hung L, Courtin J, Lumley H, Raoult D (2005) *Bartonella quintana* in a 4000-year-old human tooth. J Infect Dis 191: 607–611. JID33309 [pii]. PMID: 15655785
3. Raoult D, Dutour O, Houhamdi L, Jankauskas R, Fournier PE, Ardagna Y, Drancourt M, Signoli M, La VD, Macia Y, Aboudharam G (2006) Evidence for louse-transmitted diseases in soldiers of Napoleon's Grand Army in Vilnius. J Infect Dis 193: 112–120. JID34959 [pii]. PMID: 16323139
4. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS (1990) The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. N Engl J Med 323: 1573–1580. PMID: 2233945
5. Drancourt M, Mainardi JL, Brouqui P, Vandenesch F, Carta A, Lehnert F, Etienne J, Goldstein F, Acar J, Raoult D (1995) *Bartonella (Rochalimaea) quintana* endocarditis in three homeless men. N Engl J Med 332: 419–423. PMID: 7529894
6. Spach DH, Kanter AS, Daniels NA, Nowowiejski DJ, Larson AM, Schmidt RA, Swaminathan B, Brenner DJ (1995) *Bartonella (Rochalimaea)* species as a cause of apparent "culture-negative" endocarditis. Clin Infect Dis 20: 1044–1047. PMID: 7795048
7. Spach DH, Kanter AS, Dougherty MJ, Larson AM, Coyle MB, Brenner DJ, Swaminathan B, Matar GM, Welch DF, Root RK, Stamm WE (1995) *Bartonella (Rochalimaea) quintana* bacteremia in inner-city patients with chronic alcoholism. N Engl J Med 332: 424–428. PMID: 7529895
8. Brouqui P, La Scola B, Roux V, Raoult D (1999) Chronic *Bartonella quintana* bacteremia in homeless patients. New Engl J Med 340: 184–189. PMID: 9895398
9. Raoult D, Drancourt M, Carta A, Gastaut JA (1994) *Bartonella (Rochalimaea) quintana* isolation in patient with chronic adenopathy, lymphopenia, and a cat. Lancet 343: 977. PMID: 7909030

10. Angelakis E, Raoult D (2014) Pathogenicity and treatment of Bartonella infections. Int J Antimicrob Agents 44: 16–25. S0924-8579(14)00118-6 [pii]. doi: 10.1016/j.ijantimicag.2014.04.006 PMID: 24933445

11. Chomel BB, Kasten RW, Sykes JE, Boulouis HJ, Breitschwerdt EB (2003) Clinical impact of persistent Bartonella bacteremia in humans and animals. Ann N Y Acad Sci 990: 267–278. PMID: 12860639

12. Bonilla DL, Kabeya H, Henn J, Kramer VL, Kosoy MY (2009) *Bartonella quintana* in body lice and head lice from homeless persons, San Francisco, California, USA. Emerg Infect Dis 15: 912–915. doi: 10.3201/eid1506.090054 PMID: 19523290

13. Angelakis E, Rolain JM, Raoult D, Brouqui P (2011) *Bartonella quintana* in head louse nits. FEMS Immunol Med Microbiol 62: 244–246. doi: 10.1111/j.1574-695X.2011.00804.x PMID: 21477003

14. Tsai YL, Chang CC, Chuang ST, Chomel BB (2011) *Bartonella species* and their ectoparasites: selective host adaptation or strain selection between the vector and the mammalian host? Comp Immunol Microbiol Infect Dis 34: 299–314. S0147-9571(11)00040-3 [pii]. doi: 10.1016/j.cimid.2011.04.005 PMID: 21616536

15. Melter O, Arvand M, Votypka J, Hulinska D (2012) *Bartonella quintana* transmission from mite to family with high socioeconomic status. Emerg Infect Dis 18: 163–165. doi: 10.3201/eid1801.110186 PMID: 22257438

16. Rolain JM, Franc M, Davoust B, Raoult D (2003) Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. Emerg Infect Dis 9: 338–342. PMID: 12643829

17. Kernif T, Leulmi H, Socolovschi C, Berenger JM, Lepidi H, Bitam I, Rolain JM, Raoult D, Parola P (2014) Acquisition and excretion of *Bartonella quintana* by the cat flea, *Ctenocephalides felis felis*. Mol Ecol 23: 1204–1212. doi: 10.1111/mec.12663 PMID: 24400877

18. Angelakis E, Socolovschi C, Raoult D (2013) *Bartonella quintana* in *Cimex hemipterus*, Rwanda. Am J Trop Med Hyg 89: 986–987. ajtmh.13-0182 [pii]. doi: 10.4269/ajtmh.13-0182 PMID: 24019440

19. Rolain JM, Arnoux D, Parzy D, Sampol J, Raoult D (2003) Experimental infection of human erythrocytes from alcoholic patients with *Bartonella quintana*. Ann N Y Acad Sci 990: 605–611. PMID: 12860697

20. Sheele JM, Anderson JF, Tran TD, Teng YA, Byers PA, Ravi BS, Sonenshine DE (2013) Ivermectin causes *Cimex lectularius* (bedbug) morbidity and mortality. J Emerg Med 45: 433–440. S0736-4679(13)00510-6 [pii]. doi: 10.1016/j.jemermed.2013.05.014 PMID: 23871326

21. La SB, Fournier PE, Brouqui P, Raoult D (2001) Detection and culture of *Bartonella quintana*, *Serratia marcescens*, and *Acinetobacter* spp. from decontaminated human body lice. J Clin Microbiol 39: 1707–1709. PMID: 11325978

22. Lepidi H, Fournier PE, Raoult D (2000) Quantitative analysis of valvular lesions during Bartonella endocarditis. Am J Clin Pathol 114: 880–889. PMID: 11338477

23. Doggett SL, Dwyer DE, Penas PF, Russell RC (2012) Bed bugs: clinical relevance and control options. Clin Microbiol Rev 25: 164–192. 25/1/164 [pii]. doi: 10.1128/CMR.05015-11 PMID: 22232375

24. Eddy C, Jones SC (2011) Bed bugs, public health, and social justice: Part 1, A call to action. J Environ Health 73: 8–14. PMID: 21667718

25. Levy BA, Berenger JM, Del GP, Delaunay P, Pages F, Morand JJ (2011) Resurgence of bedbugs in southern France: a local problem or the tip of the iceberg? J Eur Acad Dermatol Venereol 25: 599–602. JDV3804 [pii]. doi: 10.1111/j.1468-3083.2010.03804.x PMID: 20704629

26. Masetti M, Bruschi F (2007) Bedbug infestations recorded in Central Italy. Parasitol Int 56: 81–83. S1383-5769(06)00129-2 [pii]. PMID: 17258934

27. Paul J, Bates J (2000) Is infestation with the common bedbug increasing? BMJ 320: 1141. PMID: 10836813

28. Hwang SW, Svoboda TJ, De Jong IJ, Kabasele KJ, Gogosis E (2005) Bed bug infestations in an urban environment. Emerg Infect Dis 11: 533–538. PMID: 15829190

29. How YF, Lee CY (2010) Survey of bed bugs in infested premises in Malaysia and Singapore. J Vector Ecol 35: 89–94. JVEC33 [pii], doi: 10.1111/j.1948-7134.2010.00033.x PMID: 20618653

30. Lee IY, Ree HI, An SJ, Linton JA, Yong TS (2008) Reemergence of the bedbug *Cimex lectularius* in Seoul, Korea. Korean J Parasitol 46: 269–271. doi: 10.3347/kjp.2008.46.4.269 PMID: 19127335

31. Suwannayod S, Chanbang Y, Buranapanichpan S (2010) The life cycle and effectiveness of insecticides against the bed bugs of Thailand. Southeast Asian J Trop Med Public Health 41: 548–554. PMID: 20578541

32. Tawatsin A, Thavara U, Chompoosri J, Phusup Y, Jonjang N, Khumsawads C, Bhakdeenuan P, Sawanpanyalert P, Asavadachanukorn P, Mulla MS, Siriyasatien P, Debboun M (2011) Insecticide

resistance in bedbugs in Thailand and laboratory evaluation of insecticides for the control of *Cimex hemipterus* and *Cimex lectularius* (Hemiptera: Cimicidae). J Med Entomol 48: 1023–1030. PMID: 21936321

33. Omudu EA, Kuse CN (2010) Bedbug infestation and its control practices in Gbajimba: a rural settlement in Benue state, Nigeria. J Vector Borne Dis 47: 222–227. PMID: 21178215
34. Delaunay P, Blanc V, Del GP, Levy-Bencheton A, Chosidow O, Marty P, Brouqui P (2011) Bedbugs and infectious diseases. Clin Infect Dis 52: 200–210. ciq102 [pii]. doi: 10.1093/cid/ciq102 PMID: 21288844
35. Goddard J, deShazo R (2009) Bed bugs (*Cimex lectularius)* and clinical consequences of their bites. JAMA 301: 1358–1366. 301/13/1358 [pii]. doi: 10.1001/jama.2009.405 PMID: 19336711
36. Nelson GS (1963) Are bed bugs vectors of filariasis? Trans R Soc Trop Med Hyg 57: 149–150. PMID: 13938040
37. Jupp PG, Lyons SF (1987) Experimental assessment of bedbugs (*Cimex lectularius* and *Cimex hemipterus*) and mosquitoes (*Aedes aegypti formosus*) as vectors of human immunodeficiency virus. AIDS 1: 171–174. PMID: 2450552
38. Webb PA, Happ CM, Maupin GO, Johnson BJ, Ou CY, Monath TP (1989) Potential for insect transmission of HIV: experimental exposure of *Cimex hemipterus* and *Toxorhynchites amboinensis* to human immunodeficiency virus. J Infect Dis 160: 970–977. PMID: 2479697
39. Bacot AW (1915) LXXXII Notes on the development of *Bacillus pestis* in bugs (*Cimex lectularius*) and their power to convey infection. J Hyg (Lond) 14: 777–792. PMID: 20474605
40. Verjbitzki DT (1906) The part played by insects in the epidemiology of plague. Journal de physiologie et pathologie générale 8: 162–208. doi: 10.1155/2014/392390 PMID: 25180180
41. Jordansky V, Klodnitzky N (1908) Conservation du bacille pesteux dans le corps des punaises. Annales de l'Institut Pasteur 22:, 455–462.
42. Jupp PG, Prozesky OW, McElligott SE, Van Wyk LA (1978) Infection of the common bedbug (*Cimex lectularius L*) with hepatitis B virus in South Africa. S Afr Med J 53: 598–600. PMID: 675426
43. Ogston CW, Wittenstein FS, London WT, Millman I (1979) Persistence of hepatitis B surface antigen in the bedbug *Cimex hemipterus* (Fabr.). J Infect Dis 140: 411–414. PMID: 501152
44. Jupp PG, McElligott SE (1979) Transmission experiments with hepatitis B surface antigen and the common bedbug (*Cimex lectularius L*). S Afr Med J 56: 54–57. PMID: 573506
45. Blow JA, Turell MJ, Silverman AL, Walker ED (2001) Stercorarial shedding and transtadial transmission of hepatitis B virus by common bed bugs (Hemiptera: Cimicidae). J Med Entomol 38: 694–700. PMID: 11580042
46. Salazar R, Castillo-Neyra R, Tustin AW, Borrini-Mayori K, Naquira C, Levy MZ (2014) Bed Bugs (*Cimex lectularius*) as Vectors of *Trypanosoma cruzi*. Am J Trop Med Hyg. ajtmh.14-0483 [pii].
47. Goddard J, Varela-Stokes A, Smith W, Edwards KT (2012) Artificial infection of the bed bug with *Rickettsia parkeri*. J Med Entomol 49: 922–926. PMID: 22897053
48. Bouhsira E, Ferrandez Y, Liu M, Franc M, Boulouis HJ, Biville F (2013) *Ctenocephalides felis* an in vitro potential vector for five *Bartonella species*. Comp Immunol Microbiol Infect Dis 36: 105–111. S0147-9571(12)00118-X [pii]. doi: 10.1016/j.cimid.2012.10.004 PMID: 23200028
49. Morick D, Krasnov BR, Khokhlova IS, Gutierrez R, Gottlieb Y, Harrus S (2013) Vertical nontransovarial transmission of Bartonella in fleas. Mol Ecol 22: 4747–4752. doi: 10.1111/mec.12408 PMID: 23875817
50. Merritt RW, Walker ED, Small PL, Wallace JR, Johnson PD, Benbow ME, Boakye DA (2010) Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 4: e911. doi: 10.1371/journal.pntd.0000911 PMID: 21179505

IV- APPLICATION DU MALDI-TOF POUR L'IDENTIFICATION DES ARTHROPODES ET DES BACTERIES ASSOCIÉES.

