CHAPTER 4

Detection of *B. henselae* and *B. quintana* in humans using Immunofluorescent Assays (IFA) targeting IgG and IgM antibodies

4.1 INTRODUCTION

Clinically-suspected CSD is usually confirmed by serological antibody assays. Immunoglobulin M (IgM) antibodies against bartonellae may be absent in the early stages of CSD, and negative results do not exclude the presence of acute disease (Marakaki *et al.,* 2003). Antibody titers of both immunoglobulin G (IgG) and IgM might be low, and diagnosis can be confirmed with rising titers in a second serum sample (Marakaki *et al.,* 2003). CSD infection typically does not respond to antibiotic therapy and clinical manifestations may be due to immunological responses within the lymphatic system (Rolain *et al.*, 2004).

Immunofluorescent assays (IFA) are used for the detection of IgG or IgM antibodies against specific antigens. *Bartonella* IgM and IgG test kits are designed to detect the antigen-antibody complexes when diluted sera are presented with *Bartonella* antigens on commercially produced slides. A fluorescein-labeled anti-IgG or IgM conjugate is responsible for visualization of reactions.

Although the IFA method is often useful and has a high sensitivity for *Bartonella* antibody detection, it has various limitations. For example; *Bartonella* IFAs have been reported to cross-react with other human pathogens, including *Coxiella burnetii*, *Chlamydia* spp., *Rickettsia rickettsii, Ehrlichia chaffeensis, Treponema pallidum, Francisella tularensis,* and *Mycoplasma pneumoniae* (Drancourt *et al.,* 1995; McGill *et al.*, 1998; McCool *et al.,* 2008). A major limitation is the subjectivity of IFA readings and time required for reading the tests. Expensive fluorescence microscopes and experienced operators are

considered necessary for result interpretation (McCool *et al.,* 2008). Results from *Bartonella* serology should ideally correlate with clinical history.

Another serological method often used for *Bartonella* detection is enzyme-linked immunosorbent assays (ELISA) where the 50s ribosomal protein L7/L12 (RplL) or a 17-kDa protein is used as antigen to which antibodies, when present will react (Loa *et al.*, 2006; McCool *et al.*, 2008). Results are read either objectively by sight, or by computerized ELISA readers.

Indirect serological methods are practical, economical and often better than direct methods which detect the actual organism (Johnson *et al*, 2003). Detection of antibodies against *Bartonella* can determine both current, acute phase, and past infections.

The objective was to determine IgG and IgM seroprevalence of *Bartonella* exposure in HIV-positive out-patients and clinically healthy volunteers tested by IFA.

4.2 MATERIALS AND METHODS

4.2.1 Samples tested

A total of 382 HIV-positive patient sera, 42 healthy volunteer sera (collected as described in Chapter 2), and 342 HIV-negative residual antenatal survey sera were screened for IgG and IgM antibodies to *B. henselae* and *B. quintana*.

Residual sera from HIV-negative antenatal women

Residual sera from the 2006 public antenatal HIV/syphilis seroprevalence survey conducted by the Department of Health were used to determine seroprevalence of *Bartonella* in HIV-negative women. Although these sera were female biased, the population groups from which these sera were initially taken matched the HIV-positive population group by age and geographical area relatively well. Three-hundred and forty-two sera (342) were selected on the basis of HIV status (HIV-negative) and age (18

through to 65). No confidential information that would trace back to antenatal patient volunteers was available and samples were relabeled with unique study numbers.

4.2.2 Immunofluorescence assays (IFA)

The prevalence of *B. henselae* and *B. quintana* in the human populations of Gauteng were determined by IFA. Commercially available kits manufactured by Focus Diagnostics (Cypress, USA) were used to screen the sera as per package insert instructions. Each kit consisted of either IgG or IgM *Bartonella* slides and included positive and negative controls (Appendix 4, Section 4.2.2).

