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Genetic Diversity of Bartonella spp. in Mexican Wild Rodents

And zoonotic risks

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Preface

Students from the faculty of Veterinary Medicine at the University of Utrecht have to fulfil a research internship. This paper is the final report of the research project at the Department of Population Health and Reproduction of the University of California at Davis and supervised by the Division of Veterinary Public Health of the Institute for Risk Assessment Sciences at Utrecht University, The Netherlands.

Research was performed to gain better understanding of the diversity and possible zoonotic risks of *Bartonella* species in Mexican wild rodents.

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1. Abstract

Objectives - The objective of this study was to identify the prevalence of *Bartonella* variants present in wild rodents from the Mexican states of Jalisco and the Yucatan and asses their implications for public health. **Materials and methods -** Hearts, blood and blood clots from a total of 100 rodents consisting of nine different species from Yucatan and Jalisco were analysed for the presence of *Bartonella* using a classical polymerase chain reaction (PCR) targeting the *gltA* (379-bp) gene region. **Results –** Blood and blood clot culture let to the isolation in 3-4 days of small gram negative bacilli which were identified as *Bartonella* spp. based on colony morphology. Although none of the 62 rodents from Jalisco were positive, rodents sampled in Yucatan showed a prevalence of *Bartonella* of 57.9% (22/38). Yucatan deer mouse (*Peromyscus yucatanicus)* was the most sampled rodent species (71%) compared to other rodents in Yucatan and accounted for 100% of the positives found in that state. Phylogenetic analysis showed that the 22 genetic variants found in this study were closely related to *Bartonella vinsonii subspecies vinsonii* (96.89% - 97.52%). One of the genetic variants also had resembling to *Bartonella vinsonii subspecies arupensis*, a zoonotic *Bartonella* subspecies. **Conclusion** - This study provides the evidence that genetic variants closely related to a non-zoonotic subspecies of *Bartonella* are present in *Peromyscus yucatanicus* rodents from San José Pituch in the Yucatan state of Mexico. To assess the implications on human health of these genetic variants of *Bartonella*, further research is necessary.

2. Introduction

The genus *Bartonella* is of increasing public health importance. These bacteria have a worldwide distribution and multiple species are capable of causing disease in both non-human animals and people. More than 33 *Bartonella* species have been described to date [1]. 15 species are confirmed as (potential) human pathogens (Table 1). The zoonotic species of these bacteria can cause severe diseases in humans such as cat scratch disease, trench fever and Carrion's disease. Some of these diseases may be fatal if not properly treated, particularly in immunocompromised individuals. The transmission of *Bartonella* bacteria occurs mainly by blood-feeding arthropod vectors.

Pets represent an important reservoir for infection with several *Bartonella* species in humans. Probably the best described *Bartonella* infection in literature is cat scratch disease (CSD), mainly caused by *B. henselae*. *Bartonella* infections in dogs have also been studied, but their role as reservoir is less clear than for cats. Dogs are more likely accidental hosts and can act as excellent sentinels for human infection [2].

In addition to pets, rodents are also recognized as an important reservoir of various *Bartonella* species. In fact, most of the described *Bartonella* species are hosted by rodents. Several of these rodent-borne *Bartonella* spp. have been associated with human illness [1].

With continued research on *Bartonella*, it is likely that more genetic variants, vectors and reservoirs will be discovered. Also considering the extensive animal reservoirs and the large number of vectors that have been implicated in the transmission of *Bartonella* species, the effects on human health could be impactful.

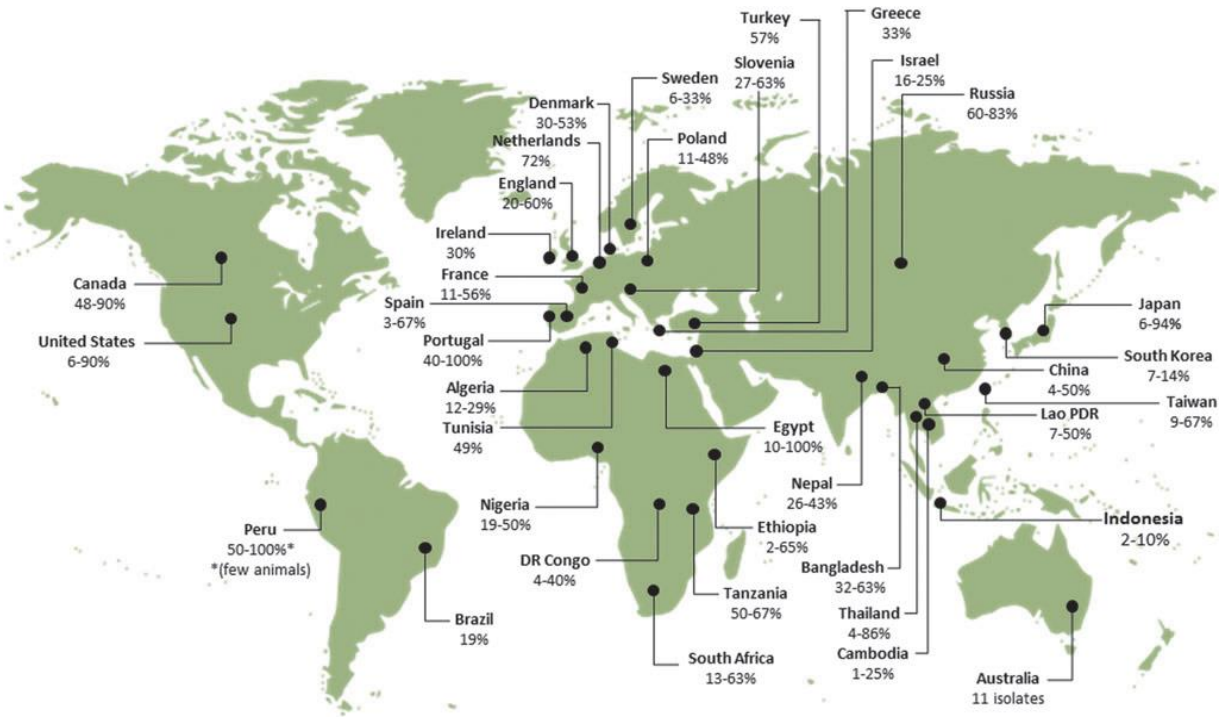

Figure 1. Range of Bartonella-infection rates in rodents reported worldwide (data from PubMed, January, 2014) [3]

Bartonella spp. are gram-negative, facultative intracellular, hemotropic bacteria. Although *Bartonella* infections are virtually ubiquitous among mammals, each *Bartonella* species or subspecies appears to be highly adapted to one or few mammalian reservoir hosts, in which it causes a long-lasting intraerythrocytic bacteraemia as a hallmark of infection. On the other hand, incidental infection of a non-reservoir host does not seem to lead to intraerythrocytic infection but can cause various clinical manifestations [4]. The bacteraemia does not appear to cause any disease in these reservoir hosts. Infection rates may reach up to around 50% in rodents or feral cats, and can be as high as 90% in wild ruminants [5]. Transmission occurs via blood-feeding arthropod vectors. Fleas are the most common known

vector for the transmission of *Bartonella* but new possible arthropod vectors have been suggested. *Bartonella* DNA has been found in multiple tick species, biting flies, keds and lice[6,7].

