CHAPTER 2

Culture of *Bartonella* species from the blood of human and animal populations in Gauteng, South Africa, 2007 – 2009

2.1 INTRODUCTION

Culture is the gold standard for diagnosis of most bacterial infections. *Bartonella* is no exception although routine diagnosis by culture is often not recommended due to the fastidious nature of the *Bartonella* bacilli (McCool *et al.,* 2008). Culture is useful for the definitive diagnosis of *Bartonella*-related infections, including fever of unknown origin, neuroretinitis, encephalitis, IE, PH, and/or BA (Hammoud *et al.,* 2008).

Several methods have been used in *Bartonella* spp. isolation, including the Septi-Chek biphasic system (Lucey *et al*., 1992; Larson *et al.,* 1994), BACTEC 460 blood culture bottles (Lucey *et al.,* 1992; Daly *et al.,* 1993, Larson *et al.,* 1994), BACTEC NR-660 infrared CO_2 detection system (Spach *et al.,* 1993; Larson *et al.,* 1994), direct plating of blood onto rabbit blood-supplemented Columbia agar (*Bartonella* media) following lysis centrifugation or freeze-thawing of a blood sample (Varela *et al.,* 1969; Regnery *et al.,* 1992, Larson *et al.,* 1994; Maggi *et al.,* 2005), and lysis-centrifugation (Koehler *et al.,* 1992; Lucey *et al*., 1992; Larson *et al.,* 1994). The most widely used methods for microbiological diagnosis of *Bartonella* spp. are direct inoculation onto *Bartonella* media or cultivation in cell-line cultures (Maurin *et al.,* 1997; Johnson *et al*., 2003). Fresh media increases the yield of *Bartonella* spp. isolation (Hammoud *et al.,* 2008).

Bartonella spp. have been found to grow best on *Bartonella* media, 5% (v/v) horse blood-supplemented Columbia media and chocolate agar. Some reports have also suggested that bartonellae grow on sheep blood agar (Lappin *et al*., 2008). Growth is dependent on humid, microaerophillic conditions (5% CO_2) at temperatures ranging between 30-37 ℃ (Maurin *et al.,* 1997; La Scola *et al*., 2001; Rolain *et al.,* 2004). Bartonellae are very slow growing organisms that require an incubation period of 1 to 6 weeks for primary isolation by culture (Johnson *et al*., 2003; Rolain *et al.,* 2004; Duncan *et al.,* 2007). As a result of the prolonged incubation period required

for culture, the media often become contaminated (Rampersad *et al.*, 2005). In order to minimize contamination by yeasts and moulds in particular, the antimicrobial amphotericin B may be added to the culture media (Maurin *et al.,* 1997).

Since *Bartonella* is intraerythrocytic, studies have suggested that bacteria can be cultured from blood specimens by freeze-thawing defibrinated blood. The rationale suggests that the blood cells will be lysed and the bacteria will be released from the erythrocytes to grow on the media (Duncan *et al.,* 2007). The blood is plated onto the *Bartonella* media and placed under microaerophillic conditions. The blood-cultures are incubated for 5 – 21 days (La Scola and Raoult, 1999).

It has been reported that lysis-centrifugation improves the speed and frequency of isolation of *Bartonella* spp. from blood cultures (Slater *et al.,* 1990; Lucey *et al.,* 1992; Schwartzman, 1992; Welch *et al.,* 1992; Larson *et al.,* 1994). However, lysis-centrifugation is said to be prone to contamination due to extensive manipulation, including centrifugation and specimen separation. As a result this method has not been adopted by diagnostic laboratories. Improvement in detection and isolation of *Bartonella* spp. from broth blood-culture systems would greatly enhance the diagnostic capacity of routine laboratories (Larson *et al.,* 1994). A novel, chemically modified insect cell culture-based liquid medium called *Bartonella* alpha-Proteobacteria growth medium (BAPG M) was found to support the growth of 7 *Bartonella* spp., where previously no suitable liquid medium was able to do so. This BAPGM pre-enrichment medium has also aided molecular detection from inoculated blood of humans and dogs (Chenoweth *et al.,* 2004; Maggi *et al.,* 2005; Cadenas *et al.,* 2007; Hammoud *et al.,* 2008).