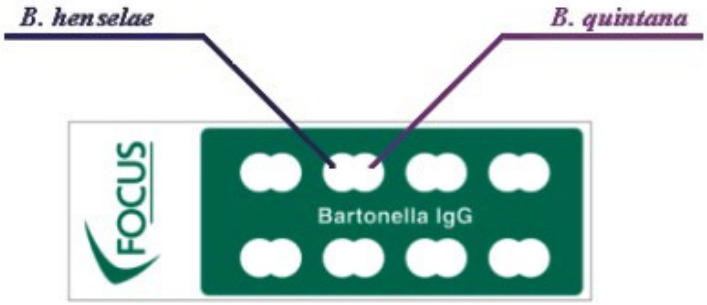

Figure 4.1 IgG commercially-purchased slide from Focus Diagnostics illustrating antigen positioning of *B. henselae* and *B. quintana* for each well. *NOTE: the positions are reversed when viewed under the microscope lens, i.e. *B. henselae* is viewed on the right and *B. quintana* on the left side of each well.

Serum was pretreated in relevant IgG or IgM diluent. The 10x IgG diluent was pre-diluted (1:10 dilution) with Phosphate buffered saline (PBS, pH 7.2) buffer to a 1x concentration prior to serum dilution. The IgM diluent was used as provided by the kit. For IgM tests, the sera were diluted 1:20 (5 µl serum: 95 µl IgM pretreatment diluent) and for IgG tests, sera were diluted to 1:64 (2 µl serum: 126 µl IgG 1x sample diluent working solution). The IgG diluent consists of PBS with 10% normal goat serum which blocks non-specific binding and unwanted background staining, whereas the IgM diluent consists of buffered isotonic solution containing anti-human IgG that removes both free and complexed IgG from the sample.

Twenty microliters (20 µl) of the pre-diluted sera, positive control, and non-reactive control was applied to allocated wells on either an IgG or IgM slide (Figure 4.1). The slides were placed into a glass petri-dish containing moist filter-paper in order to create a humid environment during incubation and prevent the slides from drying out. Slides were incubated for 90 ± 2 min at 37˚C. At the end of incubation periods, the slides were soaked in PBS for 10 min, briefly dip-rinsed in distilled water and allowed to air dry. Twenty microliters (20 µl) of either the IgM or IgG conjugate was applied to each well and was incubated as before for 30 ± 2 min. The slides were rinsed as described above and allowed to air dry. A drop of mounting medium was applied to each well and the slide wells were covered with a 24 x 50 mm coverslip. The slides were viewed at a final magnification of 400x on a fluorescence microscope.

Positive reactions were viewed as bright apple-green fluorescent bacteria against a red Vero-cell background in both IgG and IgM IFAs. Test results were validated against reactive and non-reactive controls (Figures 4.2 and 4.3).