L'identification morphologique est la méthode la plus utilisée pour identifier les arthropodes [47]. Cette technique utilise des logiciels et clés d'identification qui se reposent sur des éléments de diagnose [48-50]. On rencontre plusieurs difficultés avec cette technique à identifier des stades immatures ou des spécimens gorgés car les éléments qui permettent l'identification sont peu visible voir absent et le même problème se pose avec les spécimens altéré [51,52]. Autres problèmes peuvent se présenter avec l'identification morphologique est que cette technique nécessitent des spécialistes et qui sont de plus en plus rare. Autres techniques et méthodes d'identifications sont aussi disponible à savoir l'identification moléculaire [53], qui elle aussi malgré ses avantages, elle représente plusieurs défauts à savoir le coût élevé du consommable, le choix des amorces et marqueurs ainsi que les difficultés d'identification des nouvelles espèces. D'autres techniques et méthodes aussi sont disponible et peuvent s'avérer efficace mais représentent aussi quelques limites. Récemment l'émergence du Matrix-Assisted Laser Desorption / Ionization Time Of Flight (MALDI-TOF) qui été utilisé en routine pour l'identification des micro-organismes tel que les bactéries [54,55], virus et levures [55-57]. Récemment cette technique a commencé d'être appliquée à l'entomologie,

notamment pour l'identification des tiques [58], moustiques [59] et autres arthropodes.

Dans un premier travail (***Article N° 8***) nous avons contribué à évaluer l'approche MALDI-TOF pour l'identification de 5 espèces de puces morphologiquement identifiées par des experts. Nous avons essayé d'appliquer cette technique sur des spécimens frais mais aussi sur d'autres conservés dans l'éthanol à différentes périodes. L'utilisation des extraits des protéines de pattes (comme avec les tiques et moustiques) pour l'analyse MALDI TOF ne permettait pas d'avoir des spectres reproductibles et spécifique. Ainsi nous avons évalué les protéines extraites des spécimens privés de leur abdomen. Les résultats obtenus ont montré que le corps sans abdomen était approprié pour l'analyse MALDI-TOF des puces. Nous avons créé la base des données MALDI- TOF à partir des 5 espèces de puces fraiches, mais également nous avons testé les spécimens conservés à l'éthanol. Nous avons constaté que les puces fraiches donnaient des spectres de meilleur qualité et bonne intensité par rapport aux puces conservées dans l'éthanol. Les tests en aveugles de la base des données ont montré des correctes identifications avec des bonnes valeurs et scores d'identification.

Par la suite de ce travail nous nous sommes intéressés aux identifications des statuts infectieux des punaises de lit, notamment les *Cimex lectularius* expérimentalement infectées par *Bartonella quintana*. Des travaux réalisés dans notre équipe ont permis d'identifier par MALDI-TOF les micro-organismes (*Rickettsia slovaca* et *R. conorii*) associés aux tiques (*Dermacentor marginatus et Rhipicephalus sanguineus*) [36]. Nous avons à notre possession des punaises de lit infectées expérimentalement du projet 2 (***Article N° 7***). Nous avons pris les pattes des punaises de lit, infectées et non infectées par *Bartonella quintana* afin de les tester par MALDI-TOF et voir si cet outil permet de distinguer les punaises de lit infectées parmi les non infectées. Les résultats préliminaires obtenus n'ont pas permis de discriminer les punaises infectées parmi les non infectées. Ce travail est en cours de réalisation où nous allons essayer prochainement de tester autres parties de la punaise autre que les pattes afin de trouver des profils protéiques discriminants et qui permettent d'identifier les punaises de lit infectées par *B. quintana* parmi les non infectées.

ARTICLE 8

Article N.8. Yssouf A, Socolovschi C, **Leulmi H**, Kernif T, Bitam I, Audoly G, Almeras L, Raoult D, Parola P. **Identification of flea species using MALDI-TOF/MS**. Comp Immunol Microbiol Infect Dis. 2014 May;37(3):153-7. doi: 10.1016/j.cimid.2014.05.002. Epub 2014 May 17.

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Identification of flea species using MALDI-TOF/MS

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ABSTRACT

In the present study, a molecular proteomics (MALDI-TOF/MS) approach was used as a tool for identifying flea vectors. We measured the MS spectra from 38 flea specimens of 5 species including *Ctenocephalides felis*, *Ctenocephalides canis*, *Archaeopsylla erinacei*, *Xenopsylla cheopis* and *Stenoponia tripectinata*. A blind test performed with 24 specimens from species included in a library spectral database confirmed that MALDI-TOF/MS is an effective tool for discriminating flea species. Although fresh and 70% ethanol-conserved samples subjected to MALDI-TOF/MS in blind tests were correctly classified, only MS spectra of quality from fresh specimens were sufficient for accurate and significant identification. A cluster analysis highlighted that the MALDI Biotyper can be used for studying the phylogeny of fleas.

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an ionization technique allowing the analysis of complex molecules, such as proteins, by generating protein fingerprint signatures (spectra) from protein extracts of organisms [1]. The acquisition of spectra allows the creation of a reference spectral database that can then be used to identify species. We recently reported the use of the MALDI-TOF/MS approach as an identification tool for tick and mosquito vectors using protein extracts from the legs of ticks and mosquitoes. A database was established containing reference spectra of ticks from the South of France [2] and mosquitoes from tropical Africa [3] and Europe [4]. The accurate and robust identification obtained by blind tests allowed us to use this database in the routine identification of ticks removed from patients for entomological diagnostic purposes in our laboratory [2].

Fleas are small (2–10 mm), obligate hematophagous insects of major significance as vectors of pathogens in animals and humans. The most severe infection spread by fleas is plague, which is caused by *Yersinia pestis*. Fleas are known worldwide as vectors of *Rickettsia*, predominantly *Rickettsia typhi*, which causes murine typhus. Fleas also transmit rural epidemic typhus (*Rickettsia prowazekii*) in the USA and have recently been identified as vectors of flea-borne spotted fever (*Rickettsia felis*) [5]. Additionally,

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fleas have been reported as a *Bartonella* vector; among the 23 *Bartonella* species associated with fleas, 11 have been implicated in human diseases [5].

From a medical perspective, flea identification at the species level is also essential. Although the morphological identification of fleas is currently used, it requires extensive knowledge of flea morphology and the availability of reference information (identification keys) [6]. In addition, as a slide mount of specimens prior to stereoscopic examination is necessary for flea identification, this method is therefore time-consuming and not suitable for large-scale flea population identification. Molecular approaches, such as sequencing of the 18S gene, have been developed to identify arthropods including fleas [7], but a PCR assay to distinguish flea species or ideal PCR primer pairs to amplify the relevant gene fragments are still unavailable [2].

Thus, the aim of the present study was to investigate for the first time the applicability of MALDI-TOF/MS for the rapid identification of flea species stored under different conditions.

2. Materials and methods

The study was conducted with laboratory-reared adult flea specimens including 16 fresh (2 larva and 14 adults) and 5 adult specimens preserved in 70% ethanol of *Ctenocephalides felis* from URMITE laboratory colonies in Marseille. Other specimens originating from Pasteur Institute laboratory colonies in Algeria, including 11 *Archaeopsylla erinacei* (9 fresh and 2 specimens stored in 70% ethanol) and 14 *Xenopsylla cheopis* (10 fresh and 4 stored in 70% ethanol), were used. Additional flea specimens from 2 other species, including 2 fresh and 8 specimens stored in 70% of *Ctenocephalides canis* and 6 specimens of *Stenoponia tripectinata* stored in 70% ethanol collected in the field between 2010 and 2014, were also included. These field specimens removed from dogs or rodents and collected in Algeria were preserved in 70% ethanol prior to identification using morphological identification keys [6] under the supervision of a medical entomologist. Both genders were not available for all the flea species, and the majority of the specimens tested in this work were female.

For the sample preparation for the MALDI-TOF/MS analysis, the fresh specimens were rinsed with 70% ethanol, followed by distilled water. Three different protocols of sample preparation were tested. The legs, heads only or body of fleas without an abdomen were homogenized in 30 μl of 70% formic acid and 30 μl of 50% acetonitrile using an automatic homogenizer (FastPrep®24, MP Biomedicals).

The specimens stored in ethanol were rinsed in 70% ethanol at concentrations decreasing from 70 to 10% for 10 min each step, followed by a distilled water rinse, as previously described [2]. The sets of legs, heads only or body of fleas without an abdomen were homogenized following the protocol described above. After centrifugation, 1 μl of supernatant and 1 μl of matrix was loaded onto a target plate into four spots for each sample and dried as previously described [2]. The mass spectrometer was calibrated using the Bruker Bacterial Test Standard in the mass range of 2–20 kDa.

The protein mass profiles were acquired using a Microflex LT MALDI-TOF/MS with Flex Control software (Bruker Daltonics) using parameters previously described [2]. The spectral profiles obtained from different body sections were analyzed and compared using the Flex Analysis 3.3 software. Only the reproducible spectra were loaded into the MALDI Biotyper 3.0 to create a spectral database with 2–5 specimens for each species.

The study was validated by a blind test using new fresh laboratory samples of the species and field specimens that had been preserved in ethanol. One to three adult flea specimens of each species and fresh immature specimens were examined against the database established by calculating the similarity of 2 specimens on the basis spectra in the MALDI Biotyper 3.0 software. Each specimen was coded before beginning the blind test with the MALDI Biotyper. To determine whether the different flea species could be clustered according to the protein mass profiles, an MSP dendrogram was performed including the four fresh flea species included in our database.