The *Bartonella* genus belongs to the alpha-2-proteobacteria phylum and lies in proximity of the *Brucella* genus. Both genera are classified in the *Rhizobiales* family [8,9]. The species and subspecies of the *Bartonella* genus are subdivided evolutionary into five lineages. Linage 1 is represented by the relatively newly discovered *B. tamiae*. The ancestral linage 2 contains *Bartonella bacilliformis*. Linage 3 contains *Bartonella clarridgeiae* and *Bartonella rochalimae*. Linage 4 consists of *Bartonella schoenbuchensis* and other ruminant-specific species. The largest group is linage 5 which consists of *Bartonella henselae, Bartonella quintana, Bartonella vinsonii* and more. Linage 1, 3, 4 and 5 are considered the modern species of the *Bartonella* genus [9,10].

Bartonella henselae infections are introduced in the mammalian reservoir host by traumatic contact with infected animals or through inoculation of host skin lesions by contaminated faeces of a bloodsucking arthropod. It is known that *B. henselae* can multiply in the digestive tract of the cat flea and survive several days in the flea faeces [6,10]. Before invading the bloodstream, the bacteria take residence in a yet unrecognised primary niche which is apparently necessary and probably involves entering migratory cells. The bacteria are transported to the vascular endothelium from where they are seeded into the bloodstream leading to the establishment of a long-lasting, intra-erythrocytic bacteraemia. The *Bartonella* bacteria adhere to the erythrocytes and invade them, competent for transmission by a bloodsucking arthropod [5]. *Bartonellae* colonize not only erythrocytes in its reservoir host, there is also evidence that they can invade different host cells ranging from endothelial cells to monocytes, macrophages and dendritic cells [10].

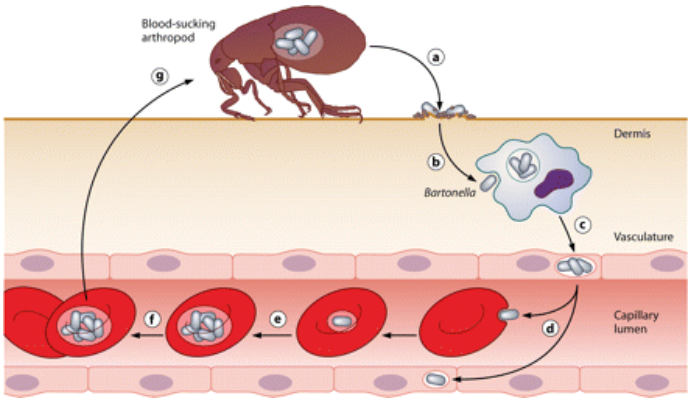

Figure 2. Common infection strategy of *Bartonellae* [5]

Although it is generally believed that the overall concept of this cycle is conserved among members of the *Bartonella* genus, a lot of differences from species to species do exist [5]. A notable difference is the presence of flagella or fimbriae. It is known that *B. bacilliformis* and *B. clarridgeiae* have flagella, which facilitates erythrocyte invasion (in case of *B. bacilliformis*), *B. tribocorum* has polar structures resembling fimbriae and other species such as *B. henselae* seem to lack flagella. *B.henselae* appears to be internalized by an actin-dependent invasome-mediated mechanism of cellular invasion based upon in vitro studies [7,11].

There are various reasons why *Bartonella* is successful as a bacterial pathogen. One is that *Bartonellae* elude the host's immune response by both passive immune evasion and active immune-modulation. Once the bacterium is inside the red blood cell, it is protected from the host's immune system [5]. More reasons are the genetic diversity, strain variability and adaptation to specific vectors and reservoirs [6]. The host specificity of *Bartonella* arises from their ability to adhere to the erythrocytes of their natural host through a host-specific adhesion process mediated by the Trw-type IV secretion system. Arthropod hosts of *Bartonella* appear to have a major impact in the diversification of *Bartonella* strains by promoting recombination. The diversity of *Bartonella* can be caused by selection, homologous recombination, lateral gene transfer or exchange of genetic material between strains. Also a host shift

of *Bartonella* species is possible due to spill over; parasite jumping from its reservoir to a non-specific host. Genetic diversity and strain variability also appear to enhance the ability of *Bartonellae* to invade not only specific reservoir hosts, but also accidental hosts [12]. Co- infection by different *Bartonella* strains, and the presence of atypical strains within vectors, may explain the jump phenomenon of nonspecific strains between hosts [9]. A co-infection with another pathogen could positively or negatively interact with *Bartonella* species. They could modulate the host's immune system or share the same resources. Co-infections of *Bartonella* previously described are with *Borrelia, Mycoplasma, Babesia and Ehrlichia* [9].

Bartonella as a zoonotic disease

According to Harms et al. 2012 the majority of human disease is caused only by three species, *B. bacilliformis, B. henselae* and *B. quintana* [5]. Other zoonotic species or subspecies are *B. elizabethae, B. grahamii, B. clarridgeiae, B. koehlerae, B. vinsonii subs. berkhoffii, B. vinsonii subs. arupensis, B. washoensis, B. alsatica, B. mayotimonensis, B. melophagi, B. rochalimae,* and *B. tamiae* [12]. For *B. bacilliformis* and *B. quintana*, humans are the main reservoir. For the others, humans are an incidental host.

Bartonella sp.	**Primary reservoir**	**Vector**	**Accidental host**
B. bacilliformis	Human	Sand fly	None
B. quintana	Human	Body louse	Cat, dog
B. elizabethae	Rat	Oriental rat flea	Human, dog
B. grahamii	Wild mice	Rodent fleas	Human
B. henselae	Cat	Cat flea	Human, dog, horse, marine animals
B. clarridgeiae	Cat	Cat flea	Human, dog
B. koehlerae	Cat	Cat flea	Human, dog
B. vinsonii subsp. berkhoffii	Coyote, dog	Unknown (ticks?)	Human, cat
B. vinsonii subsp. arupensis	White-footed mouse	Unknown (ticks? fleas?)	Human
B. washoensis	California ground squirrel	Fleas	Human, dog
B. alsatica	Rabbit	Rabbit flea?	Human
B. melophagi	Sheep	Sheep ked	Human
B. rochalimae	Canids	Fleas?	Human, dog
B. tamiae	Unknown (rat?)	Mites? Ticks?	Human
B. mayotimonensis	Bats	Unknown	Human
B. volans-like	Rodents	Unknown	Human, dog, sea otter

Table 1. Species and subspecies of *Bartonella* that are confirmed or potential human pathogens [12-16]

B. bacilliformis is the only known representative of an ancient linage that is widely separate from the more modern species of the *Bartonella* genus. Transmission is vectored by the sand fly *Lutzomyia verrucarum* [4]. An infection with *B. bacilliformis* classically shows an acute phase, better known as Oroya fever, and a chronic phase, known as verruga peruana. Oroya fever has a mortality rate from zero in treated patients to up to 88% in untreated cases do to a haemolytic anaemia which seems unique among *Bartonella* spp. Multiple vasoproliferative skin lesions are typical for verruga peruana. The combination of acute and chronic phase is classical for *B. bacilliformis* and is called Carrion's disease. This subspecies of *Bartonella* is only endemic in the high valley of the Andes [5].