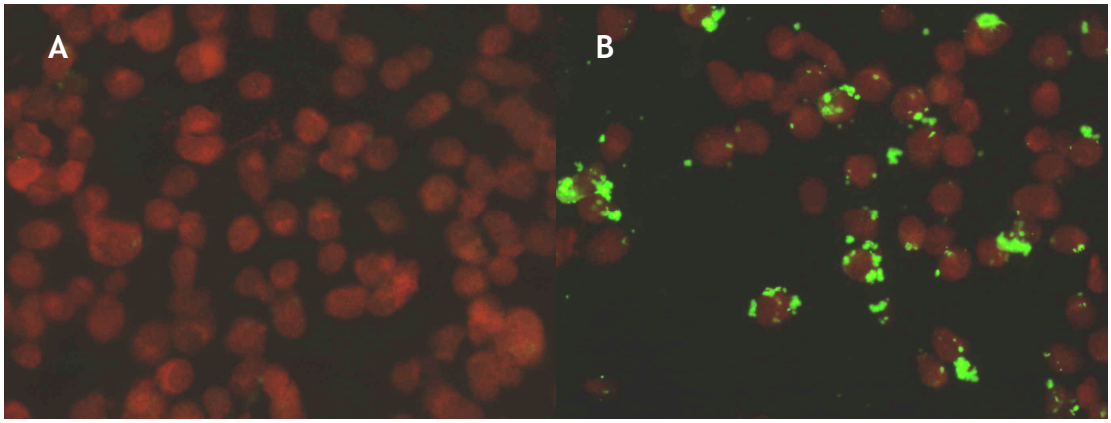

Figure 4.2 IgG internal kit-provided controls. A) non-reactive control; B) polyvalent positive control. Internal controls provide very distinct results with no background staining.

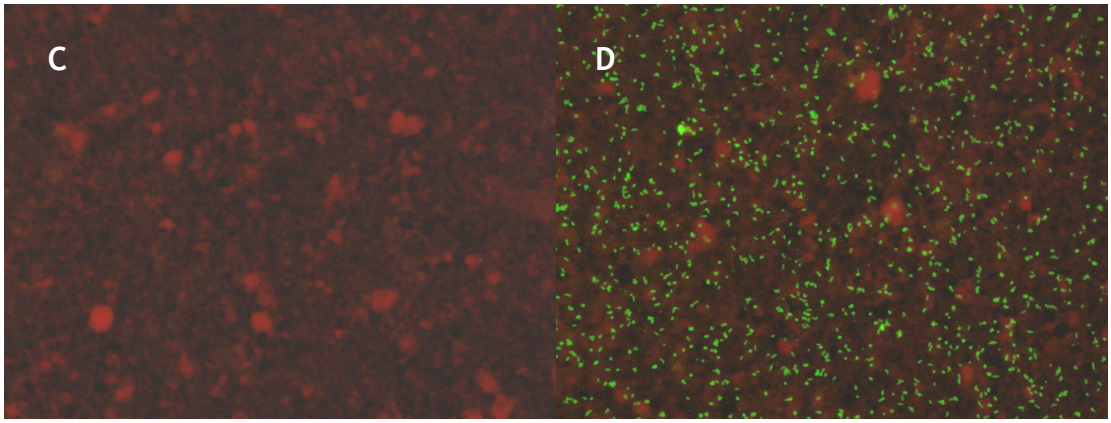

Figure 4.3 IgM internal kit provided controls. C) non-reactive control; D) polyvalent positive control.

4.3 RESULTS

4.3.1 HIV-positive immunofluorescence assays

The IgG seroprevalence for all HIV-positive sera collected was 32% (121/382) (95% confidence; 26.8 – 36.6) and the IgM seroprevalence was 14% (53/382) (95% confidence; 10.7 – 17.8). Tests were scored according to antigen fluorescence. Results were grouped into 1 of 4 categories: BH if only the *B. henselae* antigen fluoresced, BQ if only the *B. quintana* fluoresced, BHQ was scored if both antigens fluoresced equally, and NEG if neither antigen fluoresced, or if there was only background fluorescence.

Overall prevalence was calculated by adding together all positive results, i.e. BH, BQ and BHQ results collectively. Of the IgG positive samples, 73/121 (60%) were BHQ positive, 30/121 (25% overall) were BH positive, and 18/121 (15%) BQ positive. The IgM prevalence was lower for each category. Only 3% prevalences were found for both BH (12/382) and BQ (10/382) individually. The overall IgM prevalence for BHQ was 8% (Table 4.1).