3. Results

A total of 62 flea specimens were subjected to the MALDI-TOF/MS analysis. An analysis of the spectral profiles using the Flex analysis software indicated that the spectra obtained from the body of fleas without an abdomen provided consistent and reproducible spectral profiles with peaks of high intensities in the range of 2–20 kDa (Fig. 1a). The spectral profiles from legs or heads dissected and subjected to MALDI-TOF MS separately showed lower signal intensities. Therefore, the spectra of the flea body without an abdomen were used to create the database and then assessed in a blind test.

The database contains five species of 38 adult specimens including 25 fresh specimens and 13 preserved in ethanol. In the blind test, 23 adult specimens were compared with the database for species identification. All fresh flea specimens were correctly identified, and the score values were higher than 1.9, corresponding to significant identification (Table 1). Conversely, for the flea specimens stored in ethanol, despite correct identifications, the score values were low, corresponding to unreliable identification (*i.e.*, from 1.027 to 1.651) (data not shown). Overall, for the flea specimens stored in ethanol, the MS spectrum reproducibility was variable according to the duration of ethanol storage. Thus, only fresh flea specimens were used to generate the database; four flea species were used, *i.e.*, *Ct. felis*, *Ct. canis*, *Ar. erinacei* and *Xe. cheopis*. Regarding the two larvae of *Ct. felis* subjected to MALDI-TOF MS, distinct protein profiles were obtained according to their age and developmental stage (Fig. 1b). The examination of the database with the corresponding spectra resulted in matches with the adult *Ct. felis* specimens, though the score values did not reach the significant threshold value (*i.e.*, 1.9), underlining that the protein profiles evolved with developmental stage.

To control the accuracy of flea identification by MALDI biotyping, one specimen of the three fresh species was tested blindly against our in-house arthropod database including 11 tick species and 31 mosquito species [2,3].

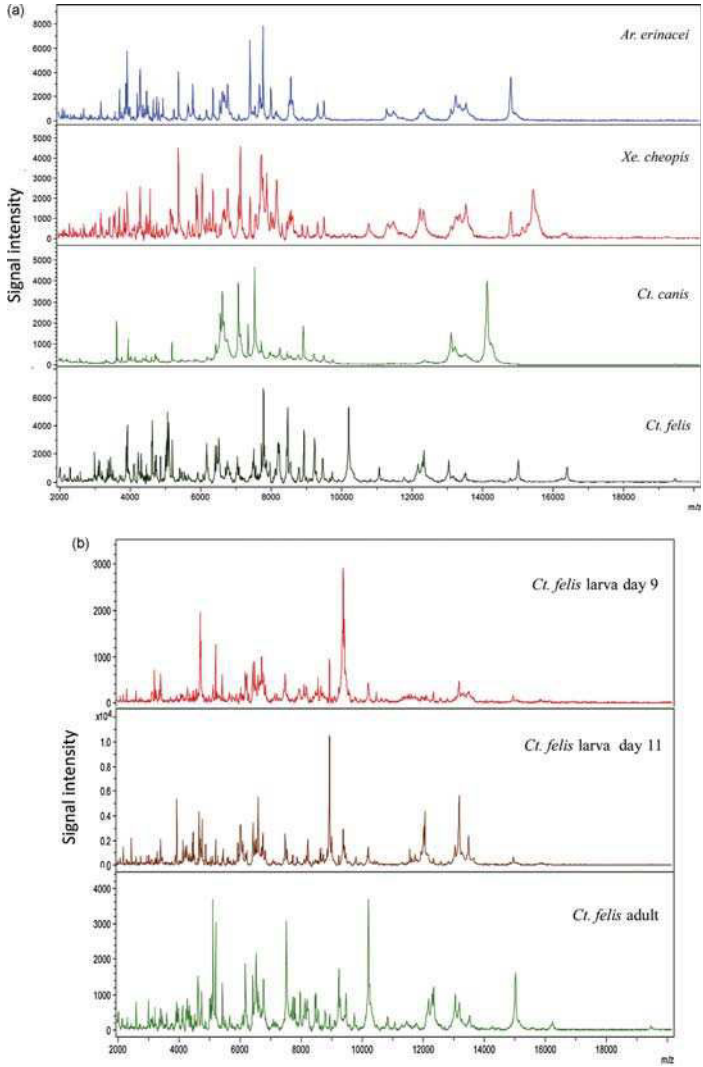

Fig. 1. MALDI-TOF MS spectral profiles from fresh flea specimens. (a) Comparison of the spectra profiles from the four fresh flea species tested. (b) Comparison of the spectral profiles from *Ctenocephalides felis* at different developmental stages.

The higher score value obtained was 0.869, highlighting a misidentification. No cross-reaction was observed at same genus level because no *Ct. felis* specimens tested matched the *Ct. canis* samples present in the database. The MSP dendrogram obtained from the spectra of two specimens of the four fresh flea species is presented in Fig. 2. The specimens were clustered according to genus and species.

4. Discussion

The fleas tested in the present work parasitize mainly domestic mammals such as cats, dogs and some rodent species. These arthropods are known as vectors of human diseases and are thus considered to be of public health importance worldwide [5].

Table 1
Fresh flea species used to evaluate the MALDI-TOF MS reference database and the score values for all the fresh samples identified by a blind test.

Flea species for database	Geographical origin/source (date of collect)	Storage condition	Number of fresh specimens used to create the database	Number of specimens used and correctly identified by the blind test procedure [low–high scores]
Ct. felis	England/LC, URMITE (2013)	Fresh	12 (6 M; 6 F)	2 F [2.422–2.616]
Ar. erinacei	Algeria/LC (2013)	Fresh	6 (2 M; 4 F)	3 F [2.02–2.27]
Xe. cheopis	Algeria/LC (2013)	Fresh	5 (3 M; 2 F)	5 F [2.035–2.881]
Ct. canis	Algeria/Field (2014)	Fresh	2	0
	Algeria/Field (2014)	Ethanol[a]	0	1 F [1.606][a]
Total			23	11

Abbreviation: LC: laboratory colony; M: male; F: Female.
[a] Due to the lack of fresh *Ct. canis* specimens for the blind test, a *Ct. canis* specimen stored in ethanol for less than three months was used.

Our results demonstrated that the use of the body of a flea without the abdomen was the best sample for distinguishing flea species by the MALDI-TOF/MS approach. These findings confirm those of previous arthropod evaluations performed on *Drosophila* [8], *Culicoides* (biting midges) [9,10], ticks [2,11], tsetse flies [12], mosquito species [3,13] and sand flies [14] using proteins extracted from either the entire body or body sections of individual specimens. In our case, the spectral profiles generated from the head or legs and subjected separately to MALDI-TOF MS were not reproducible. This lack of reproducibility could be attributed to a lack of protein abundance for obtaining intense MS spectra but also to the crushing and incomplete lysis of these body sections by the automatic homogenizer.

We observed differences at the spectral profile level between the fresh specimens and those stored in 70% ethanol of the same species, predominantly by the disappearance of a number of peaks in the 70% ethanol-stored specimens. These results are consistent with previous studies reporting that samples stored for a long duration in 70% ethanol revealed a lower average data count than specimens freshly prepared or stored for a short time in 70% ethanol [9,15].

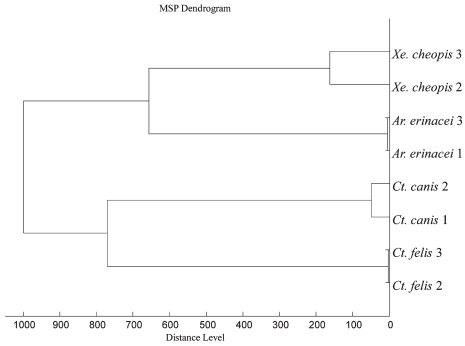

Fig. 2. Dendrogram obtained by a cluster analysis of the spectra obtained from the four flea species tested. Two fresh specimens per flea species were included in this analysis. The fleas were clustered using the MALDI Biotyper 3.0 software.

The main differences between *Ct. canis* stored in ethanol and the other flea specimens stored in ethanol was the duration. The *Ct. canis* specimens were preserved for less than three months prior to the mass spectrometry analysis, explaining the unambiguous identification even though the lower was score. In contrast, the other flea specimens were preserved in ethanol for at least two years and showed altered spectral quality and reproducibility. Indeed, long-term flea preservation in ethanol does not appear to be an adequate storage condition for further specimen identification by MALDI-TOF MS profiling. Nevertheless, for fresh flea specimens, no significant cross-recognition between flea species and the mosquito and tick species in our in-house database was observed, underlining the robustness of MALDI biotyping for accurate and rapid flea specimen identification. The MSP dendrogram function of the MALDI Biotyper 3.0 succeeded in clustering the flea species according to genus and species.

In conclusion, the present study demonstrates that MALDI-TOF/MS appears to be an efficient approach for the rapid identification of fleas. Further investigations are required to resolve definitively the problem of ethanol storage for long periods. Nevertheless, a preliminary database containing the spectra of 4 species was created, and the database will be regularly updated with new fresh flea specimens. Comparing morphological and molecular methods, MALDI-TOF/MS is relatively simple and economic after acquisition of the instrument because it does not require expensive chemicals. This approach is promising as a reference method for arthropod identification and could become a diagnostic entomological tool.

Raw data of the spectral files from the different fresh flea specimens are available on request.

References

[1] Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 2010;48(5):1549–54.
[2] Yssouf A, Flaudrops C, Drali R, Kernif T, Socolovschi C, Berenger JM, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. J Clin Microbiol 2013;51(2):522–8.
[3] Yssouf A, Socolovschi C, Flaudrops C, Ndiath MO, Sougoufara S, Dehecq JS, et al. Matrix-assisted laser desorption ionization-time of

flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors. PLOS ONE 2013;8(8):e72380.
[4] Yssouf A, Parola P, Lindström A, Lilja T, L'Ambert G, Bondesson U, et al. Identification of European mosquito species by MALDI-TOF MS. Parasitol Res 2014, http://dx.doi.org/10.1007/s00436-014-3876-y [Epub ahead of print].
[5] Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D. Fleas and flea-borne diseases. Int J Infect Dis 2010;14(8):e667–76.
[6] Beaucournu JC, Launay H. Les puces de France et du Bassin Mediterannéen. Fédération française des Societés des Sciences Naturelles. 76 ed. Faune de France; 1990.
[7] Michael FW. Phylogeny of the holometabolous insect orders based on 18S ribosomal DNA: when bad things happen to good data. EXS 2002;92:69–83.
[8] Feltens R, Gorner R, Kalkhof S, Groger-Arndt H, Von BM. Discrimination of different species from the genus *Drosophila* by intact protein profiling using matrix-assisted laser desorption ionization mass spectrometry. BMC Evol Biol 2010;10:95.
[9] Kaufmann C, Ziegler D, Schaffner F, Carpenter S, Pfluger V, Mathis A. Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for characterization of *Culicoides nubeculosus* biting midges. Med Vet Entomol 2011;25(1):32–8.
[10] Kaufmann C, Schaffner F, Ziegler D, Pfluger V, Mathis A. Identification of field-caught *Culicoides* biting midges using matrix-assisted laser desorption/ionization time of flight mass spectrometry. Parasitology 2012;139(2):248–58.
[11] Karger A, Kampen H, Bettin B, Dautel H, Ziller M, Hoffmann B, et al. Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry. Ticks Tick Borne Dis 2012;3(2):78–89.
[12] Antje H, Jayaseelan M, Burkhard B, Stephan S, Peter-Henning C, Uwe R. Identification of Tsetse (*Glossina* spp.) using matrix-assisted laser desorption/ionisation time of flight mass spectrometry. PLOS Negl Trop Dis 2013;7(7):e2305.
[13] Müller P, Pflüger V, Wittwer M, Ziegler D, Chandre F, Simard F, et al. Identification of cryptic *Anopheles* mosquito species by molecular protein profiling. PLOS ONE 2013;8(2):e57486.
[14] Dvorak V, Halada P, Hlavackova K, Dokianakis E, Antoniou M, Volf P. Identification of phlebotomine sand flies (Diptera: Psychodidae) by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Parasit Vectors 2014;7(1):21.
[15] Hubel A, Aksan A, Amy P, Chris W, Zhong X. State of the art in preservation of fluid biospecimens. Biopreserv Biobank 2011;9:210.