In immunocompetent humans ***B. henselae*** is capable of causing a regional lymphadenopathy, with or without fever or fatigue, known as cat scratch disease (CSD). Less often other symptoms are seen like headache, anorexia, splenomegaly, abscessed lymph nodes and various a-typical manifestations [6]. CSD is long lasting, though self-limiting and has a limited morbidity. Although *B. henselae* infections can cause meningitis and encephalitis, there have been a very limited number of case reports of fatal infection in immunocompetent people. In immunocompromised patients, *B. henselae* can cause more serious complications like bacillary angiomatosis and eventually damage of the skeleton, brain and inner organs with the chance of a fatal outcome. Sometimes CSD is caused by *B. clarridgeiae* or *B. quintana* [6].

Trench fever or 5-day fever is caused by ***B. quintana*** and is characterized by fever peaks, bone pain and headache accompanied by persistent bacteraemia. Trench fever is a louse-borne disease with limited morbidity. During World War I, trench fever affected approximately 800,000 soldiers from Napoleon's Grand Army. Incidence has dropped considerably since, but this disease is now re-emerging as urban trench fever, a condition associated with homelessness, alcoholism and poor conditions of health and hygiene[5]. A few suspect cases associated with *B. quintana* are bacillary angiomatosis, endocarditis and CSD. Contact with cats or cat fleas is a risk factor for becoming infected with *B. quintana* [6].

B. elizabethae was isolated from a human case of endocarditis in North America and has since been identified in wild rodents from Peru and North America. *B elizabethae*-like species are known to infect different rodent species like *Rattus* rats, *Bandicota* rats, shrews and gerbils [9].

B. grahamii was in 1994 one of the three first described rodent-associated *Bartonella* species. Currently *B. grahamii* strains are widespread among *myodes* and *microtus* voles in the northern hemisphere, *Apodemus* mice in Eurasia, and the grasshopper mice in North America. DNA of *B. grahamii* was detected in the ocular fluid of a human patient with neuroretinitis in The Netherlands in 1999 [9].

The first isolation of ***B. clarridgeiae*** was in 1995 in the USA from a pet cat of a HIV-positive patient. A prevalence of *B. clarridgeiae* from 17% to 36% in cats has been reported in literature. This species was suggested to be a minor causative agent of CSD, as antibodies against *B. clarridgeiae* were found in a suspect case of CSD and in a patient with a chest-wall abscess [6,12].

B. koehlerae was first isolated in 1999 from the blood of two domestic cats. The first human case of *B. koehlerae* endocarditis was reported in Israel in 2004 and the first canine case was a five and a half year old boxer with severe sub-aortic stenosis in 2009. Experimental infection of cats did not seem to lead to any clinical symptoms. This bacterium is very fastidious to grow, therefore culturing is difficult [6,7,12].

In 1996 ***B. vinsonii subsp. berkhoffii,*** isolated from dogs, was designated as a new subspecies[7]. In 2009 this subspecies was isolated from a cat with osteomyelitis of the carpal joint 18 months after amputation of an osteomyelitis digit in the rear leg[6]. Breitschwerdt reported a case in 2010 of a father and daughter with endocarditis, arthritis, neurological disease and vasoproliferative neoplasia, in which *B. vinsonii subsp. berkhoffii* was isolated. Vector transmission is suspected among dogs and wild canines, which are the primary reservoir hosts[17].

B. vinsonii subsp. arupensis was isolated from a cattle rancher presenting with fever and neurologic disorders in 1999. This subspecies is widespread among sylvatic rodents and infects rodents belonging to the *Peromyscus* and *Onychomys* genera, with a high prevalence in deer mice, across North America and Canada [7,9].

B. washoensis has been found in ground squirrels in the USA and China [9]. A rodent reservoir was implicated after isolation of *B. washoensis* from a patient with cardiac disease [7].

B. alsatica is a newly identified zoonotic *Bartonella* species which was initially isolated from the blood of wild rabbits in France and shown to cause endocarditis and lymphadenitis in humans [6].

Initially isolated from sheep keds and sheep blood, ***Bartonella melophagi*** was detected in the blood of two sick women in the USA one woman had a co-infection with *B. henselae* (No Reference Selected).

B. rochalimae has been found in dogs, racoons, coyotes, grey and red foxes and the fleas of the grey fox. This indicates that the natural reservoir of this zoonotic *Bartonella* species may be wild carnivores and fleas being the main vector [6]. *B. rochalimae* was isolated from an American tourist presenting with fever and splenomegaly after travelling in Peru and was later isolated from wild rats in Taiwan [9]. *B. rochalimae* has flagella and is closely related to *B. clarridgeiae*.

B. tamiae has been found in humans in Thailand. It is suggested that mites could be one of the main vectors but its reservoir and vector are being investigated [6]. *B. tamiae* has been detected in ectoparasites collected from different rodent species[9].

In 2008 a 59-year-old man presented at a hospital in Iowa, USA with endocarditis. ***B. mayotimonensis*** was identified using PCR of aortic valve tissue. This *Bartonella* species is the seventh documented to cause infective endocarditis in humans, the other six being *B. quintana, B. henselae, B. elizabethae, B. vinsonii subsp. berkhoffii, B. koehlerae* and *B. alsatica.* The reservoir has yet to be determined [18].

B. volans-like, a rodent associated *Bartonella* species have been found in dogs causing canine Bartonellosis. Breitschwerdt et al. 2009a, described a case of a 86-year-old farmer who experienced several postoperative complications after surgery for a fractured femur. Currently, the reservoir(s), mode of transmission, and putative arthropod vector(s) for these novel *B. volans-like* strains are unknown[16].

Non-zoonotic *Bartonella* species are beyond the scope of this report and will not be discussed here.

Symptoms

An infection with *Bartonellae* can occur with or without symptoms. Clinical manifestations of *Bartonella* infections range from mild symptoms to life-threatening disease. However, it does not seem to cause any disease in its reservoir host. Although the pathology of *Bartonella* bacteria requires further research, it is known that the organisms can localize to the heart valve causing an endocarditis and localization to other parts of the body causing granulomatous inflammation in lymph nodes, liver or spleen, induce central nervous system dysfunction and may contribute to inflammatory polyarthritis. Hematological abnormalities are less common but thrombocytopenia, lymphocytosis, neutropenia and eosinophillia have been reported [7,19]

Treatment

Treatment of an acute *Bartonella* bacteraemia, bacillary angiomatosis or parenchymal bacillary peliosis in humans, may consist of a two (in immunocompetent patients) to six weeks (in immunocompromised patients) administration of doxycycline, erythromycin or rifampin. Clinical improvement has also been reported using penicillin, gentamicin, ceftriaxone, ciprofloxacin and azithromycin. Cat scratch disease seems to have a lack of response to antimicrobial treatment in humans [6].

Prevention

Prevention of infection relies on arthropod control. Since the transmission of for example *Bartonella henselae* depends on fleas, the use of acaracide products to eliminate fleas from the environment is of clinical importance to decrease the risk of a *Bartonella* infection [7].