Table 4.1 *Bartonella henselae* and *Bartonella quintana* indirect immunofluorescence assay (IFA) results for HIV-positive sera (n=382)

ALL HIV-POSITIVE SERA:

IFA (IgG) results		IFA (IgM) results					
		NEG	BHQ	BH	BQ	**Grand Total**	
	NEG*	**232**	**18**	**6**	**5**	**261**	68%
	BHQ*	**58**	**10**	**2**	**3**	**73**	19%
	BH*	**24**	**3**	**3**	**-**	**30**	8%
	BQ*	**15**	**-**	**1**	**2**	**18**	5%
Grand Total		**329**	**31**	**12**	**10**	**382**	
		86%	8%	3%	3%		

*NEG, negative; BHQ, *B. henselae* & *B. quintana;* BH, *B. henselae;* BQ, *B. quintana.*

The results of IgM and IgG tests were correlated to determine how many samples were positive for both IgG and IgM screen tests. Of the 382 samples tested, 226 (60%) were negative for both IgG and IgM antibodies. When the IgG and IgM prevalence results for this sample group were compared at 95% confidence, a highly significant difference (p-value: <0.0001; chi-square statistic was 34.412 with 1 degree of freedom) was detected indicating that it is highly unlikely that results obtained were by mere chance.

HIV-positive females (n=246) showed a marginally lower *Bartonella* seroprevalence than HIV-positive males (n=136). Figure 4.4 illustrates the IgM and IgG results comparatively for male and female HIV-positive participants. Males were not significantly different to females when IgG (males: 33%; females: 31%) results were compared (p-value: 0.6877 chi-square statistic: 0.1066 with 1 degree of freedom); however, there was a non-significant difference for IgM (males: 15%; females: 14%) seroprevalences (p-value: 0.0593, chi-square statistic: 3.558 with 1 degree of freedom).

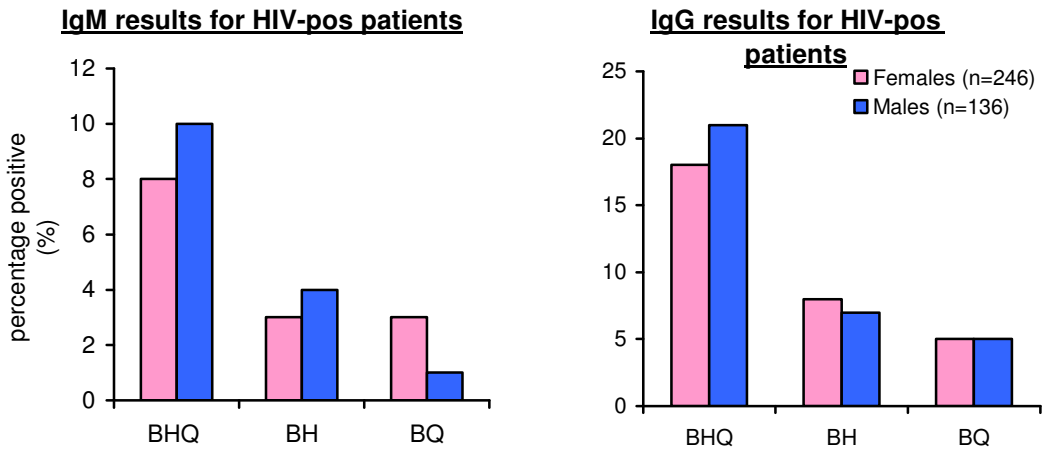

Figure 4.4 IgM and IgG *Bartonella henselae* and *Bartonella quintana* IFA results for HIV-positive males (n=136) and HIV-positive females (n=246)

In order to assess whether a difference exists in different age groups, the results were stratified by age: <25, 26 – 35, 36 – 45, 46 – 55, and >55 years. The IgM and IgG results are shown in Figures 4.5 and 4.6.