Identification of *Bartonella quintana* infected bed bugs using MALDI-TOF/MS.

Introduction

La détection récente de l'ADN de *Bartonella quintana* dans des *Cimex hemipterus* (des punaises de lit tropicales) recueillies à partir de deux prisons au Rwanda [28] ainsi que notre modèle expérimental d'infection des punaises de lit par ce pathogène (***Article N° 7***) ont permis de considérer les punaises de lit comme vecteurs potentiel de l'agent de la fièvre des tranchées, *B. quintana*. La détection des bactéries et spécifiquement *B. quintana* dans leurs vecteurs connus (le pou de corps) se réalise généralement par des méthodes moléculaires. La méthode moléculaire est une méthode spécifique et sensible mais qui exige des étapes longues de préparation des échantillons, en plus, elle est aussi couteuse en matière de consommable. Avec les recherches d'outils innovante, rapide et économique s'est émergé le MALDI-TOF.

Le but de la présente étude était de déterminer s'il est possible, d'identifier à la fois les espèces de punaises de lit et la présence d'un pathogène intracellulaire associé en un seul test. Nous avons souhaité mettre au point par l'outil MALDI-TOF la détection de

Bartonella quintana dans les punaises de lit infectées expérimentalement afin d'avoir un outil rapide pour identifier les punaises de lit mais et leur statut infectieux. Nous avons travaillé au début de ce travail sur l'analyse à partir des pattes.

MATÉRIELS ET MÉTHODES

Préparation des échantillons pour l'analyse MALDI-TOF MS

Nous avons infecté 20 punaises de lits (*C. lectularius*), via un repas sanguin infecté avec (8 x 10^5 CFU/ mL) de *B. quintana*. 20 autres punaises ont servi comme groupe control et nourries une fois par semaine avec du sang humain non infecté. Nous avons récupéré chaque 48 heures à partir du troisième jour post infection, 2 punaises de lit de chaque groupe (infectées et contrôles). Nous avons lavé chaque spécimen à l'alcool et deux fois à l'eau distillée puis nous avons disséqué sous hotte à flux laminaire, les pattes des punaises pour être analyser par MALDI-TOF. Le reste du corps ont fait l'objet d'une extraction d'ADN par EZ1 et recherche par qPCR spécifique de *B. quintana*, en ciblant deux gènes *Yop P* et *FabF3* afin de s'assurer de l'infection des punaises des lits par *B. quintana*.

Concernant l'analyse MALDI-TOF, nous avons suivi le protocole habituel d'analyse des arthropodes effectué dans notre laboratoire et consiste à extraire deux à six pattes de l'arthropode infectées et non infectées, qui seront homogénéisés manuellement puis dans 20 µL d'acide formique à 70% (Sigma, Lyon, France) et 20 µL de 100% d'acétonitrile (VWR Prolabo) en utilisant des pilons de pellets (Fischer Scientific). Tous les homogénats ont été centrifugés à 6700 xg pendant 20 s. 1 µL du surnageant a été déposé sur la cible (Bruker Daltonics) en quatre dépôts. Ensuite, 1 µL d'une suspension de matrice pour couvrir le 1 µL du surnageant a été déposé. La matrice est composée d'α-cyano-4-hydroxy-cinnamique (CHCA) saturé (Sigma), 50% d'acétonitrile, 10% d'acide trifluoroacétique (Sigma) et de l'eau ultra pure HPLC). A la suite du séchage de la matrice à température ambiante, la plaque cible a été immédiatement introduite dans la machine Microflex pour analyse MALDI-TOF MS.

Analyse des profils MS

Les profils de masse des protéines des pattes des punaises de lit infectées et/ou non ont été acquis à l'aide d'un spectromètre Microflex LT (Bruker Daltonics) avec le logiciel Flex Control (Bruker Daltonics). Les spectres ont été enregistrés de manière

linéaire, avec le mode d'ion positif avec une tension d'accélération de 20 kV, dans une plage de masse de 2,000-20,000 Da. Chaque spectre correspond à une accumulation de 240 tirs de laser à partir du même endroit dans six positions différentes. Pour contrôler la charge sur la cible, la qualité de la matrice et les performances du dispositif MALDI-TOF, la solution de matrice a été chargé en double sur chaque plaque MALDI-TO. Les profils de spectre obtenus ont été visualisés avec le logiciel Flex analysis v.3.3 et exportés vers la version v.2.2 de ClinPro Tools et MALDI-Biotyper v.3.0 (Bruker Daltonics, Allemagne).

Les comparaisons des spectres de masse des spécimens de punaises de lit infectés ou non par *Bartonella quintana*

Les spectres MALDI-TOF des extraits de protéines des pattes des punaises de lit infectées et ont été importés au ClinProTools v.2.2 (Bruker Daltonics, Allemagne) pour identifier les pics spécifiques liés à l'état de l'infection de la punaise de lit. Les paramètres d'analyse du logiciel Clin Pro Tools étaient similaires à ceux décrits précédemment [58]. Un spectre moyen a été généré pour chaque condition en utilisant l'algorithme du calcul de la moyenne de la liste de crête " average peak list calculation". Le modèle algorithme génétique (GA) du logiciel de ClinPro

Tools a ensuite été utilisé pour afficher automatiquement une liste des masses maximales discriminantes. Basé sur les masses maximales sélectionnées, les valeurs de reconnaissance des capacités (RC) et de la Croix de validation (CV) ont été déterminées [60].

RÉSULTATS ET PERSPECTIVES

Acquisition de B. quintana

Afin de s'assurer de l'infection des punaises des lit par *B. quintana* nous avons testé par qPCR les punaises exposées à *B. quintana*. Nous avons obtenu 100% d'acquisition de la bactérie et qu'elles la maintiennent jusqu'à j17 post-infection (Tableau 1) :

Tableau 1 : Total des punaises de lit testées par qPCR et MALDI-TOF

Nombre des punaises de lits testées par MALDI TOF/qPCR	**Jour de collecte (J post infection)**	**CT obtenus par qPCR**
2	**3ème**	**16**; **26**
2	**5ème**	**29**; **28**
2	**7ème**	**27** ; **29**
2	**9ème**	**29** ; **32**
2	**11ème**	**29; 33**
2	**13ème**	**24 ; 25**
2	**15ème**	**28 ; 35**

2	**17ème**	**34, Neg**
20	**Punaise contrôle**	**Neg, Neg**

Analyse par MALDI-TOF des pattes des punaises de lit infectées par *B. quintana*

Le test des pattes des punaises infectées par *B. quintana* (du tableau 1) par MALDI-TOF a permis d'identifier et avec des bons scores l'espèce de l'arthropode, comme étant des punaises de lit *Cimex lectularius* **(Figure 1).** Nous avons implémenté la base des données MALDI-TOF des arthropodes avec un spectre référence des punaises de lit infectées et on a testé en aveugle les punaises de lit infectées et non infectées (**Figure 2**), malheureusement on n'a pas pu discriminer les punaises infectées parmi les non infectées. Nous a avons confirmé par Clin Pro tools, **figure 3** montre une comparaison sur gel, les masses protéiques des punaises de lit non infectées (contrôle) et les punaises de lit infectées avec *B. quintana*, où nous n'avons pas remarqué des spectres ou masses spécifiques aux punaises infectées par rapport aux non infectées.

B. quintana a un tropisme digestif de en plus de sa localisation intracellulaire facultative, ce qui a peut-être empêché la détection de spectres discriminants. La technique MALDI-TOF/MS utilisée, détecte que des plages de masse de 2,000-20,000 Da.

D'autres variantes de MALDI-TOF/MS que nous envisageons de les faire, en l'occurrence MALDI-TOF/ MS-MS peuvent apporter des solutions et détecter peut être des masses spécifiques attribuable à l'infection par *B. quintana* ou une réponse protéique. Prochainement nous essayerons aussi de tester les autres parties du corps des punaises à savoir la moitié, tête et thorax et même l'abdomen s'il peut générer des spectres reproductibles et spécifique.

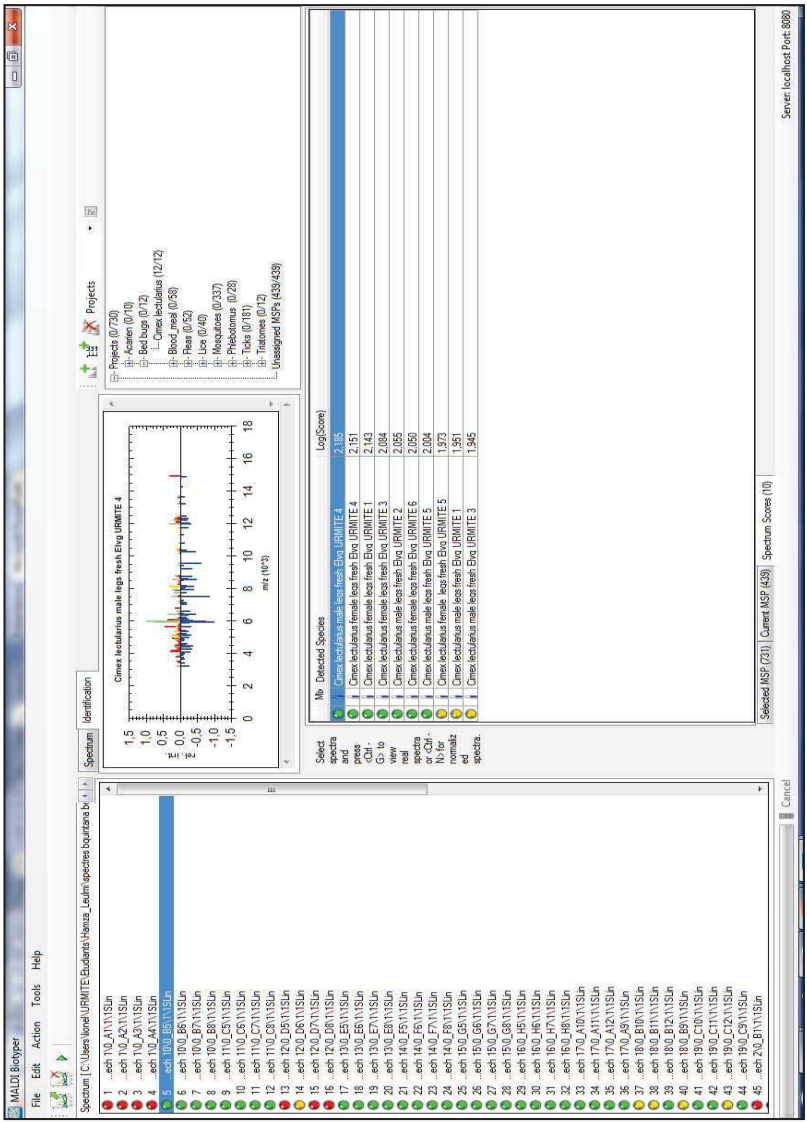

Figure 1: Identification par logiciel MALDI Biotyper les punaises de lit infectées et non infectées.