***Bartonella* infections of rodents**

From the 33 confirmed *Bartonella* species, more than half are hosted by rodents and at least five of these are pathogenic to humans (*B. grahamii, B. elizabethae, B. vinsonii subsp. arupensis, B. washoensis* and *B. tamiae)*. They can cause symptoms like endocarditis, fever, lymphadenitis and hepatitis. The transmission of *Bartonellae* between rodents appears to be mainly accomplished by fleas and compared with human- or cat-associated *Bartonella sp.,* the rodent-associated species have a higher number of host-adaptability genes. Geographically, it seems that rodent-associated *Bartonella sp*. are structured according to the distribution of their rodent host. For example, all *Rattus* rat-adapted *Bartonella sp*. have a worldwide distribution, whereas others are only found in one area [9]. According to Breitschwerdt et al. 2000, the prevalence of bacteraemia caused by *Bartonella* within rodent populations can be quite high. In the United Kingdom and the south eastern United States a prevalence of 62.2% and 42.2% respectively, are documented [7]. Other reported prevalence of *Bartonella* in rodents are 82.4% in Colorado [20], 57% in Canada [21], 42.2% in Southeastern U.S. [22], 42.9% in Brazil [23], 23.7% in Lithuania [24], 12.5% - 35.7% in Thailand [25] and 57.7% on the Heixiazi islands in China [26]. In a recent article of Rubio et al. 2014, wild rodents from north-western Mexico were investigated. Different *Bartonella* strains were cultured from 50.1% of these samples. Of these isolates 23 genetic variants were found including 15 novel variants [1].

The impact of rodent-borne *Bartonella* infections on human health may be underestimated. In 2010 Kosoy et al. reported that in 4.6% of 261 human patients with fever of unknown origin from Thailand, DNA of rodent-borne *Bartonella sp.* was present. All patients had contact with rodents prior to illness [27]. The route of infection and the mechanisms of transmission to humans are currently unknown [9].

Since rodents often play a crucial role in the transmission of zoonotic disease, such as leptospirosis, Lyme borreliosis and plague [9] and are recognised as a reservoir of *Bartonella sp.,* the impact of *Bartonella* bacteria on human health is important to assess.

Objectives / Aim of the study

The objective of this study is to identify which *Bartonella* strains are present in rodents of the Jalisco and Yucatan states of Mexico and to determine their prevalence, especially for the zoonotic species. Since the prevalence of infection and public health and veterinary importance vary according to species and geographical region, we believe that with this information the implications of *Bartonella* sp. in rodents for human health in Mexico can be assessed. It is expected to find rodent strains like *B. vinsonii* subsp. *vinsonii, B. vinsonii* subsp. *arupensis* and *B. washoensis* as previously described by Rubio et al. in 2014 [1].

3. Materials and Methods

Ethics statement

This study and sampling protocol were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine of the University of California, Davis (approval number 18367).

Study area

Rodent blood, blood clots and hearts were collected from five different sites located in two states. In early December 2013 blood, blood clots and hearts were collected from rodents caught in the localities Peto (20°4´51´´N 88°54´47´´ W), Bokobá (21°0´ 23´´N 88°54´47´´ W) and San José Pituch (20°59´0´´ N 88°42´12´ W) in the Yucatan state of Mexico. Rodent hearts were collected from the localities Emiliano Zapata (19° 22.883' N 104° 58.743' W) and Puente Cuitzmala (19° 24.616' N 104° 57.492' W) in the Jalisco state of Mexico in June 2012. The vegetation is characteristic of tropical savannah.

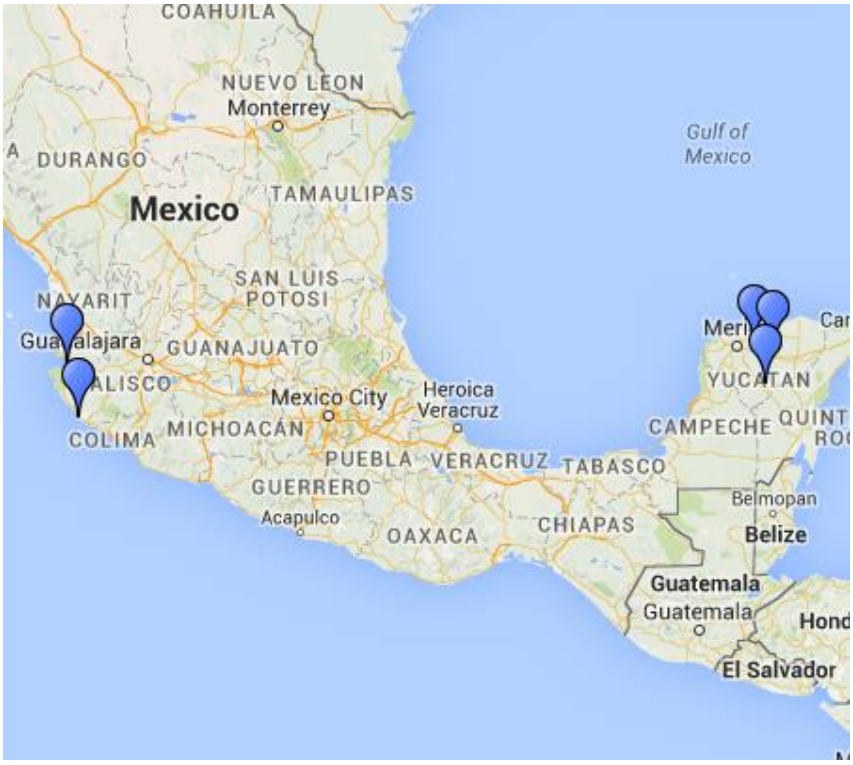

Figure 3. Sampling sites (blue marks) in Mexico. (assembled in Google maps)

Sampling procedures

To collect the rodents, Sherman traps (7.6cm x 8.9cm x 22.9cm; H.B. Sherman Traps, Inc., Tallahasse, FL) were placed every 10 meters on each site of a transect. A peanut butter and oat mix was placed in the back of the trap as bait for the rodents. The traps were placed at dusk and checked and emptied at dawn. The live trapped rodents were euthanized and transferred to a field lab for necropsy. Rodents were identified to species based on external morphology.

From the rodents in Jalisco, half hearts were collected and from the rodents of the Yucatan, blood, blood clots and hearts were collected. The samples were sent to the Chomel laboratory in the Department of Population Health and Reproduction at the University of California, Davis. All samples were stored in -80°C freezer.

Rodent species from Yucatan

Rattus rattus

Peromyscus yucatanicus

Heteromys gaumeri

Ototylomys phyllotis

Rodent species from Jalisco

Oryzomys couesi

Peromyscus perfulvus

Mus musculus

Osgoodomys banderanus

Liomys pictus

Bartonella culture of rodent blood and blood clots

Blood and blood clot samples were stored in a freezer as cell lysis by freezing to plating, facilitates bacterial isolation from blood. Although bacteria of the *Bartonella* genus are fastidious and slow growing, they can be cultured successfully on agar plates [6]. For culture of rodent blood, a total of two hundred microliters of thawed EDTA blood and M199 growth medium (1:1) was plated onto fresh (<1 week old) 5% defibrinated rabbit blood agar plate as previously described[28]. For culture of the blood clots twenty microliters of M199 growth medium was plated in the centre of a rabbit blood agar plate and was mingled with a small blood clot using a disposable loop and spread out on the plate.