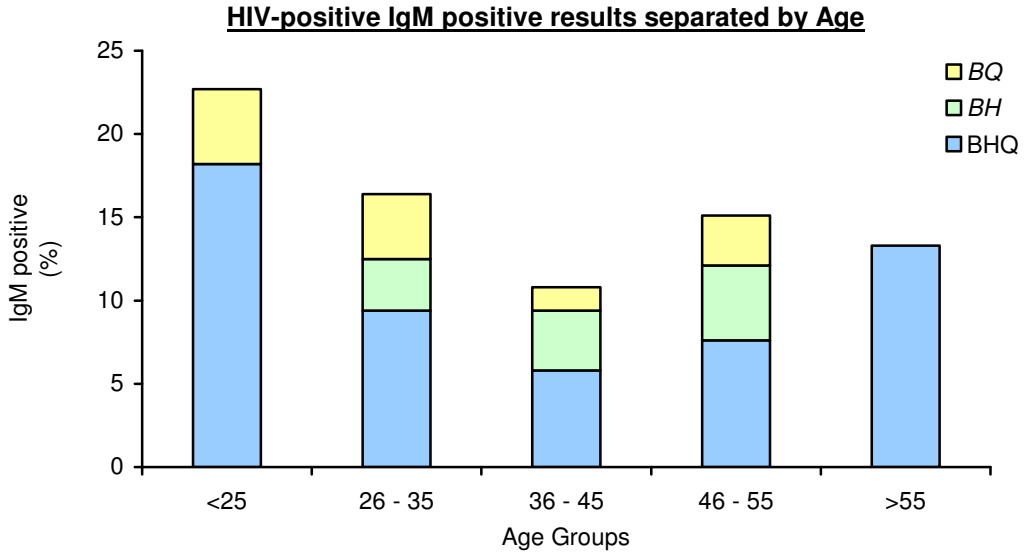

Figure 4.5 IgM results stratified by age. The sum of positives for BHQ, BH, and BQ gives the total positive prevalence for the following age groups: <25, 26 – 35, 36 – 45, 46 – 55, and >55 years.

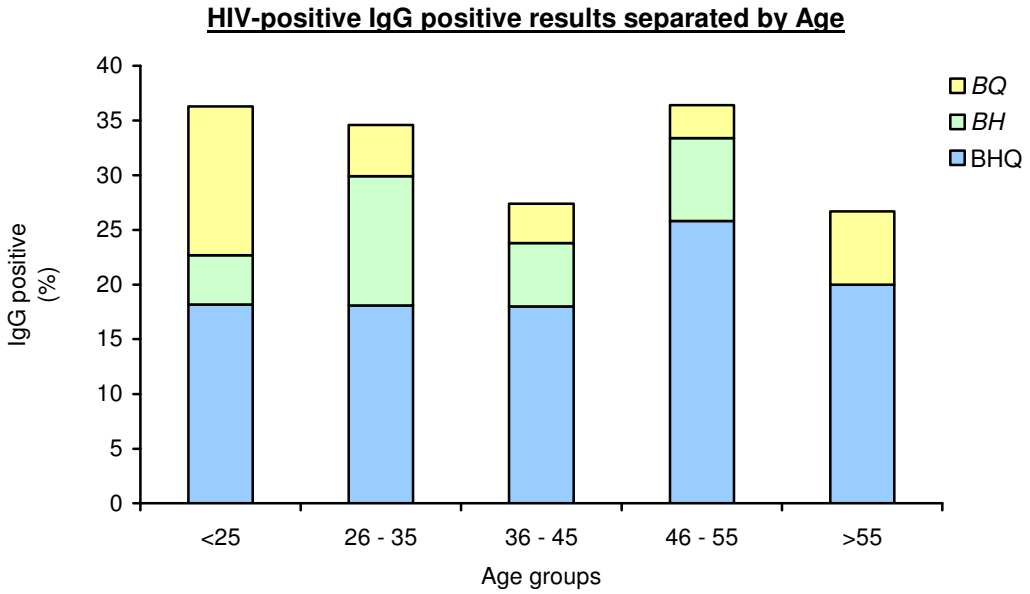

Figure 4.6 IgG results stratified by age. The sum of positives for BHQ, BH, and BQ gives the total positive prevalence for each age group.