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

HIV-positive patient recruitment and blood handling

Ethical approval was granted (protocol number: M070637) by the Human Research Ethics Committee (medical) of the University of the Witwatersrand (Wits), Johannesburg (Appendix 2; Section 2.2.1a) for the use of 384 HIV-positive patient volunteers and 384 clinically healthy

volunteers. Permission was obtained from the relevant authorities to recruit volunteer patient participants and collect blood from them at the HIV-clinic. A convenience sample of 382 HIV-positive patients were recruited from the Chris Hani Baragwanth Hospital (Soweto, Gauteng), HIV-clinic. HIV-status was known as the clinic only treats HIV-positive patients. Specimen collection started on the 14th September 2007 and completed end of January 2008.

Survey objectives and consent forms were explained to prospective participants by an available HIV-counselor employed at the clinic, a laboratory aide or by the phlebotomists drawing the blood samples. Patients were assured that the study was voluntary and results would not directly benefit nor disadvantage them in any way. Informed consent forms (Appendix 2; Section 2.2.1b) were completed by the participating patients before blood collection. The recruited volunteers were provided with an information sheet containing the contact details of the Special Bacterial Pathogens Reference Unit (SBPRU) at the National Institute for Communicable Diseases (NICD) should they have any queries. Results were returned to practicing clinicians at the clinic to do with as they saw fit for management of participating patients.

Two 4 ml tubes of blood were collected from each volunteer, 1 tube containing the anticoagulant ethylenediaminetetra-acetic acid (EDTA) and the other a plain red-top tube in which the blood was allowed to clot. Collected blood was numbered and temporarily stored in a polystyrene cooler box for transport. Signed consent forms were numbered and filed. Between 20 and 45 patients' specimens were collected per day. Sampling in 2007 took place weekly on a Wednesday, or Thursday, or Friday as time permitted and later increased to 3 times a week in the month of January 2008, on a Wednesday, Thursday and Friday for 2 weeks. Blood samples were transported to the laboratory. Clotted blood samples were spun down at 4000 rpm for 10 min at room temperature separating the serum from the blood clot. The serum was aliquoted into 2 ml tubes and stored at minus 20 °C. The EDTA blood was not spun down, but was aliquoted into 2 ml tubes and frozen at minus 20 °C until use.

Clinically healthy volunteer recruitment and blood collection

Convenience samples of assumed healthy volunteers were recruited from the NICD and the animal shelter from which felid and canine specimens were collected. Due to ethical considerations, the volunteers were not asked their HIV-status. Health was assumed on the basis of volunteers being at work on the day of blood collection with no observable signs of illness.

Informed consent (Appendix 2; Section 2.2.1c) was obtained and 2 tubes (4 ml each) of blood, 1 EDTA and 1 whole clotted blood were drawn by a qualified clinician. The clotted blood specimens were spun at 4000 rpm for 10 min. The sera was separated from the blood and stored in a 2 ml tube at minus 20 ℃. The EDTA blood was handled differently to the HIV-positive samples as the HIV-positive samples were the first to be collected and the processes involving sample handling were later refined. These were spun down at 4000 rpm for 50 – 60 min; the plasma was separated from the whole blood, stored in a 2 ml tube and frozen at minus 20 ℃. The EDTA blood was not freeze-thawed prior to use and was stored at 4 ℃ for no longer than 1 week before being processed for testing. Processed samples were stored at minus 20 ℃.

Collection of cat and dog blood specimens

Ethical approval was granted (clearance certificate number: 2007-112) by National Health Laboratory Service Animal Ethics Committee (NHLS-AEC) (Appendix 2; Section 2.2.1d) and by the Animal Ethics Screening Committee (AESC) of Wits University (Clearance certificate number: 2008/56/03) for the use of 384 cat and 384 dog blood specimens (Appendix 2; Section 2.2.1e). Permission was also granted by the governing body of a national animal welfare organisation. Specimens were obtained from animals undergoing euthanasia, surgery or sterilization procedures at a Gauteng animal shelter.