The plates were incubated at 35°C in an aerobic atmosphere of 5% carbon dioxide in a high humidity chamber for 4 to 5 weeks. Bacterial growth was monitored every day the first week and then on alternate days for up to three weeks. After three weeks the plates were checked twice a week. Bacterial colonies were presumptively identified as *Bartonella* spp. based on their morphology. Suspected *Bartonella* colonies were plated from the original plates onto secondary rabbit blood agar plates, and incubated at the same conditions. When growth showed on the secondary plates, a part of the colonies were harvested and stored in a freeze medium at -80°C and another part of the colonies were suspended in Tris buffer and kept in a refrigerator till DNA isolation.

Morphology of _Bartonella_ spp.

Bartonella colonies are up to 1mm small rounds that have a lucent to opaque greyish colour. They rise in the middle and are flatter on the edges. The edges can be irregular or smooth. Depending on the size of the colonies, they become more opaque and the edges are more irregular.

Figure 4. Original plate with *Bartonella* colonies

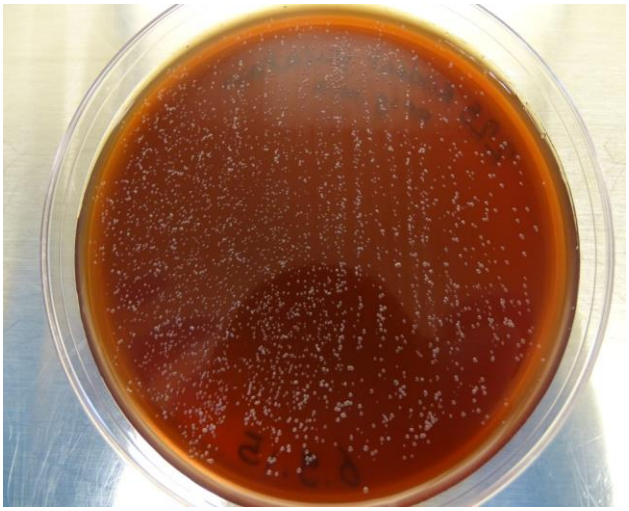

Figure 5. Secondary plate with *Bartonella* colonies

DNA extraction from _Bartonella_ cultures

A suspension of Tris buffer and harvested colonies was heated for 15 minutes at 100°C followed by a vortex for 10-15 seconds. After 5 minutes of cooling down, the samples were centrifuged at 12,500 rpm at 4°C for 10 minutes. 100µl of the supernatant containing the genomic DNA to be used as DNA template in the PCR was then pipetted into a sterile 1.5ml (stock) vial containing 100µl of 2xTE buffer. A ''working solution'' of 1:10 was made by adding 5µl of the stock vial into a new 1.5 vial containing 45µl of TE buffer. These vials were stored in a -20°C freezer.

DNA extraction of heart tissue

DNA extraction from rodent heart tissue of 98 rodents from the Mexican states of Jalisco and the Yucatan were performed using a Qiagen DNeasy tissue and blood kit (Qiagen, Valencia, CA) according to manufactures instructions. See Appendix for the DNA extraction protocol used in this research project.

Conventional PCR

A PCR amplification of the extracted DNA from the heart tissue and cultures was performed using primers CSH1F and CSH1137 to generate a 379-bp amplicon of the *Bartonella* citrate synthase gene (*gltA*) (Norman et al., 1995). Sequencing *gltA* is effective in clearly distinguish each (sub)species and according to Buffet et al. this is the method by choice [9].

For the suspected positive cultures not only a PCR on the *gltA* fragment has been done, the *ftsZ, rpoB* and *16S* rRNA gene regions have also been amplified[29-32]. Primer sets targeting these other regions have not been applied on heart tissue because of experiences previously. The PCR programs that are used in this research project can be found in the Appendix.

	gltA	***ftsZ***	***rpoB***	***16S* rRNA**
Primer	CSH1F and BhCS1137	APT0257 and APT0258	APT0244 and APT0245	fD1 and rP2
PCR program	Bart 6,57	Bart 63	Bart 62	Bart 63

Table 2. Primers and PCR programs used for the specific gene region

Sequencing and phylogenetic analysis

The PCR products were cleaned up to obtain a purified DNA product using a Qiagen QIAquick PCR Purification Kit according to manufactures instructions. The purified PCR products were sequenced at the College of Biological Sciences UCDNA Sequencing Facility at the University of California, Davis. Phylogenetic trees were constructed from *gltA* sequences using the MrBayes plugin in Geneious version 8.1.7 with a 1,100,000 MCMC and 100,000 burn-in length[33,34].

4. Results

Animals

In the Yucatan, *Peromyscus yucatanicus* was the most prevalent rodent species and accounted for 71% (n=27) of the rodents captured. *Rattus rattus, Ototylomys phyllotis* and *Heteromys gaumeri* were also collected and accounted for 5.26% (n=2), 5.26%, and 18.42% (n=7), respectively, of all trapped animals in the Yucatan. A total of 35 hearts, 13 blood samples and 32 blood clots were made available for this research project. The total of 38 rodents caught at three different sites in the Yucatan state consisted of four different rodent species.

Site / rodent species	*Rattus rattus*	*Ototylomys phyllotis*	*Heteromys gaumeri*	*Peromyscus yucatanicus*	**Positives/total**
Peto	2	0	3	0	**0/5**
Bokobá	0	0	2	3	**0/5**
San José Pituch	0	2	2	24	**22/28**
Positive/total	**0/2**	**0/2**	**0/7**	**22/27**	

Table 3. Number and type of species caught at different sites in the Yucatan state of Mexico.

In the state of Jalisco *Oryzomys couesi* was the most prevalent animal species and accounted for 56.45% (n=35) of the rodents captured. *Mus musculus, Liomys pictus, Osgoodomys banderanus* and *Peromyscus perfulvus* were also collected and accounted for 25.8%, 8%, 3.2% and 3.2%, respectively, of all trapped animals in the Jalisco state. A total of 62 rodent hearts were collected for this research project.

In the state of Jalisco a total of 62 rodents from five different species were studied, originating from two different sites within the state.

Site / rodent species	*Peromyscus perfulvus*	*Osgoodomys banderanus*	*Liomys pictus*	*Mus musculus*	*Oryzomys couesi*	Unknown species	**Positives/total**
Emiliano Zapata	1	0	0	1	13	1	**1/16**
Puente Cuitzmala	1	2	5	15	22	1	**0/46**
Positive/total	**0/2**	**0/2**	**0/5**	**0/16**	**1/35**	**0/2**	

Table 4. Number and type of species caught at different sites in the Jalisco state of Mexico.

Of the seven *Peromyscus yucatanicus* for which both blood and blood clots were available, three were culture positive and three were culture negative for both samples. One culture was positive for only the blood clot for one rodent. There were 30 rodents for which we had both a blood clot and a heart sample (Table 5). Of these, 15 (50%) were culture and PCR positive on both samples, four (13.3%) were only culture positive from the blood clot and eleven (36.7%) were negative for both samples. All PCR positive heart samples were also positive by culture of whole blood or blood clot. The only isolate close to *B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *arupensis* was positive from the blood sample, but negative from the heart and blood clot.

***Bartonella* culture of rodent blood and blood clots**

Rodent blood and blood clots were only collected in the Yucatan area. A total of 38 rodents were caught in this area from which 13 blood samples and 32 blood clot samples were obtained. From the total of 13 blood samples 30.8% (n=4) showed *Bartonella* suspected colonies and of the 32 blood clots collected 65.6% (n=21) was suspected *Bartonella* positive after culturing.