The <25 year age group illustrated the highest prevalence to *Bartonella* antibodies for IgM (22.7%) and second highest for IgG (36.4%). The >55 age group had the lowest IgG prevalence (26.7%) and the second lowest IgM prevalence (13.3%). The lowest IgM prevalence was found in the 36 – 45 age group and the highest IgG prevalence was in the 46 – 55 year age group (Data tables in Appendix 4, Section 4.3.1a).

4.3.2 Healthy volunteer seroprevalence of *Bartonella* antibodies

The IgM overall prevalence was 17% (7/42) (95% confidence; 7.5 – 32.0) and the overall IgG prevalence was 19% (8/42) (95% confidence; 9.1 – 34.6) for the healthy volunteers (Table 4.2). At 95% confidence, it was found that there was a non-significant difference between IgG and IgM prevalences (p-value: 0.7757; chi-square statistic: 0.081 with 1 degree of freedom) therefore the 2% difference between IgG and IgM results was attributed to chance.

Table 4.2 *Bartonella henselae* and *Bartonella quintana* indirect immunofluorescence assay (IFA) results for healthy volunteer sera (n=42)

ALL HEALTHY VOLUNTEER SAMPLES:

IFA (IgG) results	IFA (IgM) results				Grand Total	
	NEG	BHQ	BH	BQ		
NEG	**31**	**2**	**-**	**1**	**34**	81%
BHQ	**2**	**2**	**-**	**-**	**4**	10%
BH	**1**	**-**	**2**	**-**	**3**	7%
BQ	**1**	**-**	**-**	**-**	**1**	2%
Grand Total	**35**	**4**	**2**	**1**	**42**	
	83 %	10%	5%	2%		

Seven of the 42 clinically healthy volunteers were recruited from the animal welfare shelter providing us with cat and dog blood specimens. These volunteers are potentially at high risk of *Bartonella* exposure due to their occupation, and although the sample number was small, it was found that 3 of the 7 volunteers were antibody positive for *Bartonella*. A need to screen more individuals with increased exposure to domestic and feral animals is thus highlighted.

Due to the small sample group and noticeable bias towards the feminine, it was difficult to draw any conclusions about the difference in prevalences between healthy males (n=9) and healthy females (n=33).

4.3.3 HIV-negative Immunofluorescence assays from antenatal survey residual sera

The antenatal survey residual samples illustrated an IgM and IgG seroprevalence of 18% (95% confidence; 11.9 – 22.7) (Table 4.3).

Table 4.3 *Bartonella henselae* and *Bartonella quintana* indirect immunofluorescence assay (IFA) results for antenatal survey residual sera (n=342)

ALL HIV-NEGATIVE ANTENATAL SURVEY SERA:

IFA (IgG) results	IFA (IgM) results: NEG	BHQ	BH	BQ	Grand Total	
NEG	**227**	**21**	**19**	**12**	**279**	82%
BHQ	**32**	**1**	**2**	**1**	**36**	10%
BH	**15**	**1**	**2**	**-**	**18**	5%
BQ	**6**	**2**	**-**	**1**	**9**	3%
Grand Total	**280**	**25**	**23**	**14**	**342**	
	82%	7%	7%	4%		

All residual antenatal sera were female thus it remains unknown whether HIV-negative males share a similar seroprevalence, although, it was speculated that males would share a similar seroprevalence to the female population.

When results for the HIV-positive patient volunteers were compared to the HIV-negative sample group, a highly significant difference was found for IgG (p-value: <0.0001; chi-square statistic: 16.724 with 1 degree of freedom) but not for IgM (p-value: 0.1179; chi-square statistic: 2.444 with 1 degree of freedom). The IgM prevalence for HIV-positives was lower than that of the HIV-negative sample group, although non-significantly different. It is hypothesized that due to decreased immunocompetence of the HIV-positive volunteers, although infection rates may be higher, the patients fail to elicit a significant immunological response.