Two 1.5 ml blood samples were obtained from each cat and dog being euthanized at the shelter's animal hospital. The information accompanying the samples was: type of animal, age, region from which it was rescued, and whether the animal was feral or not. Upon receipt, the samples were numbered and spun down at 4000 rpm. The clotted blood was spun down for 10 min at room temperature and the serum was separated from the blood. The EDTA blood was spun for 50 – 60 min at room temperature separating the plasma from the whole blood. All samples were stored at 4 ℃ for no longer than 1 week before being tested. Specimens were stored at minus 20 ℃ once testing was complete.

Rodent blood and tissue collection

Rodent specimens were prospectively sourced from a Gauteng pest control company. Ethical clearance was obtained (clearance certificate number: 2007-112) from National Health Laboratory

Service Animal Ethics Committee (Appendix 2; Section 2.2.1d) and from AESC of Wits University (Clearance certificate number: 2008/56/03) for the use of 384 rodents (Appendix 2; Section 2.2.1e). Rodents from Ekhuruleni Metropolitan were live trapped, anaesthetised using CO_2, and exsanguinated on the same day. Trapping was done by trained municipal pest control officers, and rodents were caught primarily for pest control and plague surveillance programmes. Wire cage traps (Figure 2.1) baited with grain and other food scraps were set out in the afternoons in predetermined problematic areas. Traps were checked for rodents early the next morning. Cages containing rodents were placed into containers and transported to the handling sites. Upon arrival at the handling site, the laboratory was notified that rodents had been delivered.

Figure 2.1 Wire cages used for rodent trapping. (Picture source: trapman.co.uk)

At the handling sites, the cages containing rodents were placed into a large clear plastic bag which was sealed at the opening. CO_2 was fed into the bag and the activity of the rodents was monitored. Once the rodents were unconscious, they were removed from the cage and handled one at a time. Rodents were bled by cardiac puncture until exsanguinated. Depending on size of the rodent, 2 to 8 ml of blood was drawn and aliquoted into a red-top plain tube and an EDTA tube. Specimens were numbered and handled as described for the felid and canine specimens.

The blood samples were separated by centrifugation at 4000 rpm. Clotted-blood was spun for 10 min, and EDTA blood was spun for 50 – 60 min. Separated samples were stored at 4 °C until testing (within 1 week of collection) and the plasma and sera were frozen at minus 20 °C. EDTA blood samples were also stored at minus 20 °C once the testing had been completed. The following information was gathered for each rodent: gender, sexual condition, mass, body length, tail length, left hind foot length, right ear length, and species of rodent.

2.2.2 Culture of control strain and selection of media

The American Type Culture Collection (ATCC) control strains were imported from the USA and propagated according to the product propagation procedure included with the organism. The following control stains were used: *B. henselae* Houston I (ATCC 49882), *B. clarridgeiae* (ATCC 70095), *B. grahamii* (ATCC 700132), *B. vinsonii* subsp. *berkoffii* (ATCC 51672), and *B. elizabethae* (ATCC 49927).

All *Bartonella* culture work was carried out in a class 2 biosafety cabinet in order to minimize the possibility of specimen contamination. Culture vials from ATCC were hydrated with 500 µl of brain heart infusion (BHI) broth (Diagnostic Media Products, Gauteng). Ten microliters of the reconstituted culture mixture was inoculated onto the appropriate media (Table 2.1) and placed into rectangular anaerobic containers. Inoculated media was incubated at 37 °C and under appropriate atmospheric conditions created by CO_2 AnaeroPack (Mitsubishi Gas Chemical Co. Inc.) for at least 5 days.

After the minimum 5 days incubation period, cultures were assessed for signs of bacterial growth. The inoculated media were re-incubated under the same conditions for a further week if bacterial growth was not observed. If growth occurred the culture was sub-cultured onto fresh media and stored in tryptic-soy broth (TSB) and 10% (v/v) glycerol (Diagnostic Media Products, Gauteng). The TSB and 10% (v/v) glycerol mixtures were stored at minus 80 °C.