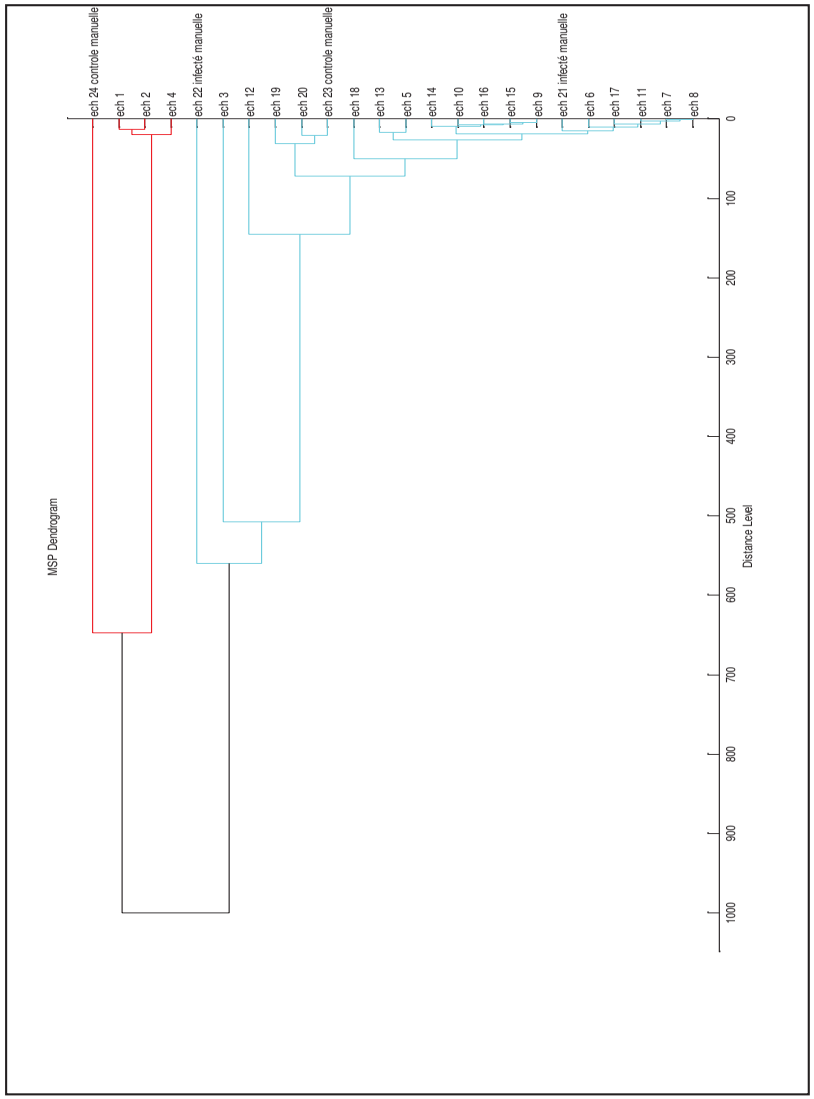

Figure 2: Dendrogramme des punaises de lit infectées et contrôle.

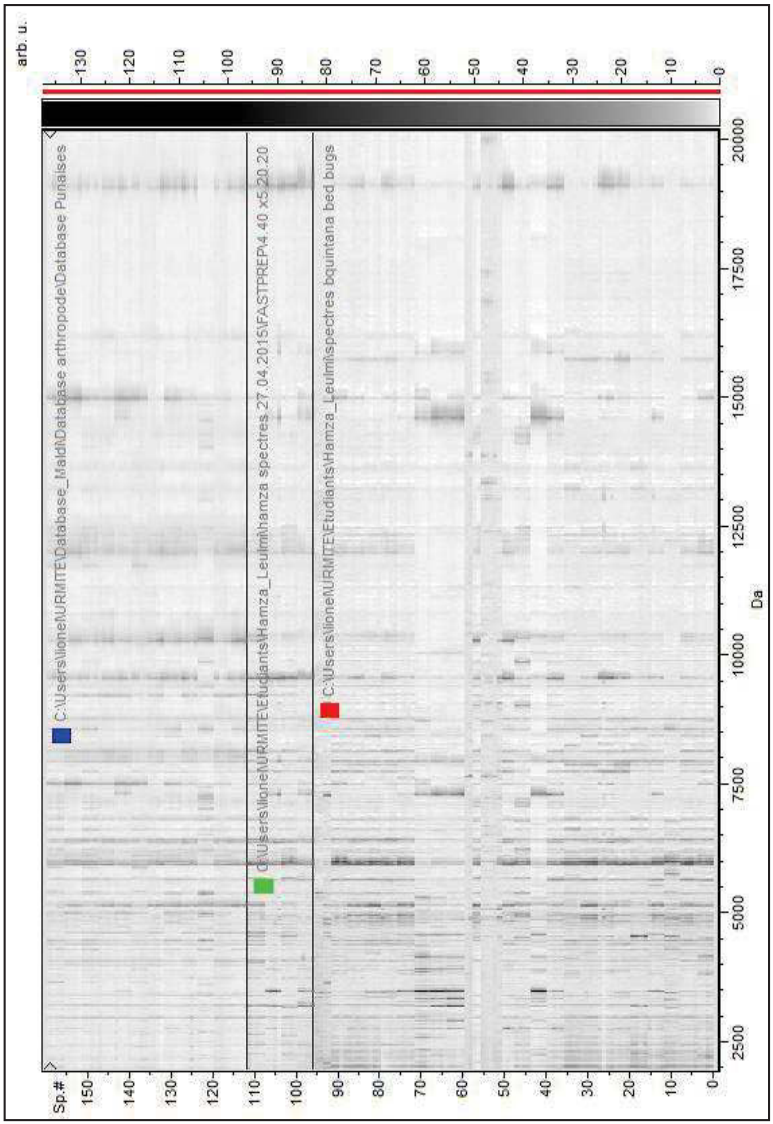

Figure 3: Comparaison sur gel des masses protéiques entre les punaises contrôles (de la base des données) et les punaises infectées par *B. quintana*.

IV- CONCLUSION GENERALE & PERSPECTIVES

La première partie de cette thèse a porté sur des investigations épidémiologiques notamment sur les puces de rongeurs en Afrique subsaharienne où, nous avons identifié pour la première fois l'agent du typhus murin *Rickettsia typhi* et *Bartonella* sp au Bénin, l'agent de la peste *Yersinia pestis*, *Rickettsia felis* et *Bartonella sp* au RD Congo ainsi que *Rickettsia typhi* et *Rickettsia felis* en Tanzanie. Par la suite de ce travail, nous nous sommes intéressés aux investigations en Algérie principalement sur les tiques, puces et tissus (rates) d'animaux domestiques et sauvages. Dans une première partie nous avons détecté pour la première fois la présence d'un agent de borréliose de lyme, *Borrelia garinii* en Algérie. Nous avons confirmé également la détection de *Rickettsia monacensis* dans ces tiques. Nous avons mené également une enquête sur les ectoparasites des mammifères et / ou la rate des animaux sauvages à El Tarf et Souk Ahras, Algérie. Nous avons détecté par qPCR*, Bartonella tamiae*, *Coxiella burnetii*, *R. slovaca, R. massiliae*, *R. aeschlimannii* dans des tiques. Enfin *R. felis* a été également détecté dans des *Archaeopsylla erinacei* et *Ctenocephalides felis* puces collectées sur des hérissons. Une dernière partie a intéressé la détection d'*Erhlichia canis, Coxiella burnetii* et *Bartonella henselae* sur les rates des chiens et chat, ainsi que sur les tiques

et puces de ces hôtes capturés au niveau de la fourrière canine d'Alger.

Les résultats obtenus ont contribué à élargir les connaissances sur le répertoire des bactéries associées aux puces et aux tiques présentes en Afrique. Dans notre étude, nous avons illustré également le rôle de ces arthropodes dans les enquêtes entomologiques sur les vecteurs de maladie, et ainsi permet aux cliniciens de confirmer les étiologies de fièvre inexpliquée chez les patients africains. Les résultats obtenus de cette partie permettront aussi de sensibiliser les autorités sanitaires et les médecins des pays concernés de la présence des maladies vectorielles afin de rechercher et traiter les patients exposés aux arthropodes. D'autres études sur les rickettsioses, bartonelloses et autres maladies vectorielles doivent se faire en Afrique pour évaluer leur prévalence et épidémiologie ainsi que leur pertinence clinique dans les zones tropicales et subtropicales, afin d'établir des programmes de lutte anti vectorielle.

Concernant le deuxième volet de cette thèse, nous avons étudié les modèles expérimentaux d'infection des puces et des punaises par l'agent de la fièvre des tranchées, *Bartonella quintana*, et cela pour évaluer leurs compétences vectorielles.

Bartonella quintana est une bactérie transmise naturellement par les fèces infectées des poux de corps. Récemment, cette bactérie a été détectée dans les puces de chat (*Ctenocephalides felis*). Suivant deux essais séparés nous avons montré que les puces acquièrent la bactérie et l'éliminent vivante dans leur fèces. Nous avons remarqué que les puces maintiennent la bactérie jusqu'au J11/J13 post infection. Un deuxième modèle expérimental d'infection des punaises de lit par *B. quintana* a été effectué suivant deux essais séparés. Récemment, cette bactérie a été détectée dans d'autres familles d'arthropodes y compris les punaises de lit, ce qui pose la question de leur implication dans cette transmission. Bien que plusieurs agents pathogènes infectieux ont été signalés et suggérer d'être transmis par les punaises de lit, mais aucune preuve concernant cette compétence vectorielle n'est éclaircie. De ce fait, des punaises de lit (adulte et larves) ont été exposées à *B. quintana* via un repas sanguin infecté. A J3 post infection, nous avons obtenu 100% d'acquisition de la bactérie. Le suivi de *B. quintana* au niveau des excréments a montré que la bactérie était détectable dès le 3ème jour post infection et a persisté jusqu'au 18 ± 1 jour post infection. Bien que des dosages immunohistochimiques localisent les bactéries dans le tractus gastro-intestinal de la punaise de lit, mais la détection de *B. quintana* dans les premiers

et seconds stades larvaire a suggéré une transmission verticale non transovarienne de la bactérie.