4.4 DISCUSSION

The HIV-positive group results were compared with the healthy volunteer group at a 95% confidence. There was a non-significant difference between IgG results for HIV-positive compared to clinically healthy volunteers (p-value: 0.0913; chi-square statistic: 2.850 with 1 degree of freedom). There was no significant difference for the IgM results

of the two groups (p-value: 0.6221; chi-square statistic: 0.243 with 1 degree of freedom), thus differences were likely due to chance.

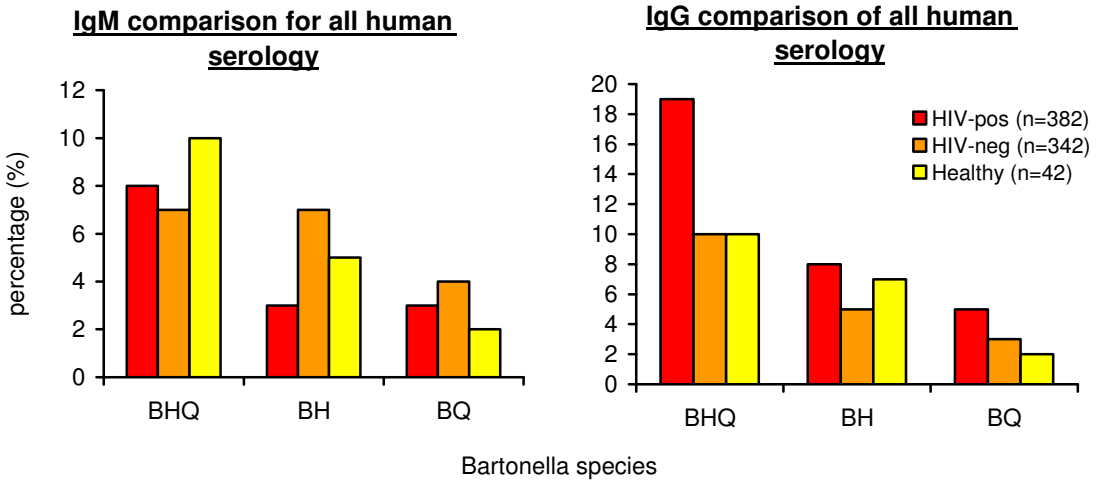

Figure 4.7 Bar graphs illustrating overall population group comparisons for IgM and IgG antibody seroprevalence.

Figure 4.7 illustrates the 3 sample groups: HIV-positive, HIV-negative, and clinically healthy volunteers where IgM and IgG results were compared. Sera fluorescing positive for both species made up the highest proportion of the positive results for IgG and IgM tests, for all tested groups. *B. henselae* has been isolated in cats and dogs, suggesting that dogs and cats may potentially be *Bartonella* reservoirs for human infection (Kim *et al*., 2009). The expectation was that *B. henselae* would be the most commonly isolated species due to its association with domestic animals and isolation from domestic cats in Gauteng. Rats and mice share overlapping niches with humans, and are likely to transmit rodent *Bartonella* spp. to humans. Particularly homeless and intravenous drug users living in streets in the USA (Comer *et al.,* 1996; Childs *et al.,* 1999; Ellis *et al.,* 1999; Comer *et al.,* 2001; Boulouis *et al*., 2005) or outdoor activities such as with Swedish elite orienteers (McGill *et al*., 2001; Boulouis *et al*., 2005) have increased human contact with rodents.

The IFA used for the determination of seroprevalence had various limitations which allowed this test to be an adequate screen test, but a suboptimal diagnostic tool.

According to the manufacturer, IFA kits were reported to have high sensitivity, and low specificity, with some cross-reactivity. The IgG kit suggested 97% sensitivity and 90% specificity for *Bartonella*, with cross-reactivity reported as negative 90% of the time. The IgM kit reported a lower sensitivity (42%), but boasted 100% specificity for *Bartonella*. The implication of this is that approximately 58% of the *Bartonella* infections are missed. Furthermore the kit insert suggests that this is the case or the *B. henselae*, however no work was done on the *B. quintana* for which antigen is presented on the slides. Cross-reactivity was reported negative 95% of the time when other taxonomically related organism antibodies were present in the sera (Regnery *et al.,* 1992b). Reading IFA results were subjective and time consuming, requiring both specialized microscopes and operator expertise (McCool *et al*., 2008).