Table 2.1 *Bartonella* spp. media, growth conditions and morphology

CULTURE ID:	RECONSTITUTION MEDIA:	GROWTH MEDIA:	DAYS INCUBATED:	CONDITIONS:		MORPHOLOGY:
				Temp:	Atmosphere:	
B. henselae (ATCC 49882)	Brain-heart infusion broth	*Bartonella* media*	5-10	37 ℃	5% CO_2	• Small • metallic sheen • some pitting into media • non-hemolytic
B. clarridgeiae (ATCC 70095)	Brain-heart infusion broth	*Bartonella* media **biphasic slant***	7–10	37 ℃	3 – 5% O_2, & 10% CO_2	• Small • metallic sheen • non-hemolytic
B. grahamii (ATCC 700132)	Brain-heart infusion broth	*Bartonella* media *	2–3	37 ℃	5% CO_2	• Small • metallic sheen • smooth colonies • non-hemolytic
B. vinsonii* subsp. *berkoffii (ATCC 51672)	Brain-heart infusion broth	*Bartonella* media *	2–3	37 ℃	5% CO_2	• Small • metallic sheen • smooth colonies • non-hemolytic
B. elizabethae (ATCC 49927)	Brain-heart infusion broth	*Bartonella* media *	2–3	37 ℃	5% CO_2	• Small • metallic sheen • smooth colonies • non-hemolytic

***in-house laboratory media production (See Appendix 2; Section 2.2.2)**

2.2.3 Blood culture method

All HIV-positive, healthy volunteer, cat, dog, and rodent blood was cultured for the presence of *Bartonella* spp. Culturing was done in a class 2 biohazard safety cabinet to reduce contamination. EDTA blood was used for culture unless the EDTA specimen was insufficient (especially for cat and dog samples), in which case some of the separated clotted EDTA blood was used. Approximately 100 – 150 µl of each whole blood sample was inoculated onto fresh *Bartonella* media and spread over the surface using sterile glass spreaders. Inoculated media were placed under 5% CO_2 at 37 ℃ for 7±2 days before being checked for growth.

If growth was present, the colonies were inspected for the following characteristics: pitting into the media, small dry colonies, and a slight metallic sheen, resembling the appearance of the ATCC culture strains. Cultures were assessed for amount of growth. Media with faint or no growth were re-incubated as previously stated to a maximum of 2 months with periodic examinations for growth. If growth resembling the control cultures was observed, colonies were Gram stained, and plated out onto fresh media. The initial isolation plates were re-incubated with the freshly sub-cultured ones for a further 7 days.

All blood cultured isolates were confirmed by molecular methods (covered in Chapter 3) and stored in TSB with 10% (v/v) glycerol at minus 80 ℃.

2.3 RESULTS

2.3.1 Culture of control strain and selection of media

All ATCC control strains except *B. clarridgeiae* were successfully cultured on *Bartonella* media. The controls were grown on the recommended media (tryptic soy agar with 5% (v/v) sheep blood); however, it was found that the growth was better on in-house laboratory-produced *Bartonella* media. *B. henselae*, *B. elizabethae*, and *B. vinsonii* subsp. *berkoffii* all grew within 2 – 4 days of inoculation whereas *B. grahamii* took up to 7 days to grow. The *B. clarridgeiae* was the most difficult to culture as it was the only species that required a broth-agar slope in order to grow and required nearly 2 weeks to demonstrate growth.

Morphologies of the cultures, except *B. clarridgeiae*, were very similar and colonies were very small even after a prolonged incubation period (Figure 2.2).