La méthodologie adoptée dans ces deux modèles expérimentaux notamment pour l'infection artificielle des punaises de lit par *B. quintana* peut être exploité dans la compréhension d'autres interactions arthropodes-pathogènes.

Prochainement nous allons essayer d'évaluer la compétence vectorielle des punaises de lit vis à vis d'autres bactéries en l'occurrence (*Yersinia pestis*, *Coxiella burnetii et Rickettsia prowazekii*) qui étaient étudiées dans un siècle en arrière mais avec des biotechnologies limitées, ce qui a restreint l'aboutissement de ces travaux. J'ai mis au point jusqu'à présent le protocole expérimental d'infection des punaises de lit avec *Rickettsia prowazekii*. Je dispose d'un élevage artificiel et opérationnel de punaises de lit avec plus de 200 femelles et 200 stades immatures. Nous avons au laboratoire de niveau de sécurité 3 les cultures de *R. prowazekii* et autres pathogènes. J'ai été habilité à travailler sur les micro-organismes hautement pathogène et agents de bioterrorisme par l'Agence nationale de sécurité du médicament et des produits de santé et par la direction de l'inspection DI - 650. Prochainement nous projetons d'infecter les punaises de lit en premier lieu avec l'agent du

typhus épidémique avec *R. prowazekii* suivant le protocole suivant:

Nous allons mettre, 50 punaises de lit dans 4 cellules (50 *C. lectularius* **x** 4) (2 cellules d'adultes et larves L1 contrôles et 2 infectées d'adultes et L1). 2 cellules de punaises de lit seront infectées trois fois pendant 5 jours via un repas sanguin contenant une suspension de *Rickettsia prowazekii*. Pendant chaque procédé d'infections, nous s'assurons par qPCR de la présence de la bactérie dans l'inoculum. Les punaises contrôles seront maintenues en élevage par un repas sanguin non infecté. A j3 post infecté nous récupérons 5 punaises de chaque lot afin de les testées par qPCR et voir l'acquisition de la bactérie, les fèces et les œufs seront de même testés ainsi que cultivé pour voir la voie d'élimination. L'immunohistochimie sera pratiquée sur trois autres punaises afin de localiser la bactérie dans le corps des punaises. Des tests MALDI-TOF seront aussi pratiqués sur les pattes des punaises testés par qPCR afin de trouver des spectres spécifiques et attribuables à l'infection par *R. prowazekii*. Chaque 48h Nous suivons par qPCR, MALDI-TOF et culture, le maintien de l'infection, l'acquisition et l'élimination de la bactérie. Une fois que nous avons des résultats des modèles expérimentaux, nous procéderons par la suite à l'application sur un modèle animal. Nous allons utiliser deux

hamsters, le premier sera infecté par voie intraveineuse par une concentration modérée de *R. prowazekii* et nous mettons dessus des punaises de lits naïves (non infectées). Les punaises de lit seront testées par qPCR et MALDI-TOF pour voir l'acquisition et le maintien de la bactérie. Concernant le second hamster, nous allons infectées expérimentalement des punaises de lit via un repas infecté et nous mettons les punaises de lit en contact avec la peau de le hamster. Le sang du hamster sera testé chaque 24h par qPCR afin de trouver une éventuelle transmission.

Concernant la dernière partie de cette thèse qui a intéressé l'application de la technologie MALDI-TOF à l'entomologie. Nous avons identifié 5 espèces de puces par cet outil protéomique, nous avons montré que les spécimens frais donnent de meilleurs résultats et identification par rapport aux spécimens conservés dans l'éthanol. D'autres travaux sont nécessaires pour améliorer cette technique et essayer de palier le problème des échantillons conservés dans l'éthanol. Par la suite de ce travail nous avons essayé d'identifier les punaises de lit infectées par *Bartonella quintana*, les résultats préliminaires du test des pattes n'ont pas permis de discriminer les punaises infectées parmi les non infectées, probablement par ce que *B. quintana* est une bactérie intracellulaire facultative, et dotée d'une localisation digestive comme on a montré dans les articles 6 et 7. Nous

envisageons prochainement de tester autres parties du corps de la punaise autre que les pattes et essayer de trouver des profils discriminants attribuable à *B. quintana* et cela même en cherchant des protéines associées à la réponse immunitaire de la punaises vis-à-vis *B. quintana* et autres pathogènes associés.

RÉFÉRENCES

References

1. Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE et al.: **Update on tick-borne rickettsioses around the world: a geographic approach.** *Clin Microbiol Rev* 2013, **26:**657-702.

2. Chikeka I, Dumler JS: **Neglected bacterial zoonoses.** *Clin Microbiol Infect* 2015, **21:**404-415.

3. Greer A, Ng V, Fisman D: **Climate change and infectious diseases in North America: the road ahead.** *CMAJ* 2008, **178:**715-722.

4. Mediannikov O, Fenollar F: **Looking in ticks for human bacterial pathogens.** *Microb Pathog* 2014, **77:**142-148.

5. Baneth G: **Tick-borne infections of animals and humans: a common ground.** *Int J Parasitol* 2014, **44:**591-596.

6. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D: **Fleas and flea-borne diseases.** *Int J Infect Dis* 2010, **14:**e667-e676.

7. Eisen RJ, Gage KL: **Transmission of flea-borne zoonotic agents.** *Annu Rev Entomol* 2012, **57:**61-82.

8. Delaunay P, Blanc V, del GP, Levy-Bencheton A, Chosidow O, Marty P, Brouqui P: **Bedbugs and infectious diseases.** *Clin Infect Dis* 2011, **52:**200-210.

9. Parola P, Raoult D: **Tropical rickettsioses.** *Clin Dermatol* 2006, **24:**191-200.

10. Kernif T, Socolovschi C, Bitam I, Raoult D, Parola P: **Vector-borne rickettsioses in North Africa.** *Infect Dis Clin North Am* 2012, **26:**455-478.

11. Chomel BB, Boulouis HJ, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, Birtles RJ, Koehler JE, Dehio C: **Ecological fitness and strategies of adaptation of Bartonella species to their hosts and vectors.** *Vet Res* 2009, **40:**29.

12. Mogollon-Pasapera E, Otvos L, Jr., Giordano A, Cassone M: **Bartonella: emerging pathogen or emerging awareness?** *Int J Infect Dis* 2009, **13:**3-8.

13. Chomel BB, Kasten RW: **Bartonellosis, an increasingly recognized zoonosis.** *J Appl Microbiol* 2010, **109:**743-750.

14. Harms A, Dehio C: **Intruders below the radar: molecular pathogenesis of Bartonella spp.** *Clin Microbiol Rev* 2012, **25:**42-78.

15. Maurin M, Raoult D: **Q fever.** *Clin Microbiol Rev* 1999, **12:**518-553.

16. Angelakis E, Mediannikov O, Socolovschi C, Mouffok N, Bassene H, Tall A, Niangaly H, Doumbo O, Znazen A, Sarih M et al.: ***Coxiella burnetii*-positive PCR in febrile patients in rural and urban Africa.** *Int J Infect Dis* 2014, **28:**107-110.

17. Drancourt M: **Plague in the genomic area.** *Clin Microbiol Infect* 2012, **18:**224-230.

18. Raoult D, Mouffok N, Bitam I, Piarroux R, Drancourt M: **Plague: history and contemporary analysis.** *J Infect* 2013, **66:**18-26.

19. Bitam I, Ayyadurai S, Kernif T, Chetta M, Boulaghman N, Raoult D, Drancourt M: **New rural focus of plague, Algeria.** *Emerg Infect Dis* 2010, **16:**1639-1640.

20. Neerinckx S, Bertherat E, Leirs H: **Human plague occurrences in Africa: an overview from 1877 to 2008.** *Trans R Soc Trop Med Hyg* 2010, **104:**97-103.

21. Cabanel N, Leclercq A, Chenal-Francisque V, Annajar B, Rajerison M, Bekkhoucha S, Bertherat E, Carniel E: **Plague outbreak in Libya, 2009, unrelated to plague in Algeria.** *Emerg Infect Dis* 2013, **19:**230-236.

22. Andrianaivoarimanana V, Kreppel K, Elissa N, Duplantier JM, Carniel E, Rajerison M, Jambou R: **Understanding the persistence of plague foci in Madagascar.** *PLoS Negl Trop Dis* 2013, **7:**e2382.

23. Bertherat E, Thullier P, Shako JC, England K, Kone ML, Arntzen L, Tomaso H, Koyange L, Formenty P, Ekwanzala F et al.: **Lessons learned about pneumonic plague diagnosis from two outbreaks, Democratic Republic of the Congo.** *Emerg Infect Dis* 2011, **17:**778-784.

24. Stanek G, Wormser GP, Gray J, Strle F: **Lyme borreliosis.** *Lancet* 2012, **379:**461-473.

25. Bouattour A, Ghorbel A, Chabchoub A, Postic D: **[Lyme borreliosis situation in North Africa].** *Arch Inst Pasteur Tunis* 2004, **81:**13-20.

26. Rolain JM, Franc M, Davoust B, Raoult D: **Molecular detection of *Bartonella quintana, B. koehlerae, B. henselae, B. clarridgeiae, Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France.** *Emerg Infect Dis* 2003, **9:**338-342.

27. Rolain JM, Bourry O, Davoust B, Raoult D: ***Bartonella quintana and Rickettsia felis* in Gabon.** *Emerg Infect Dis* 2005, **11:**1742-1744.

28. Angelakis E, Socolovschi C, Raoult D: ***Bartonella quintana in Cimex hemipterus*, Rwanda.** *Am J Trop Med Hyg* 2013, **89:**986-987.

29. Raoult D, Drancourt M, Carta A, Gastaut JA: ***Bartonella (Rochalimaea) quintana* isolation in patient with chronic adenopathy, lymphopenia, and a cat.** *Lancet* 1994, **343:**977.

30. Drancourt M, Moal V, Brunet P, Dussol B, Berland Y, Raoult D: ***Bartonella (Rochalimaea) quintana* infection in a seronegative hemodialyzed patient.** *J Clin Microbiol* 1996, **34:**1158-1160.

31. Slowik TJ, Lane RS: **Feeding preferences of the immature stages of three western north American ixodid ticks (Acari) for avian, reptilian, or rodent hosts.** *J Med Entomol* 2009, **46:**115-122.

32. Burch RL: **The progress of humane experimental technique since 1959: a personal view.** *Altern Lab Anim* 1995, **23:**776-783.

33. Balls M: **To be credible, success in "reducing the use of animals in scientific research" must involve the use of fewer animals.** *Altern Lab Anim* 2015, **43:**75-77.

34. Thomas JA, Bailey DL, Dame DA: **Maintenance of *Anopheles albimanus* on frozen blood.** *J Am Mosq Control Assoc* 1985, **1:**538-540.