IgM indicates acute infection and IgG a secondary immune response. The length of time for which IgG antibodies remain in an exposured individual is not definitively known, although it has been suggested that IgG antibodies may remain present in the blood for up to a year following infection. In initial stages of infection, it is possible for there to be both IgG and IgM antibodies present in the serum, thus it remains difficult to determine infection onset, and whether or not the infection is still active, particularly in HIV-positive individuals (Florin *et al*., 2008). Other limitations include: IgM antibody production in acute infections differs depending on the immunocompetance of the host, bacterial load, incubation period for infection, immune response, and non-species specific response of the host's immune system and consequent cross-reactivity. All limitations considered, IFA remains the most practical, albeit expensive, tool for *Bartonella* detection (Florin *et al*., 2008).

IFA seroprevalence testing has been carried out in many countries assessing both HIV-positive and clinically healthy populations. In a study carried out in Catalonia, Spain, 340 HIV-positive volunteers were tested for *Bartonella* IgG seroprevalence. The overall antibody seroprevalence for HIV-infected people was reported as 22.4%. Approximately 26% of the females (n=82) and 74% of the males (n=258) tested IgG positive for *Bartonella* (Pons *et al.,* 2008). Our study illustrated a significantly (p-value: 0.0026; with 1 degree of freedom) higher overall antibody seroprevalence for HIV-positive volunteers. In a retrospective study carried out in Sweden 59 serum samples (23/59 HIV-positive) taken at autopsy from intravenous drug abusers during 1987-1992 were examined. IFA

tests were carried out to assess the seroprevalence of 6 pathogenic *Bartonella* spp. The overall seroprevalence was reportedly 39% (23/59) and 48% (11/23) for the HIV-positive individuals (McGill *et al.,* 2003). A German study conducted with a sample group of 270 clinically healthy student volunteers reported a 30% seroprevalence of IgG antibodies to *B. henselae*. This paper did however express the concern that it was uncertain whether the high seroprevalence in healthy individuals was evidence of previous infection, acute infection, or due to cross-reactivity with other unrelated agents, for example *Coxiella burnetii* (Sander *et al*., 1998). In another Swedish study, 16.1% overall prevalence for a group of 498 healthy blood donors tested by IFA was reported. Antibodies for 6 pathogenic species were tested for including: *B. elizabethae, B. grahamii, B. henselae (Houston-1), B. henselae (Marseille), B. quintana*, and *B. vinsonii* subsp. *vinsonii*. It was found that *B. elizabethae* accounted for the highest seroprevalence (14%) and *B. vinsonii* subsp. *vinsonii* for the least (0%) (McGill *et al.,* 2005). Healthy veterinary students in Japan were tested by IgG and IgM IFA and yielded 10.9% (14/129) prevalence for *B. henselae* IgG tests and 0.8% (1/129) prevalence for IgM antibodies against *B. henselae.* It was found that CSD-suspected patients had significantly higher *B. henselae* IgG and IgM positive rates and that the majority of the volunteers who were positive for *B. henselae* antibodies were in recent contact with cats. Interestingly, it was also found that female volunteers had a significantly higher infection rate than male volunteers, and that the younger groups accounted for the highest seropositive rates (Kikuchi *et al*., 2002). Serum from 140 healthy blood donors in New Zealand were tested and found to have a seroprevalence of 3.6% for *B. henselae* antibodies (Zarkovic *et al.,* 2007).

The results of this study were generally in line with the results obtained internationally and indicated that there is a need for further study into the immunological responses of *Bartonella* infection on exposed and susceptible populations, particularly the immunocompromised.