The rodent-borne *Bartonella* colonies took three to four days to appear on the agar plates.

PCR on extracted DNA

From rodent blood and blood clot samples 58% (n=22) out a total of 38 rodents revealed a positive reaction using the primer set targeting the *gltA* gene region of the *Bartonella* bacterium. This percentage was identical when the *16S* rRNA region was targeted instead of the *gltA*, but was 55% (n=21) when *ftsZ* and *rpoB* primer sets were used.
From the Jalisco state one out of 62 (1.6%) rodent heart samples tested positive targeting the *gltA* gene region of *Bartonella*. In rodent hearts from the Yucatan state a higher prevalence was found, here 40% (n=14) out of 35 rodents showed a positive result using primers targeting the *glt*A gene. Previous experience showed negative results using other primer regions on heart tissue explaining why only *gltA* primers have been used in this research for the PCR of heart tissue.

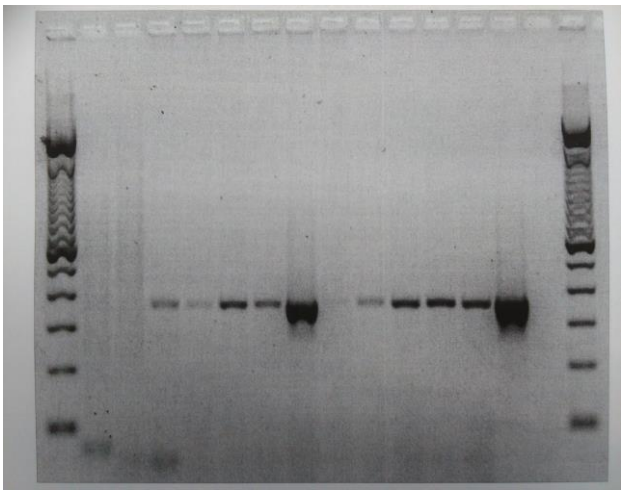

Figure 6. Photo of a gel electrophoreses with two 100 bp ladders on the sides and a negative and positive control in the most right lanes next to the ladder. The bands showing on the same height as the positive control are positive samples obtaining the *Bartonella* DNA.

Sequencing

All PCR positive heart and culture samples tested with the primer set targeting the gltA gene region were sequenced. The sequence of the single PCR positive sample from the Jalisco area was indistinct so this sample was considered to be negative, leaving no positive samples for the Jalisco area. A phylogenetic analysis by neighbour joining method has been performed on the sequencing data of the 22 positive Yucatan samples as showed in figure 7.

Figure 7. Phylogenetic tree based on neighbour joining method. Red: sequencing results in this research project, blue: data from Rubio et al. 2013, green: data from Billeter et al. 2014, **black**: acknowledged *Bartonella* species.

Rodent number	Blood clot	Blood	Heart
208	N	Y	Y
209	N	Y	Y
210	N	Y	Y
211	N	Y	Y
212	N	Y	Y
213	N	Y	N
214	Y	Y	Y
215	Y	N	Y
216	Y	Y	Y
217	Y	N	Y
218	Y (+)	Y (+)	Y (+)
219	Y (+)	N	Y (+)
220	Y	Y (+)	Y
221	Y (+)	N	Y (+)
222	Y (+)	N	Y (+)
223	Y	N	Y
224	Y (+)	N	Y (+)
225	Y (+)	Y (+)	Y
226	Y	N	Y
227	Y	N	Y
228	Y (+)	N	Y (+)
229	Y (+)	Y (+)	Y (+)
230	Y (+)	N	Y (+)
231	Y (+)	N	N
232	Y (+)	N	Y (+)
233	Y (+)	N	Y (+)
234	Y (+)	N	Y (+)
235	Y	N	Y
236	Y (+)	N	Y
237	Y (+)	N	Y (+)
238	Y (+)	N	N
239	Y (+)	N	Y
240	Y (+)	N	Y (+)
241	Y (+)	N	Y
242	Y	Y	Y
243	Y (+)	N	Y
244	Y (+)	N	Y (+)
245	Y	N	Y

Table 5. Overview of samples originating from rodents from the Yucatan state.
Y = sample available, N = no sample available, (+) = PCR positive

5. Discussion

This study demonstrated the presence, prevalence and genetic diversity of *Bartonella* bacteria in rodents from regions in two different states of Mexico. To our knowledge, there are only two previously published articles that describes the genetic diversity of *Bartonellae* present in Mexican rodents and their fleas [1,35]. The prevalence of *Bartonella* in rodents reported by Rubio et al. (50.1%) seems to correspond to the prevalence found in this study (57.9%). The percentages are also consistent with the characteristics described in other literature [7,21,26].

Phylogenetic analyses of the *gltA* sequence revealed that *Peromyscus yucatanicus* rodents from the Yucatan state harbour diverse genetic variants of the *Bartonella* genus. All of the genetic variants found in this research are in close proximity to *Bartonella vinsonii* subsp. *vinsonii.* This subspecies is not pathogenic to humans in contrast to the two other subspecies in the group of *Bartonella vinsonii* (*B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii*). It is assumed that, since most of our positives were closely related to *B. vinsonii* subsp. *vinsonii*, these genetic variants are not capable of causing disease in humans. The sequencing result of a single sample that was deviating from the other genetic variants identified in the present study, revealed to be in close proximity of *B. vinsonii* subsp. *arupensis*, a zoonotic *Bartonella* subspecies but was still slightly closer to *B. vinsonii subsp. vinsonii*. It can therefore be concluded that this genetic variant could be a zoonotic risk. *B. vinsonii* subsp. *arupensis* and *B. vinsonii subsp. vinsonii* were also detected in rodents and their fleas from areas in Northern Mexico (Chihuahua) [1,35]. It is beyond the scope of this study to examine the threat of these specific genetic variants on human health and so unless further research is being conducted, it will remain uncertain whether these genetic variants are capable to cause any disease in humans.

In this study there was a notable difference in the number of rodent species caught within the Yucatan state. Although it was not surprisingly that *Peromyscus yucatanicus* had the most positives in absolute numbers because it was the most prevalent species, it was expected to find *Bartonella spp.* in the other rodents species as well. It is known that especially rodent-borne *Bartonella* species are host specific. This could explain the high prevalence in just one rodent species. Further studies are required to confirm this observation, considering that our sample size was relatively small. The *Bartonella* strains isolated from the Yucatan rodents were quite similar to strains isolated from rodents in the western USA[20,36] and Northern Mexico[1].

It was surprisingly that the samples from the Jalisco state were all negative, because prevalence similar to the pattern observed in the Yucatan was expected. A possible explanation could be that the presence of an inhibitory substance in the heart tissue was present which prevented detection of DNA. Another possible cause could be that the quality of the tissue was not good enough to isolate the *Bartonella* bacteria.

Most rodent borne *Bartonella* spp. are flea-borne[37], and likely the rodents captured in this study must have been flea and tick invested. For instance, 40.4% of the fleas collected from rodents captured is north-eastern Mexico were *Bartonella* spp. PCR positive[35].