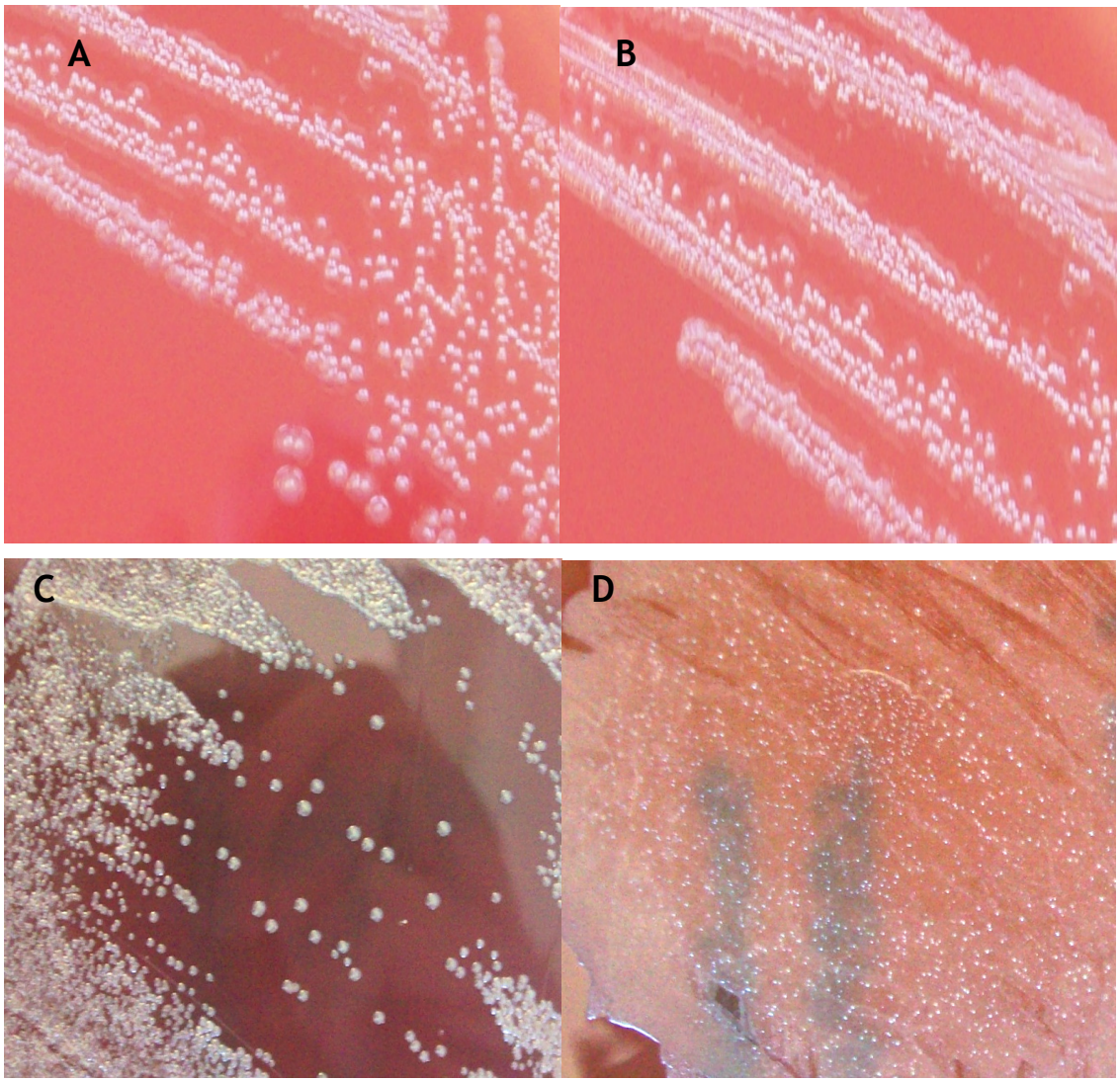

Figure 2.2 ATCC control cultures grown on 5% (v/v) rabbit blood-supplemented Columbia agar incubated at 37 ℃ in 5% CO_2 for 7 days; A, *B. henselae* (ATCC 49882), B, *B. elizabethae* (ATCC 49927), C, *B. vinsonii subsp. berkoffii* (ATCC 51672), and D, *B. grahamii* (ATCC 700132).

2.3.2 HIV-positive patients

A total of 382 HIV-positive patients attending the HIV clinic volunteered to participate in the study. Two-hundred and forty-six (246 i.e. 64%) were women and 136 (36%) were men. Volunteers attended the clinic for antiretroviral treatment (ART), baseline testing, viral load testing, co-infection follow-ups, and administration of broad-spectrum antibiotics against opportunistic infections.

EDTA blood samples (whole blood including plasma) were plated out after having been freeze-thawed from minus 20 °C. Blood cultures from this sample group yielded no *Bartonella* spp. isolates. Approximately a third (130/382) of the blood cultures were contaminated by fungal or environmental bacteria growth due to the prolonged incubation periods. In order to decrease the amount of contamination, amphotericin B was included in the blood culture technique. To assess the effect of amphotericin B on the growth of *Bartonella*, the *B. henselae* ATCC strain was inoculated into BHI broth with 5% (w/v) amphotericin B and plated out onto fresh *Bartonella* media. Upon assessment, it was observed that although amphotericin B does reduce the amount of fungal contamination, it also suppresses the growth of the control culture. The addition of amphotericin B was therefore discontinued. Due to the low circulating bacterial load often observed in natural infection, the risk of suppression, although slight, was not one worth taking. If a culture became contaminated with fungal growth, the culture was repeated.