35. Seng P, Rolain JM, Fournier PE, La SB, Drancourt M, Raoult D: **MALDI-TOF-mass spectrometry applications in clinical microbiology.** *Future Microbiol* 2010, **5:**1733-1754.

36. Yssouf A, Almeras L, Terras J, Socolovschi C, Raoult D, Parola P: **Detection of *Rickettsia* spp in ticks by MALDI-TOF MS.** *PLoS Negl Trop Dis* 2015, **9:**e0003473.

37. Yssouf A, Parola P, Lindstrom A, Lilja T, L'Ambert G, Bondesson U, Berenger JM, Raoult D, Almeras L: **Identification of European mosquito species by MALDI-TOF MS.** *Parasitol Res* 2014, **113:**2375-2378.

38. Karger A, Kampen H, Bettin B, Dautel H, Ziller M, Hoffmann B, Suss J, Klaus C: **Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry.** *Ticks Tick Borne Dis* 2012, **3:**78-89.

39. Thomas RE, Macaluso KR, Azad AF: **Chapter 52:Care, Maintenance, and Experimental Infection of Fleas (Siphonaptera).** *Biology of disease vectors / edited by William C Marquardt [et al -2nd ed Elsevier Academic Press* 2005,727-732.

40. Ferdowsian HR, Beck N: **Ethical and scientific considerations regarding animal testing and research.** *PLoS One* 2011, **6:**e24059.

41. Bitam I, Dittmar K, Parola P, Whiting MF, Raoult D: **Fleas and flea-borne diseases.** *Int J Infect Dis* 2010, **14:**e667-e676.

42. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, Gage KL, Leirs H, Rahalison L: **Plague: past, present, and future.** *PLoS Med* 2008, **5:**e3.

43. Bonilla DL, Kabeya H, Henn J, Kramer VL, Kosoy MY: ***Bartonella quintana* in body lice and head lice from homeless persons, San Francisco, California, USA.** *Emerg Infect Dis* 2009, **15:**912-915.

44. Angelakis E, Rolain JM, Raoult D, Brouqui P: ***Bartonella quintana* in head louse nits.** *FEMS Immunol Med Microbiol* 2011, **62:**244-246.

45. Tsai YL, Chang CC, Chuang ST, Chomel BB: **Bartonella species and their ectoparasites: selective host adaptation or strain selection between the vector and the mammalian host?** *Comp Immunol Microbiol Infect Dis* 2011, **34:**299-314.

46. Angelakis E, Socolovschi C, Raoult D: ***Bartonella quintana* in *Cimex hemipterus*, Rwanda.** *Am J Trop Med Hyg* 2013, **89:**986-987.

47. William CBI, Leonard EM: **Molecular Taxonomy and Systematics of Arthropod Vectors.** *BIOLOGY OF DISEASE VECTORS* 2004, **33:**505-528.

48. Estrada Pena A.: *Ticks of domestic Animals in the Mediterranean Region.University of Zaragoza.131 pp.* 2004.

49. Faran ME, Linthicum KJ: *A handbook of the Amazonian species of Anopheles (Nyssorhynchus)(Diptera: Culicidae).* 1981.

50. Walker A, Bouattour A, Camicas J, Estrada-Peñan A, Horak I, Latif A, Pegram R, Preston P: *Ticks of Domestic Animals in Africa : a Guide to Identification of Species.* 2003.

51. Kent RJ, Deus S, Williams M, Savage HM: **Development of a multiplexed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to identify common members of the Subgenera Culex (Culex) and Culex (Phenacomyia) in Guatemala.** *Am J Trop Med Hyg* 2010, **83:**285-291.

52. Zapata MA, Cienfuegos AV, Quiros OI, Quinones ML, Luckhart S, Correa MM: **Discrimination of seven Anopheles species from San Pedro de Uraba, Antioquia, Colombia, by polymerase chain reaction-restriction fragment length polymorphism analysis of its sequences.** *Am J Trop Med Hyg* 2007, **77:**67-72.

53. Robert H C: **Molecular markers for the phylogenetics of mites and ticks.** *Systematic & Applied Acarology* 2002, **7:**3-14.

54. Antony C, Guy P, Gilbert G: **Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology.** *FEMS Microbiol Rev* 2012, **36:**380-407.

55. Martin W, Edward RBM: **Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology.** *Systematic and Applied Microbiology* 2011, **34:**2-11.

56. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G: **Performance of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of Bacterial Strains Routinely Isolated in a Clinical Microbiology Laboratory.** *Journal of clinical Microbiology* 2010, **48:**1549-1554.

57. Seng P, Rolain JM, Fournier PE, La Scola B, Drancourt M, Raoult D: **MALDI-TOF-mass spectrometry applications in clinical microbiology.** *Future Microbiol* 2010, **5:**1733-1754.

58. Yssouf A, Flaudrops C, Drali R, Kernif T, Socolovschi C, Berenger JM, Raoult D, Parola P: **Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors.** *J Clin Microbiol* 2013, **51:**522-528.

59. Yssouf A, Socolovschi C, Flaudrops C, Ndiath MO, Sougoufara S, Dehecq JS, Lacour G, Berenger JM, Sokhna CS, Raoult D et al.: **Matrix-assisted laser desorption ionization--time of flight mass**

spectrometry: an emerging tool for the rapid identification of mosquito vectors. *PLoS One* 2013, **8:**e72380.

60. Calderaro A, Gorrini C, Piccolo G, Montecchini S, Buttrini M, Rossi S, Piergianni M, Arcangeletti MC, De CF, Chezzi C et al.: **Identification of Borrelia species after creation of an in-house MALDI-TOF MS database.** *PLoS One* 2014, **9:**e88895.

ANNEXES

Article N.10. Bérenger J-M, Almeras L, Leulmi H, Parola P. **A High-Performance Vacuum Cleaner for Bed Bug Sampling: A Useful Tool for Medical Entomology**. DOI: http://dx.doi.org/10.1093/jme/tjv019 first published online: 19 March 2015.

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A High-Performance Vacuum Cleaner for Bed Bug Sampling: A Useful Tool for Medical Entomology

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ABSTRACT Arthropods can be captured by two modes: a passive mode using traps or an active mode mainly based on the use of mouth or powered aspirators. These apparatuses are useful tools for collecting large numbers of crawling, flying, resting, or jumping arthropod specimens, particularly small specimens, such as mosquitoes or sandflies, for laboratory experiments or breeding. Different aspirator models are used to collect various arthropod specimens. However, to our knowledge, no specific system is currently available for the reliable sampling of live bed bugs in the field. Thus, we described a new system based on a classic autonomous house aspirator that requires few modifications for the collecting bed bugs. The low weight and size of this apparatus is advantageous, and it provides for rapid and secure bed bug sampling for medical entomology purposes.

KEY WORDS bed bug, vacuum system, arthropod sampling, medical entomology

In the past two decades, a worldwide resurgence of bed bug infestations has occurred (Baumblatt et al. 2014). The re-emergence of bed bugs has been attributed to the banning of effective pesticides, such as dichlorodiphenyltrichloroethane (DTT), widespread pesticide resistance, and increased human travel (Adelman et al. 2011, Doggett et al. 2012). Victims of bed bug infestations frequently suffer from psychological distress (Goddard and de Shazo 2012). The need for bed bug amelioration requires increased bed bug monitoring and control. To increase monitoring and control levels, laboratory research on this pest insect is required for the development of innovative strategies and tools to eliminate bed bug infestations. Prior to developing laboratory experimental protocols to control bed bugs, field collection of this insect is necessary. A high rate of bed bug survival is necessary for the establishment of laboratory colonies; therefore, collection systems should not damage the insects. Although there is no clear evidence that bed bugs can transmit disease, recent experimental studies have reported that bed bugs could be potential vectors of human infectious pathogens, such as arboviruses (Doggett et al. 2012), bacteria (Goddard et al. 2012, Angelakis et al. 2013), or parasites (Salazar et al. 2014). The collection system should protect the collector from potential pathogen transmission, and cleaning of the collection system should be simple.

Mouth aspirators (i.e., pooters) are frequently used by entomologists (Wiens and Burgess 1972, Farr 1989, Vazquez-Prokopec et al. 2009). These systems present advantages including that they are simple to construct, they are light weight and easy to manipulate in the field, and they are inexpensive. Pooters are not recommended for the field collection of arthropod vectors because they place the collector at risk of exposure to potentially infected arthropods.

Different vacuum aspirator models have been designed to capture insects, and they usually target specific arthropod families (Schauff 1997, Gibb and Oseto 2006, Upton and Mantle 2010). Vacuum aspirators are useful for collecting large quantities of arthropods for laboratory experimentation or colony establishment. Battery-powered autonomous electrical aspirators are well adapted for the indoor collection of arthropods (Governatori et al. 1993, Vazquez-Prokopec et al. 2009). However, significant modification to some of these designs is necessary to avoid damage to the arthropod specimens. For delicate flying insects, such as mosquitoes, specific aspirators have been developed, such as the BioQuip C-cell aspirator (Catalog reference number: 2809A, BioQuip Products, Rancho Dominguez, CA). Based on our own experience, we observed that the BioQuip C-cell aspirator is not efficient at capturing insects, such as bed bugs, which are strongly attached to the seams of mattresses or hidden in the crevices of bed frames or walls.

The absence of a dedicated auto-powered vacuum system for bed bug collection led us to develop an apparatus for the rapid sampling of a large number of specimens. Among the battery-powered aspirators available, the Dyson DC34 hand vacuum model (Dyson France, Paris, France) was selected based on

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the following characteristics: it is a portable system with 10 to 15 continuous minutes of battery power; a high suction power with two speeds (38 or 65 air watts); low weight (1.3 kg), which is ideal for situations requiring the extension of the arms while using the device; and a reasonable price (approximately 160 €).

The Dyson DC34 has a two-part design. Part 1 is composed of plastic components that include a tube holder and a plastic jar with a trapdoor for cleaning. Part 2 is composed of the engine and the battery (Fig. 1E and F). The extracted air passes through a filter that is easy to wash and is located between Part 1 and Part 2 (Fig. 1D). Part 1 is made entirely of plastic that is easily cleaned (Fig. 1B).

To preserve the integrity of bed bugs, which could be damaged in the collection jar, the original factory tube was replaced by a BioQuip sampling vial (Catalog reference number: 2809V, BioQuip) and a BioQuip nozzle (Fig. 1A). The diameter of the BioQuip vial matches the tube holder of the Dyson DC34. The flap valve of the pick-up tube (Fig. 3) allows the retention of collected arthropods in the sampling vial. At the opposite side of the sampling vial, a metallic wire mesh facilitates the retention of the collected arthropods in the sampling vial. To prevent the loss of small arthropods, a fine mosquito net (mesh < 1 mm) is glued onto the metallic wire grid (Fig. 3, bottom). When the sampling vial is one-quarter full, it is replaced by another vial. Switching sample vials is simple, rapid, and certain because of the flap valve.