6. Conclusion

This study provides evidence that various genetic variants of *Bartonella*e are present in Yucatan deer mice (*Peromyscus yucatanicus)* rodents in the Yucatan state of Mexico. A potential zoonotic genetic variant of *Bartonella*, closely related to *B. vinsonii* subsp. a*rupensis* and *B. vinsonii subsp. vinsonii,* is present in wild rodents from Mexico. Different presumably non-zoonotic genetic variants have also been identified and were in close proximity to *B. vinsonii* subsp. *vinsonii*. It brings a better understanding of the distribution of this rodent-borne bacterium among rodents in North and Central America. To assess the implications on human health of these genetic variants of *Bartonella*, further research is required.

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Among the thousands of things I learned from this experience, one sentence reflects best what is important to keep in mind when conducting research: failure is not the opposite of success, it is part of it.

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9. Appendix

Whole blood culture protocol

Samples must first undergo freeze-thaw cycle in the -80°c freezer before being cultured.

- Take samples out of -80°c freezer and let them thaw. Once liquid, shake vigorously until there are no more chunks/blobs
- Supplies:
    - Culture notebook, write in what you're culturing
    - Sterile filtered P200, P1000 pipet tips, and their respective pipettes.
    - Sterile 1.5 mL vials.
    - M199 growth medium
    - Yellow loops
    - Hazardous sharps/hazardous waste containers, 15mL tube to for liquid waste.
    - Fresh blood agar culture plates (expire 9 days after receiving).
- Place plates lid side down in the hood. Label: sample ID, animal, original mix, date plated and plate A or B.
- Pipet 200µl of M199 into a sterile vial.
- Pipet 200µl of blood sample into same vial and mix with pipet by pipetting up and down. If there is less than 200µl of blood, use what is available and make notion in notebook.
- Pipet 200µl of the mix onto plate A and 200µl onto plate B. (Place drops uniformly throughout the plate, avoiding the edges: looks like chocolate chip cookie).
- If there is enough, save at least 25µl of your sample in case you need to re-culture.
- With yellow loop: use a circular motion to spread drops. Once spread, zigzag across plate.
- Once done with all samples, place in jar and label. Light candle and put it in the jar. Close the jar and let the candle burn out. Then place in incubator at 35°C.
- Clean BSC (biological safety cabinet) with micro-cam and alcohol.

DNA extraction protocol

Qiagen DNeasy blood + tissue kit

Amount of tissue needed:

- Spleen: 10 mg
- Liver: 25 mg
- Heart: 25 mg

Day 1:

- Thaw tissue from -80°C freezer
- Heat up water bath to 56°C (put ATL buffer in immediately)
  - Plug in machine and turn power switch
  - Make sure water level isn't too low (should be at 4th notch)
  - ATL buffer is kept in Qiagen kit. Place in small jar and don't entirely submerge
- Prepare steril 1,5 ml tubes in BSC.
  - Turn on and sterilize the BSC
  - Poor uncapped sterilized tubes from jar
  - Put closed tubes that are not needed in blue tray in drawer
- Obtaining tissue
  - First label and weigh scaled 1,5ml tubes. Write weight on paper. (Unlock Mettler by pulling red piece and turn dial to ''1'' position. It's now ready to use.)
  - Put in BSC: sharps container, 1,5 tubes and samples in rack, disposable forceps, scalpels, loops and petridishes.
  - Use half petridish to cut tissue into small pieces and put pieces in corresponding 1,5ml tube. Weigh the tube (and add more pieces till right weight is obtained).
- Get ATL from water bath and shake by inverting. Add 180 µl ATL with and push tissue that is against the wall in the ATL at bottom of vial. Do the same with all of the vials
- Add 20 µl of proteinase K and directly vortex
- Pulse vortex all samples again when proteinase K is added to all vials
- Place caps on vials and put in floating rack in water bath
- Pulse vortex all samples again after 1 hr (and again after 2 hrs)

Day 2:

- Warm AL buffer at 56°C in water bath
- Heat hot block to 95°C
- Set up rack with all tubes that are needed
  - Collection tubes 2ml from Qiagen kit (amount of samples times 2)
  - Collection tubes 1,5 ml. (Cut off the lids from as many tubes you will need but leave lids on). (Amount of samples times 2)
  - Small 1ml tubes. (Same amount of samples)
- Get samples from water bath and centrifuge all samples in small (purple) centrifuge
- Add 200 µl of AL to all samples. (Vortex each sample immediately after adding AL)
- Put caps back on tubes and place in hot block for 10 min.
- Let samples cool for a few minutes. Then remove caps
- Centrifuge samples in small centrifuge
- Add 200 µl of ethanol. (Vortex immediately after adding ethanol)
- Centrifuge samples in small centrifuge
- Transfer mixture to a spin column from Qiagen kit using a P1000
- Centrifuge in large centrifuge at 8000 rpm for 1 min.
- Move spin column (inner tube with filter) to a new 2ml collection tube
- Add 500µl of buffer AW1 for first wash
- Centrifuge in large centrifuge at 8000 rpm for 1 min.

- Move spin column to a new 2ml collection tube
- Add 500µl of buffer AW2 for second wash
- Centrifuge in large centrifuge at 14000 rpm for 3 min.
- Move spin column to a new collection tube (1,5 ml tubes with lids cut off)
- Centrifuge in large centrifuge at 14000 rpm for 1 min.
- Move spin column to a new collection tube (1,5 ml tubes with lids cut off)
- Add 50µl of AE buffer (from aliquots from Rick) directly on the filter
- Let it incubate at room temperature for 1 minute
- Centrifuge in large centrifuge at 8000 rpm for 1 min.
- Don't change collection tube and repeat 50µl of AE buffer directly on filter
- Let it incubate at room temperature for 1 minute
- Centrifuge in large centrifuge at 8000 rpm for 1 min.
- Transfer all mixture from collection tube into small (properly) labelled 1µl tube
- Put 1µ vials in -20 freezer

PCR protocol for extracted/isolated DNA

Preparation:

- Put UV light on in PCR hood for 15 minutes.
- Get an ice bucket and fill halfway with ice.
- Get your PCR reagents: Taq polymerase, Magnesium, Primers (forward and reverse), 10x Gold Buffer, DNTP, PCR H_2O
- Ice bucket immediately: Taq polymerase, Magnesium
  Ice bucket after thawed (20-30 minutes): Primers, 10x Gold Buffer, DNTP, PCR H_2O.
- Get DNA samples and positive control from freezer and put in the fridge.
- In your notebook, make a list of your reagents and calculate total amounts needed. For every 10 samples, make 1 extra.
- Switch gloves (since you've touched your samples now and you don't want to contaminate your reagents)

In PCR hood:

- Use the forceps to pull out the microvials you need. Loosely cap them. Write the number on the top and write the date on the side. You'll need one for each sample plus 2 (+ and – controls).
- Add calculated amount of reagents to your one sterile 1.5 mL vial in the following order:
  (Place your stock solutions back into the ice bucket after you're done adding them)
  1. PCR H_2O (27.75 µl per microvial)
  2. Gold buffer (5 µl per microvial)
  3. Magnesium (3 µl per microvial)
  4. dNTP (4 µl per microvial)
  5. Primer (forward) (2.5 µl per microvial)
  6. Primer (reverse) (2.5 µl per microvial)
  7. TAQ gold (polymerase) (0.25 µl per microvial)
- Pulse vortex PCR mix vial 3 times for 3 seconds. Then centrifuge a couple seconds.
- Pipet 45 µl of your created solution into all of your labelled PCR microvials.