No HIV-positive patient blood cultures yielded *Bartonella* spp. To ensure that *Bartonella* was not missed, any cultured organisms were Gram-stained. Any Gram-negative pleomorphic bacilli were sub-cultured and *Bartonella* was excluded by PCR (Chapter 3).

2.3.3 Healthy volunteers

Healthy volunteers were required to determine the normal infection rates in healthy individuals. A total of 42 healthy volunteers were recruited; 7 specimens were obtained from staff working at the animal shelter from which the cat and dog samples were obtained and 35 samples were collected from staff of the NICD. All specimens were freshly cultured onto the *Bartonella* media. Only 1 of the 42 blood specimens illustrated growth indicative of *Bartonella* spp. The morphology and Gram-stain (Gram-negative, pleomorphic bacilli) result was consistent with *Bartonella*. Unfortunately, the culture failed to grow on sub-culture and final confirmation was done by PCR of DNA extracted from the blood of the volunteer.

The 7 animal shelter volunteers have daily exposure to many different animals including stray/feral cats and dogs. Cats and dogs have been associated with *Bartonella* spp. infections (Barnes *et al*., 2000; Ketring *et al.,* 2004). It was therefore expected that that this group would be most likely to demonstrate *Bartonella* bacteraemia. This was not the case in this study, although it would be necessary to obtain a larger sample population in order to obtain a better prevalence estimate.

2.3.4 Cats and dogs

No dog (n=179) blood specimens yielded *Bartonella* isolates.

Cat blood cultures (n=98) yielded 5 (~5% culturable prevalence) culture and PCR-confirmed *Bartonella* spp. It was concerning that the culturable prevalence was so low and that 3 of the 5 isolates came from a single batch of 7 feline blood samples received from the animal shelter, whereas preceding batches had not yielded culture isolates. To exclude the possibility of cross-contamination, the blood samples were repeated and DNA was extracted. It was confirmed that the blood samples were positive for the *Bartonella* spp. on culture and confirmed by nested PCR (discussed in Chapter 3).

Samples taken from euthanized animals were stored in the fridge for up to 3 weeks before being processed. It is therefore hypothesized that *Bartonella* bacilli (if present) die in the blood stored at 4 ºC before being cultured. Supporting this hypothesis is the fact that isolates were obtained when the blood specimens were plated out within 1 week of collection from the animals. Furthermore, the fact that PCR detected *Bartonella* DNA in the blood, illustrates that *Bartonella* spp. were obviously infecting the cats and dogs from which we received specimens.

2.3.5 Rodents

A total of 124 rodent samples taken from *Rattus norvegicus* and *R. rattus* were cultured on *Bartonella* media. Only 16 (13% culturable prevalence) *Bartonella* isolates were cultured and confirmed by PCR. All isolates were Gram-negative, pleomorphic bacilli that illustrated morphology consistent with the ATCC control cultures. Primary isolation occurred between 5 and 9 days, with sub-cultures growing within 4-5 days. All isolates were obtained from the blood of adult *Rattus rattus* and *Rattus norvegicus*, with no apparent bias for gender of rodent, infecting 8 female and 8 male rats. Rodent weights ranged from 127.7 g to 449.7 g with a mean weight of 318.8 g.

There were at least 2 different morphologies observed for the rodent isolates and these were later identified by sequencing.

Morphology type 1: colonies were tiny, pin-point, smooth, moist, and slightly metallic. This species grew on the *Bartonella* media surface rather than pitting into it and were highly adhesive, and quite difficult to emulsify in sterile water.

Morphology type 2: colonies were drier and rougher, with variable degrees of pitting into the media. They too had a metallic sheen and were tiny pin-point colonies, but were far more difficult to scrape off the media, although emulsification of the bacteria in sterile water was easier.