We tested whether the commercial collecting vial could be replaced by a homemade collection vial using a 50-ml tube (Fig. 2, bottom). The 50-ml tube was modified to obtain a pick-up tube with a nozzle on one side and a fine mosquito net on the other side, as is presented in Figure 3. For sample transport to the laboratory, we recommend to put the pick-up tube containing the specimens into a closed flask to preclude any risk of insect release. The advantages of this aspirator are that the modifications (i.e., replacement of the original factory tube with a sampling vial) needed for the vacuum are minimal and could be purchased from a supplier or could be home-made with very inexpensive materials, that the collector is protected from direct contact with the insects during collection and transfer, and the capability for complete disinfection of the unit.

The recent finding that bed bugs could be a vector of *Trypanosoma cruzi* (Salazar et al. 2014) highlights the importance of collection and monitoring of this arthropod for human health. Bed bug control measures require frequent visits to apartments or houses suspected of insect infestation. The collection of numerous specimens from different areas is required for experimental studies, such as establishing bed bug breeding programs for laboratory assays of new control strategies (e.g., pesticides and pest control material), testing vector competence with an experimental model, detecting pathogens, and genetically analyzing sample populations. For these collections, a portable aspirator that possesses high autonomy and high power is needed because some arthropods are securely attached to walls or fabric.

The modified version of the Dyson DC34 hand vacuum presented here was assessed in field conditions in the collection of bed bugs from the *Cimex lectularius* species, which expanded globally in the previous decade (Davies et al. 2012, Doggett et al. 2012). For the collection of specimens in apartments or houses, we collaborated with different groups that specialize in pest control. The success of this collaboration is based on the short time required to execute bed bug collection prior to the physical, mechanical, and chemical treatment used by pest groups.

In March 2012, we were informed by our pest research collaborators that an apartment in Aix-en-Provence in southern France was infested by bed bugs.

Fig. 1. Modified Dyson DC 34 vacuum cleaner for bed bug collection: (1) A detailed view of the movable parts—(A) BioQuip sampling vial; (B) plastic jar; (C) trapdoor; (D) filter; (E) digital motor; (F) battery. (2) A view of the modified Dyson DC 34 vacuum cleaner ready to be used for bed bug collection. A BioQuip sampling vial is set, and a home-made plastic vial is presented below (falcon 50 ml). (3) A detailed view of the BioQuip sampling vial apertures with front and back views. (4) The protective clothing of the manipulator in field conditions using the modified Dyson DC 34 vacuum cleaner in a bed bug-infested apartment is shown.

Using the modified Dyson DC34 hand vacuum system, >700 bed bug specimens were successfully captured in less than 15 min (a collection of eggs, immature, and adult specimens). This method appears to not damage the insects (Fraval 2002), and we noticed a high survival rate (>90%) even for the eggs and immature stages. The high power of this vacuum cleaner enables removal of the insects attached to walls, wood, and fabrics, as well as from wall crevices (Fig. 2). After use, the movable parts of this vacuum cleaner are easily cleaned and disinfected. To kill all the arthropods or eggs potentially present in the vacuum, it could be placed entirely, except for the battery, in a freezer (−20°C) for 72 h (Olson et al. 2013). This system could be applied for the collection of other arthropods, including body lice or fleas. Because these arthropod families have been reported to be vectors of human diseases, we recommend that the collector wear a biosafety level 3 mask, gloves, and hazmat suit to prevent a risk of contamination by dust, feces, or bites (Fig. 2). Additionally, if an infection is verified, gas decontamination (hydrogen peroxide fumigation) of the vacuum and filter is possible.

In conclusion, bed bugs are a public health problem, and laboratory research on monitoring, control, and pesticide resistance requires bed bugs collected in the field. The lack of a dedicated system for bed bug collection in the field led us to adapt a standard vacuum for the collection of specimens. The modified Dyson DC 34 hand vacuum presented here has numerous advantages and enables the rapid and secure collection of live bed bugs.

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References Cited

Adelman, Z. N., K. A. Kilcullen, R. Koganemaru, M. A. Anderson, T. D. Anderson, and D. M. Miller. 2011. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PLoS ONE 6: e26228.

Angelakis, E., C. Socolovschi, and D. Raoult. 2013. Bartonella quintana in Cimex hemipterus, Rwanda. Am. J. Trop. Med. Hyg. 89: 986–987.

Baumblatt, J. A., J. R. Dunn, W. Schaffner, A. C. Moncayo, A. Stull-Lane, and T. F. Jones. 2014. An outbreak of bed bug infestation in an office building. J. Environ. Health 76: 16–18.

Davies, T. G., L. M. Field, and M. S. Williamson. 2012. The re-emergence of the bed bug as a nuisance pest: implications of resistance to the pyrethroid insecticides. Med. Vet. Entomol. 26: 241–254.

Doggett, S. L., D. E. Dwyer, P. F. Penas, and R. C. Russell. 2012. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25: 164–192.

Farr, D. V. 1989. Pump-aspirator for collecting small insects. N. Z. Entomol. 12: 84–85.

Fraval, A. 2002. Captures et collections. II. Aspirateurs. Insectes 124: 27–28.

Gibb, T. J., and C. Y. Oseto. 2006. Arthropod collection and identification – Laboratory and field techniques. Academic Press, Elsevier, p. 311.

Goddard, J., and R. de Shazo. 2012. Psychological effects of bed bug attacks (*Cimex lectularius* L.). Am. J. Med. 125: 101–103.

Goddard, J., A. Varela-Stokes, W. Smith, and K. T. Edwards. 2012. Artificial infection of the bed bug with Rickettsia parkeri. J. Med. Entomol. 49: 922–926.

Governatori, M., C. Bulgarini, F. Rivasi, and S. Pampiglione. 1993. A new portable aspirator for culicidae and other winged insects. J. Am. Mosq. Control Assoc. 9: 460–462.

Olson, J. F., M. Eaton, S. A. Kells, V. Morin, and C. Wang. 2013. Cold tolerance of bed bugs and practical recommendations for control. J. Econ. Entomol. 106: 2433–2441.

Salazar, R., R. Castillo-Neyra, A. W. Tustin, K. Borrini-Mayori, C. Naquira, and M. Z. Levy. 2014. Bed Bugs (Cimex lectularius) as Vectors of Trypanosoma cruzi. Am. J. Trop. Med. Hyg. pii: 14–0483.

Schauff, M. E. 1997. Collecting and preserving insects and mites: Techniques and tools. Systematic Entomology Laboratory, USDA, National Museum of Natural History, Washington, DC, p. 68.

Upton, M. S., and B. L. Mantle. 2010. Methods for collecting, preserving and studying insects and other terrestrial arthropods, 5th ed. Australian Entomological Society, Canberra, Australia, p. 81.

Vazquez-Prokopec, G. M., W. A. Galvin, R. Kelly, and U. Kitron. 2009. A new, cost-effective, battery-powered aspirator for adult mosquito collections. J. Med. Entomol. 46: 1256–1259.

Wiens, J. E., and L. Burgess. 1972. An aspirator for collecting insects from dusty habitats. Can. Entomol. 104: 1557–1558.

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Acarologie

IDENTIFICATION AND SEASONAL DYNAMICS OF TICKS ON WILD BOAR (*SUS SCROFA*) IN THE EXTREME NORTH-EAST OF ALGERIA

par

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During a period of two years, from April 2011 to March 2013, and for the first time in Algeria, a study was undertaken to identify ticks on wild boar in the extreme northeast of Algeria. In total, 3266 tick specimens were collected. These were obtained from 111 boars killed by approved hunting associations, 80 of which were found infested with ticks, giving an infestation rate of 72.1 %. The highest rates of infestation were observed in summer and spring. The infestation of wild boar depends very significantly on season and altitude (P<0.00001). The highest rate of infestation was observed for the species *Rhipicephalus turanicus* (62.2 %), followed by *Dermacentor marginatus* (36.0%), *Hyalomma marginatum* (6.3 %) and finally *Ixodes ricinus* (3.6 %). Taken together, these results add to our knowledge of wild ixodid populations in Algeria and should help to improve control methods.

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Identification et dynamique saisonnière des tiques chez le sanglier (*Sus scrofa*) dans l'extrême nord-est algérien

Durant une période de deux ans allant du mois d'avril 2011 au mois de mars 2013, et pour la première fois en Algérie, une étude a été entreprise pour identifier les tiques chez le sanglier dans l'extrême nord-est algérien. Un total de 3266 tiques qui ont été collectées et conservées dans des tubes contenants de l'éthanol à 70°. Ainsi, 111 sangliers ont été abattus par des associations de chasses agrées, ces derniers ont fait l'objet de cette étude, dont 80 ont été révélés infestés par les tiques donnant ainsi un taux d'infestation de 72,1 %. Les taux d'infestation les plus élevés ont été observés en été et printemps. L'infestation des sangliers dépend très significativement de la saison et de l'altitude et (P<0.00001). Le taux d'infestation le plus élevé a été observé pour l'espèce *Rhipicephalus turanicus* (62,2 %) suivie par l'espèce *Dermacentor marginatus* (36,0 %), *Hyalomma marginatum* (6,3 %) et enfin *Ixodes ricinus* (3,6 %). L'ensemble de ces résultats participe à l'amélioration des connaissances de la faune Ixodidienne en Algérie afin d'améliorer les moyens de lutte.

Mots-clés : Sanglier, Algérie, tiques, *Rhipicephalus turanicus*, *Dermacentor marginatus*, *Hyalomma marginatum*, *Ixodes ricinus*.

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Monsieur Hamza Lecheb

La Médaille d'Argent

DE LA JOURNÉE INFECTIOPÔLE SUD 2014

Pour ses travaux de recherche

Avec les félicitations du Jury, de la Présidente et du Directeur de la Fondation,
et du Président du Conseil Régional,

Michel Vauzelle
Yolande Obadia
Didier Raoult

Jean-Louis Mège
Eric Delaporte
Bruno Pradines

MÉDITERRANÉE INFECTION

Région
Provence
Alpes
Côte d'Azur

Infectiopôle Sud
fondation de coopération scientifique

La Fondation Méditerranée Infection

Faculté de Médecine, 27, Boulevard Jean Moulin, 13 385 Marseille Cedex 5 - France

Décerne à :

Monsieur Hamza Leulmi

La Médaille d'Argent

DE LA JOURNÉE INFECTIOPÔLE SUD 2015

Pour ses travaux de recherche

Avec les félicitations du Jury, de la Présidente et du Directeur de la Fondation,
et du Président du Conseil Régional,

Yolande Obadia
Didier Raoult

Christian Devaux
Michel Drancourt
Raymond Ruimy

MÉDITERRANÉE INFECTION

ESCCAR

International congress on Rickettsia and other intracellular bacteria

June 13-16, 2015 – CHUV, Lausanne, Switzerland

CERTIFICATE OF ATTENDANCE

As Poster Presenter

for

Hamza LEULMI

DETECTION OF BARTONELLA TAMIAE, COXIELLA BURNETII AND RICKETTSIAE IN ARTHROPODS AND TISSUES FROM WILD AND DOMESTIC ANIMALS IN THE NORTHERN EAST OF ALGERIA

Gilbert Greub
Chairman of the Scientific Committee and
Vice-chairman of ESCCAR Committee

Sébastien Aeby
Chairman of the Organizing Committee

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