- Pipet 5 µl PCR H_2O into the negative control microvial and 3 µl PCR H_2O into the positive control microvial.
- Put away all your reagents now, make sure to tap out ice from bottom if there is any.
- Get your samples and positive control (B. henselae U4 10^{-3}) from the fridge.
- (make dilutions of your samples if needed. 10µl AE and 10µl of sample)
- Pipet 5 µl positive control into your positive control microvial.
- Pipet 5 µl of your samples into all your microvials.
- Put away your samples and the positive control in freezer.
- Thermocycler:
- Try to evenly spread out your microvials(samples and controls) in the block.
- Close the block and loosely tighten.
- Turn on the machine. "RUN". Hit proceed. Now scroll through your programs until you find "BART 6,57''. Use select arrow to select that. Then hit proceed. TUBES, proceed. Total volume: 50 µl, proceed. Use heated lid: Yes. Wait about 4 hours.
- Spray and wipe PCR hood with alcohol and put UV light on for 15 minutes.

Next day:

- Thermocycler should be at 4.0°C. Check for error messages.
- Hit proceed, go to next step and proceed again. Machine says program complete. Hit proceed again and you'll see initial menu.
- Open up block and take out samples with gloves. Leave the block open for a couple hours. Turn off the machine. Put samples in the fridge. They are now ready to run the gel with these.

PCR Programs:

Bart 6,57			**Bart 62**			**Bart 63**	
Time:	Temperature:		Time:	Temperature:		Time:	Temperature:
10 min.	95℃		5 min.	95℃		5 min.	95℃
30 sec.	94℃		30 sec.	95℃		30 sec.	95℃
1 min.	57℃		1 min.	58℃		15 sec.	55℃
2 min.	72℃		1 min.	72℃		1 min.	72℃
5 min.	75℃		10 min.	72℃		10 min.	72℃
∞	4℃		∞	4℃		∞	4℃

Gel electrophoresis protocol

Making the gel:

- Turn on the water bath to 56°C. Make sure water level is high enough.
- Get 250 mL erlemyer flask and put skinny big stirrer bar in the flask.
- Get 100 mL grad. cylinder and pour 80 mL of 0.5x TBE into grad. cylinder. Then pour that into the 250 mL flask with stir bar and place on stirring plate and stir at 4.5-5.
  (If you need to make more 0.5x: add 900 mL of the sterilized dH2O water from jug and then 100 mL of the 5x TBE.)
- Add 1.6g LE Agarose to the flask. Put on foil cap and turn on the heat to 10. Get orange gloves and warning signs out. (Will boil in 5-10 minutes).
- Once boiling, remove from hotplate. Directly pipet in 5 µl "1:10 Gel Red". Place back on hotplate for 5-7 seconds. Immediately place in the big beaker in the water bath for 20 minutes.
- Turn off and unplug hot plate. Leave signs out.
- Set up agar tray. Make sure it's level with the bubble leveler.
- After 20 minutes: get hot water running in the sink. Take your flask out of the water bath, pour it into your tray and immediately clean the flask with hot water. Let your solution in the tray solidify for 30-45 minutes.
- If you are not using the tray on the same day, put it in the big fridge in a zip lock bag with a wet towel.

In PCR hood:

- Use forceps to get 4+[# of samples] 0.5 sterile vials.
- Get "LOAD" buffer 1.5 ml vial from room temperature reagents box and get out "100 BP" 0.5 vial from -20 freezer.
- Label your vials with the numbers you have in your notebook. The last 2 are labeled ''STD''. Pipet 7 µl of LOAD buffer in all vials.
- Pipet in 5 µl of samples(your pcr'd tubes) into their respective vials and pipet 5 µl of the "100 BP" vial into the 2 STD vials. Every vial has 12 microliters total now.
- Put the 100 BP vial back in the freezer. Put the "LOAD" buffer back into the room temperature box. Put PCR'd samples in the fridge.

Set up gel electrophoresis:

- Get 1800 mL erlemyer flask and 250 mL grad. cylinder. Pour 160 mL stock solution (5x TBE) into grad cylinder. With the huge grad cylinder fill erlemyer flask with 1000 mL and then 440 mL of the best quality water. Swirl the flask 10 sec. (Good for 5/6 uses or 1 week)
- Level electrophoresis box with leveler. Place box in right direction (current will go from – (black) to + (red) so place comb on black side)
- Get out your gel tray and flood it. (make sure the tray gets into the notch)
- Remove the comb gently and rinse with Di water. Put black paper under box to see wells beter.
- Pipet 12 microliters into each well. Use sterile unfiltered (yellow) tips and P20 pipet.
- Put the top on (red on red and black on black) and plug in the wires. Hit the power switch. First screen displays V(volts), should be 90. The 2nd parameter, mA is set at 300. Set time at 2 hours. Show display as "actual" and hit the on / off button. Lights "timer on" "v at limit" will go on. Now you want to make sure bubbles are rising at the black electrode. Double check in a few minutes to make sure green dyes are migrating in right direction and turning into yellow and blue dyes.
- After 2 hours: Turn rocker switch off, unplug wires, take off top. Use your fingers to hold the gel so it doesn't fall out of its holder. Place it on a paper towel while still in the tray.

Reading the gel:

- Turn on computer (Optiplex 9020) and log in.
- Get UV tray and put gel on the tray (wells away from you). Push off the gel slowly to force out any bubbles.
- Open up camera chamber, put tray with gel in chamber and close. Flip power switch on side of camera chamber.

- Open Image Lab program and choose Protocol 1 (or make new protocol). Press ‘’Run’’.
- Click save as. Save into your folder.
- Go to image tools, choose invert data. Go to ‘’crop’’ and adjust the lines. Print the image (2 pages on one) and paste into notebook.
- Cleanup: Turn off power to camera chamber. Gel goes into regular trash. Clean UV tray with Di water and dry with Kim Tech wipes. Close chamber. You’re done.

DNA purification protocol

Qiagen purification kit

Needed:

- 1 set 2µl tubes
- 2 sets 1.5µl tubes
- 2 sets 0.5µl tubes
- Qiagen purification kit

In PCR hood:

- UV PCR hood for 15 min.
- Get all supplies and samples
- Label one set (#of your samples) of 0.5µl tubes
- Open the tubes and add 250µl PB buffer w/ P1000
- Centrifuge all samples on small purple machine
- Add samples to vials with PB buffer with P100 on 50µl and mix with pipet
- Transfer mixture to labelled spin columns
- Centrifuge spin columns for 1 min, 13000 rpm at room temperature
- Take the 2µl tubes and snap off the lids
- Transfer filter to these 2µl tubes
- Add 700µl PE buffer w/ P1000 (not directly on filter)
- Centrifuge for 1 min, 13000 rpm at room temperature
- Transfer filter to 1.5µl tubes and don't add anything
- Centrifuge for 1 min, 13000 rpm at room temperature
- Transfer filter into second 1.5µl tubes
- Add 30µl EB directly on filter w/ P100 and let it incubate for 1 min.
- Centrifuge for 1 min, 13000 rpm at room temperature
- Take out the filter and transfer liquid to labelled 0.5µl tubes w/ P200
- Put clean PCR products in box in -20°C freezer.

Clean up and UV hood for 15 min.