2.4 DISCUSSION

Bartonella remains one of the most difficult organisms to detect via blood or tissue culture. A comparative study performed on HIV-positive in- and outpatients in the San Francisco Bay- area hospitals illustrated 2.9% (11/382) culture prevalence (Koehler *et al*., 2003). Frean *et al.* (2002) looked at the prevalence of *Bartonella* in HIV-positive out-patients at several hospitals in Johannesburg and found a 10% PCR prevalence of *Bartonella*. This indicates current bacteraemia since the PCR targets bacterial DNA. A conservative expectation was to find at least 10% culture-positive specimens for *Bartonella* spp. Immunosuppression means that contraction of opportunistic infections (e.g. *Bartonella* spp.) is highly possible and as a precaution, broad-spectrum antibiotic treatments are periodically prescribed for HIV-positive patients. The effect of broad-spectrum antibiotics on *Bartonella* infections lacks clarity. Research has been conducted on *in vivo* and *in vitro* antimicrobial sensitivities of *Bartonella* to various drug classes. *In vitro* testing has shown bactericidal efficacy, but *in vivo* tests have demonstrated mostly bacteristatic activity, with the exception of aminoglycosides which are bactericidal (Florin *et al*., 2008). The bacteriostatic activity of the broad-spectrum drugs may be suppressing already low circulating bacteremia to undetectable levels for culture in some specimens.

The hypothesized major cause of low cultured isolate yield is the freeze-thawing of blood samples (particularly HIV-positive specimens) killing off the circulating bacteria before opportunity is had for the samples to be inoculated onto the culture media. Published methodologies were followed for the initial starting point and it was later discovered that the method of fresh inoculations of sample blood onto the *Bartonella* media yielded more promising results. The lack of cultured *Bartonella* in human blood does not indicate that the prevalence is zero, since *Bartonella* spp. were detected by PCR (Chapter 3) and IgM IFA (Chapter 4).

Various studies carried out globally to determine the culture-prevalence of *Bartonella* spp. in felines have suggested that the prevalence can be from 1.6% to as high as 53%. A study carried out in Nancy, France looked at the stray feline population from 10 colonies within and around the town. Of the 94 stray cats that were enrolled, 50 blood samples (53%) were positive by culture (Heller *et al*., 1997). In another culture-based study carried out in the UK, 9.4% (n=360) cats where positive for *B. henselae* (Birtles *et al*., 2002). A Canadian study reported a *B. henselae* culture prevalence of 1.6% (14/896) in healthy cats (Kamrani *et al*., 2008), whereas another study reported that the culture technique used failed to yield any meaningful data due to the degree of contamination of the media (Rampersad *et al*., 2005). In an Australian study, the culture prevalence was reported as 35% (27/77) positive for *B. henselae*. This study further suggested that feral cats had a higher culture-prevalence (24/59) than domestic cats (3/18) (Branley *et al*., 1996).

When dog populations were assessed for *Bartonella*, the following culture-prevalences were reported: in Bristol, UK, no *Bartonella* spp. were isolated from the blood of 211 dogs (Birtles *et al*., 2002), and in Grenada, 1.4% (1/73) (Yabsley *et al*., 2008) was found positive for *Bartonella* spp. Very little has been done on determining the culture-prevalence of canine *Bartonella* spp.

Rodent populations have also been tested for *Bartonella* culture-prevalences. In Taiwan,10.3% (6/58) *Rattus* spp. rats were found to be infected with *Bartonella* (Lin *et al*., 2008) and 41.5% (137/330) from various other rodent spp. (Bai *et al*., 2009), whereas a Chinese study reported 43.5% (57/131) *Bartonella* spp. culture prevalence in rodents from the Yunnan province (Ying *et al.,* 2002). The culture prevalence of *Bartonella* spp. in *R. rattus* was studied and the following was reported: Tel Aviv, Israel, 16% (10/62) (Harrus *et al*., 2009), Dhaka, Bangladesh 32.3% (32/99) (Bai *et al*., 2007), and in southern China 12.5% (3/24) (Castle *et al*., 2004) culture prevalence.

The general consensus for *Bartonella* spp. is that it remains one of the most difficult organisms to culture. Although culture is regarded as the gold standard for detection, it is limited and problematic. The diagnosis of bartonellae is fast moving towards molecular techniques. This is described in the next chapter.